Characterisation of the interactions and biological impacts of cisplatin analogues and metallo-drug complexes with DNA

A thesis submitted in fulfilment for the degree of Doctor of Philosophy by

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(Accepted Response to Examiners – v2.1)
Statement of Authenticity

I, Mark William Burgess, declare that this thesis contains no material that has been accepted for the award of any other degree or diploma and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except when due reference has been made in the text of this thesis.

Mark William Burgess

March 2013
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<tr>
<td>2D</td>
<td>Two dimensions</td>
</tr>
<tr>
<td>32P</td>
<td>Phosphorus isotope 32</td>
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<tr>
<td>3478MEEN</td>
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<tr>
<td>56MESS</td>
<td>([(5,6\text{-}\text{dimethyl-1,10-phenanthroline})(1S,2S\text{-diaminocyclohexane})\text{platinum(II)})]^{2+}</td>
</tr>
<tr>
<td>A2780</td>
<td>Human ovarian epithelial cell line</td>
</tr>
<tr>
<td>AAdUTP</td>
<td>AminoAllyl deoxyuridine triphosphate</td>
</tr>
<tr>
<td>BER</td>
<td>Base excision repair</td>
</tr>
<tr>
<td>Bp</td>
<td>Base Pair</td>
</tr>
<tr>
<td>CE-CC</td>
<td>Capillary Electrophoresis in the Critical Conditions</td>
</tr>
<tr>
<td>CE-LIF</td>
<td>Capillary Electrophoresis with Laser Induced Fluorescence</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>(\text{cis-diamminedichloroplatinum(II)})</td>
</tr>
<tr>
<td>CTR-1</td>
<td>Copper transporter-1 protein</td>
</tr>
<tr>
<td>Cu56MESS</td>
<td>([(5,6\text{-}\text{dimethyl-1,10-phenanthroline})(1S,2S\text{-diaminoethane})\text{copper(II)})]^{2+}</td>
</tr>
<tr>
<td>DACH</td>
<td>dichloro(1R,2R\text{-diaminocyclohexane})\text{platinum(II)}</td>
</tr>
<tr>
<td>DCBP</td>
<td>dichloro(2R,2′R\text{-bipyridine})\text{platinum(II)}</td>
</tr>
<tr>
<td>DCRP</td>
<td>(\text{cis-dichlorobis(pyridine)}\text{-platinum(II)})</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Media</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>dsDNA</td>
<td>Double stranded DNA</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>EcoRI</td>
<td>Restriction endonuclease with sequence recognition for 5′-G\text{\textbullet}AATTC-3′</td>
</tr>
<tr>
<td>E. coli DH5α</td>
<td>Escherichia coli strain DH5α</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene-diamine-tetra-acetic acid</td>
</tr>
<tr>
<td>eof</td>
<td>Electro-osmotic flow</td>
</tr>
<tr>
<td>ERCC1</td>
<td>Excision repair cross-complementation group 1 protein</td>
</tr>
<tr>
<td>EtBr</td>
<td>Ethidium bromide, 3,8-Diamino-5-ethyl-6-phenylanthridinium bromide</td>
</tr>
<tr>
<td>FAM</td>
<td>Carboxyfluorescein, also referred to as “5FAM” or “5-FAM” which is used as a 5′ modification to DNA for fluorescent detection.</td>
</tr>
<tr>
<td>gDNA</td>
<td>Genomic DNA</td>
</tr>
<tr>
<td>GSH</td>
<td>Reduced glutathione, also referred to as “glutathione”</td>
</tr>
<tr>
<td>GSH : r_b</td>
<td>Glutathione (moles) to drug (moles) to nucleotide ratio</td>
</tr>
<tr>
<td>GSSG</td>
<td>Oxidised glutathione, also referred to as “glutathione disulfide”</td>
</tr>
<tr>
<td>HEK293</td>
<td>Human embryonic kidney 293 cell line</td>
</tr>
<tr>
<td>HeLa cell line</td>
<td>Immortal human cervical cancer cell line</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>IAA</td>
<td>Isoamyl alcohol</td>
</tr>
<tr>
<td>IC50</td>
<td>Half maximal inhibitory concentration</td>
</tr>
<tr>
<td>ICL</td>
<td>Interstrand cross link</td>
</tr>
<tr>
<td>Kbp</td>
<td>Kilo base pair (may also be abbreviated as “Kb”)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>L1210 cell line</td>
<td>Mouse lymphocytic leukaemia cell line</td>
</tr>
<tr>
<td>LA</td>
<td>Linear amplification</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Broth</td>
</tr>
<tr>
<td>LD</td>
<td>Linear Dichroism</td>
</tr>
<tr>
<td>LIZ500</td>
<td>LIZ fluorophore labelled molecular weight standard consisting of 16 single-stranded labelled fragments of 35, 50, 75, 100, 139, 150, 160, 200, 250, 300, 340, 350, 400, 450, 490, and 500 bases</td>
</tr>
<tr>
<td>m².V⁻¹.s⁻¹</td>
<td>Mobility, meters squared per volts per second</td>
</tr>
<tr>
<td>MCF-7 cell line</td>
<td>Human breast cancer cell line</td>
</tr>
<tr>
<td>MMR</td>
<td>Miss match repair</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Centre for Biotechnology Information</td>
</tr>
<tr>
<td>NER</td>
<td>Nucleotide excision repair</td>
</tr>
<tr>
<td>NP-40</td>
<td>Nonyl phenoxypolyethoxyl-ethanol type 40</td>
</tr>
<tr>
<td>pBR322</td>
<td>Plasmid Bolivar-Rodriguez series 322</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>pDNA</td>
<td>Plasmid DNA</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>phen</td>
<td>1,10-phenanthroline</td>
</tr>
<tr>
<td>pUC19</td>
<td>Plasmid University of California series 19</td>
</tr>
<tr>
<td>PvuII</td>
<td>Restriction endonuclease with sequence recognition for 5’-CAG↓CTG-3’</td>
</tr>
<tr>
<td>r²</td>
<td>Coefficient of determination</td>
</tr>
<tr>
<td>R6G</td>
<td>5-carboxyrhodamine 6G</td>
</tr>
<tr>
<td>R6GdUTP</td>
<td>5-carboxyrhodamine-6G-aminoallyl-deoxyuridine-triphosphate</td>
</tr>
<tr>
<td>r_b</td>
<td>Drug (mole) to nucleotide ratio</td>
</tr>
<tr>
<td>RO</td>
<td>Reverse osmosis</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RuP1</td>
<td>( \Delta{[\text{Ru}(\text{phen})_2(3-[(\text{methyl}-4-p-1H-imidazole-2-carbonyl}-\text{amino}}]-\text{butyrylamino})-1\text{H-pyrrole}-2\text{-carbonyl}-\text{amino}}]-\text{propionic acid})))(PF₆)₂</td>
</tr>
<tr>
<td>RuP2</td>
<td>( \Delta{[\text{Ru}(\text{phen})_2(3-[(\text{methyl}-4-4-\text{[1-methyl-4-}3-\text{[(dipyrido[6,7-d:2',3'\text{-f}][quinoxaline}-2\text{-carbonyl}]amino}]\text{-hexanoylamino}}]-1\text{H-pyrrole}-2\text{-carbonyl}-\text{amino})]-1\text{H-pyrrole}-2\text{-carbonyl}-\text{amino}}]-\text{propionic acid})))(PF₆)₂</td>
</tr>
<tr>
<td>RUVBL2</td>
<td>Human gene coding for a AAA+ protein</td>
</tr>
<tr>
<td>Scal</td>
<td>Restriction endonuclease with sequence recognition for 5’-AGT↓ACT-3’</td>
</tr>
<tr>
<td>SD, StdDev</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>ssDNA</td>
<td>Single stranded deoxyribonucleic acid</td>
</tr>
<tr>
<td>SYBR</td>
<td>Also known as SYBR Green I</td>
</tr>
<tr>
<td>N’,N’-dimethyl-N-[4-[(E)-(3-methyl-1,3-benzothiazol-2-ylidene)methyl]-1-phenylquinolin-1-ium-2-yl]-N-propylpropane-1,3-diamine</td>
<td></td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate-EDTA</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-borate-EDTA</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N’,N’-Tetramethylethane-1,2-diamine</td>
</tr>
<tr>
<td>( \beta )-globin</td>
<td>Human gene for beta globin</td>
</tr>
</tbody>
</table>
List of publications produced from this thesis

**Lorne Genome Conference 2012 – Poster Presentation**

**Lorne Cancer Conference 2012 – Poster Presentation**

**Combio Conference 2012 – Poster Presentation**
Abstract

The discovery of the anti-cancer properties of *cis*-diaminedichloroplatinum(II) (cisplatin) resulted in a surge of metallo-drug research with the aim of discovering new compounds which could deliver maximum therapeutic outcomes whilst minimising drug resistance and toxic side effects for cancer patients. It is widely believed that the primary mechanism for cisplatin-induced cancer cell death arises from its ability to form covalent adducts with DNA, primarily at purine bases. It has been shown that the formation of cisplatin-DNA adducts leads to a disruption of biological functions such as protein/DNA binding, DNA replication and transcription which causes cell death.

Although cisplatin has proven useful in the clinic, there are a range of side effects associated with its administration that include nephrotoxicity, neurotoxicity, ototoxicity and gastrointestinal tract toxicity. Additionally, there is a range of cellular resistance issues associated with cisplatin treatment. For example, the metallo-drugs high affinity to bind to the thiol groups of proteins and the peptide glutathione (GSH), establishes a competitive mechanism that titrates the drug away from binding to DNA. Inorganic chemists continue to address these issues by employing a rational drug design strategy that is based on modifying the ligand groups or the central metal atom. This approach has lead to the synthesis of many hundreds of cisplatin analogue compounds and other novel metallo-drugs which many are currently under investigation as potential anti-cancer drug candidates.

The work described in this thesis develops and applies several methodologies to determine the potential of cisplatin analogues and novel metallo-drugs as anti-cancer compounds. These methodologies centred around three approaches that elucidate the DNA binding properties of these metallo-drugs. The first approach characterises the mode of DNA binding and measures the metallo-drug effect on modifying DNA structure. The second approach analysed both the ability of the metallo-drugs to inhibit DNA replication and determined the DNA binding
sequence specificity. The third approach compared the binding affinity of metallo-drugs for GSH or DNA.

The metallo-drugs tested include cisplatin, which served as a positive control since its interactions with DNA have been well studied. The cisplatin analogues tested included carboplatin, oxaliplatin, DCBP, DCRP, DACH, and metallo-intercalators 56MESS and 3478MEEN. Other metallo-drugs such as the copper(II) 56MESS analogue Cu56MESS and the ruthenium(II) compounds RuP1 and RuP2 were also studied. Finally, the well defined DNA strand breaker phleomycin was included to serve as a positive control in for compounds that may exhibit endonuclease activity.

Data presented in this thesis demonstrated that the novel application of free solution CE-CC was effective to determine metallo-drug induced structure modifications on a linear dsDNA substrate. Furthermore, metallo-drugs were able to be differentiated into their DNA binding mode by combining CE-CC data with plasmid mobility data on a 2D scatter plot analysis.

The 2D scatter plot analysis of metallo-drug/DNA structure modification showed that the ruthenium(II) groove binder RuP2, with an additional pyrrole ligand compared to the RuP1 analogue, could substantially increase the molecular weight of a linear dsDNA substrate. Furthermore, the 2D scatter plot DNA structure analysis revealed the platinum(II) metallo-intercalator 3478MEEN adopted exhibited duel covalent binding and π-π base stacking intercalation with DNA. The covalent binding activity associated with 3478MEEN was also evident in an interstrand crosslink assay which involved a novel application of a urea-based denaturing agarose slab gel electrophoresis and 5′FAM linear dsDNA substrate.

The Linear Amplification (LA) reaction was employed to characterise metallo-drug induced inhibition of DNA replication in addition to identifying the test compound’s sequence binding specificity. A novel application of Rhodamine 6G labelled nucleotides was used to quantify metallo-drug induced inhibition of DNA replication by a DNA polymerase. Furthermore, the
application of a 5′FAM labelled primer in a LA reaction together with separation of the products by Capillary Electrophoresis with Laser Induced Fluorescence (CE-LIF) was performed. From these data, sites of adduct formation were identified that corresponded to the known sequence specificity of cisplatin, in addition the binding specificity of other metallo-drugs were determined.

A kinetic study was employed to measure metallo-drug thiol bond reactivity with GSH. It was demonstrated that the thiol bond reactivity with the control platinum(II) compounds cisplatin, oxaliplatin and carboplatin were in agreement with published data, in addition novel reactivity data were obtained for compounds not previously studied. Finally, an in vitro competitive assay and analysis protocol was developed to quantitatively analyse the binding preference of the metallo-drug between DNA and GSH. A 2D scatter plot analysis of metallo-drug reactivity with GSH and DNA revealed that Cu56MESS and the DNA strand breaker, phleomycin, exhibit a similar DNA and GSH reaction profiles in the absence of a reducing agent which warrants further investigation.

Overall, developed methodologies produced data that showed that the platinum(II) metallo-intercalator 3478MEEN adopted dual covalent binder and π-π base stacking DNA binding modes. The copper(II) metallo-drug Cu56MESS showed negligible DNA strand breaking activity in the absence of a reducing agent like H2O2, however, it did exhibit GSH reactivity suggesting that the need for a reducing agent may not be necessary for reactions with other biological substrates. Finally, the ruthenium(II) groove binder RuP2 with its additional pyrrole ligand compared to RuP1 could substantially increase the molecular weight of a linear dsDNA substrate.
Chapter 1: General Introduction

1.1 The epidemiology and fiscal impact of cancer on society

Cancer is one of the most prevalent diseases in the human population with 12.7 million new diagnoses and 7.6 million cancer related deaths recorded in 2008 (Jemal et al., 2010). Cancer is the primary cause of death in the developed world and the second biggest cause of death in the developing world (Mathers et al., 2008). For males, lung cancer is the most prevalent cancer diagnosis at 17%, while for woman the most prevalent cancer diagnosis is breast cancer at 23% (Jemal et al., 2011).

A wide variety of risk factors contribute to the increased incidence of cancers in the human population. In the developed world, an ageing population has been identified as a major contributor to the increases in cancer diagnosis. In Australia, the ageing population is expected to contribute to a 70% increase in the number of new cancer diagnoses to 170,000 per annum between 2007 and 2025 (Baade et al., 2012). Rates of cancer morbidity and mortality in the Australian population have doubled between 1982 and 2007, with the morbidity rate now at 108,368 per year and mortality rate increase to 27% which now contributes to approximately 1 in 5 of all disease occurrences in Australia (AIHW, 2010).

Several factors have been identified that may mitigate the risk of acquiring cancer. Most cancers in the developed world are diagnosed as preventable and brought about by life style activities and environmental conditions such as the smoking of tobacco products, diet and nutrition, alcohol consumption, physical activity, occupational hazards and sun exposure (Kushi et al., 2006). Furthermore, only 5 to 10% of all cancers are caused by genetic factors such as inheritance of high risk genes (Anand et al., 2008).
Prostate, colo-rectal, lung and breast cancers are the most prevalent cancers in Australia (Baade et al., 2012). These diseases translate to a severe economic cost to the Australian health system whereby the direct cost of cancer in the 2007 to 2008 financial year was over $3.8 billion dollars (AIHW, 2009). The high socio-economic costs to government health systems and the community has prompted governments to develop policies and benchmarks that are targeted at improving cancer survival rates over defined time periods such as those legislated in the New South Wales and Victorian state jurisdictions of Australia (Coleman et al., 2011). As a result of these government health initiatives and the prioritisation of cancer research in Australia, there has been a reported increase in survival rates for colorectal, lung, breast and ovarian cancers between 1995 and 2007 (Coleman et al., 2011).

1.2 Cancer biology

Cancer is a class of over 100 diseases characterised by the uncontrolled proliferation of aberrant cells due to multiple changes in the genome that result in the loss of cell cycle control (Hanahan and Weinberg, 2000). The classic pathology of cancer is the abnormal growth of tumour tissue although this is not necessarily the case for all cancers. For example, leukaemias can have many phenotypic variations that are traditionally identified by cyto-morphological examinations (Haferlach et al., 2005).

Recently, more sophisticated genome-wide analysis techniques have been employed to more accurately diagnose and classify different types of cancers (Ramaswamy et al., 2001, Golub et al., 1999, Haferlach et al., 2005). The importance of accurate identification of cancer cells is critical for devising an effective treatment strategy and to deliver positive health outcomes for patients in the clinic (Golub et al., 1999). It is generally believed that the successful transformation of a cell into a carcinogenic derivative requires multiple alterations to its genetic material (Hanahan and Weinberg, 2000, Evan and Vousden, 2001).
1.2.1 Multiple DNA mutations are required for cancer cell proliferation

For a cell with multiple mutations to successfully progress to carcinogenesis, it needs to acquire several traits. Such traits include the need for cancer cells to be self sufficient in the production of growth signals, produce angiogenesis factors for additional blood supply, resist anti-growth and apoptosis signalling, acquire unlimited replication potential, and have the ability to invade and metastasise in distal tissue (Hanahan and Weinberg, 2000, Evan and Vousden, 2001).

The ability of a cell to detect mutations in its genetic material is an important mechanism for preventing the development of carcinogenesis. One of the most well defined mechanisms for the detection of DNA mutations is the p53 tumour suppressor protein which works to prevent cancer cell development by monitoring the genetic integrity and cell cycle progression. p53 is a transcription factor that binds to cognate response elements and activates the expression of genes in response to many cancer associated conditions such as chromosomal aberrations and DNA damage, shortening of telomeres, hypoxia, nutrition deprivation and the inappropriate activation of oncogenes (Oren, 2003, Kastan et al., 1995, Lengauer et al., 1998). Upon detection of an irregularity in the cell’s genome, gene expressions induced by p53 can enable cell cycle arrest at G1 phase mediated by cell survival signalling until appropriate repairs to the damaged genetic material can be completed (Kerr et al., 1994, Evan and Vousden, 2001, Oren, 2003). However, if repair mechanisms fail to correct the error in the gene sequence, p53 can also initiate apoptosis signalling which eventually results in cell death (Oren, 2003, Kerr et al., 1994, Evan and Vousden, 2001).

The loss of cell cycle control and failure of a cell to undergo apoptosis in relation to carcinogenesis has been extensively documented and linked to the p53 tumour suppressor protein (Hartwell and Kastan, 1994, Evan and Vousden, 2001, Kastan et al., 1995, Hanahan and Weinberg, 2000). p53 mutations range from single base changes to complete deletions of
the gene and have been observed in over 50% of all human cancers (Hanahan and Weinberg, 2000, Oren, 2003, Oren, 1992). This contributes to the disabling of the cells’ tumour-suppressive function and may lead to carcinogenesis (Kerr et al., 1994, Hartwell and Kastan, 1994, Hollstein et al., 1991, Kastan et al., 1995).

1.2.2 Cancer cell metabolism

Work by Warburg in the 1920’s determined that most cancer cells preferred to metabolise glucose by glycolysis in the presence of oxygen, termed aerobic glycolysis, which creates a paradox as ATP production is more efficient by oxidative phosphorylation metabolism (Warburg, 1956). The molecular basis for cancer cell preference to undergo aerobic glycolysis for the metabolism of glucose is unclear and has been extensively reviewed (Kim and Dang, 2006, Hsu and Sabatini, 2008, Gatenby and Gillies, 2004). Furthermore, the high glucose uptake by cancer cells forms the basis of a tumour diagnostic tool for patients in the clinic where a fluorescent glucose analogue 2-[fluorine-18]-fluoro-2-deoxy-d-glucose is detected by PET scan (Schirrmeister et al., 2001).

Regulation of native cell populations are normally controlled by growth factors that dictate rates of nutrient uptake from the surrounding micro-environment (Turner and Grose, 2010). However, acquired genetic mutations of tumour suppressor genes and/or the activation of oncogenes such as c-myc, ras, p53 and src in cancer cells enable the bypass of growth factor mediated control pathways and facilitate the increased uptake of glucose from the surrounding micro-environment (Osthus et al., 2000, Aaronson, 1991, Turner and Grose, 2010, Hue and Rousseau, 1993, Vizan et al., 2005, Bensaad et al., 2006). Furthermore, many of the genes that are involved in glycolysis, such as hexokinase-2 and lactate dehydrogenase-A, are transcriptional targets of oncogenes, such as c-myc, which have previously been shown to be up-regulated in cancer cells (Kim and Dang, 2006).
The heightened rates of cancer cell metabolism which result in increased cell proliferation have also been correlated to increased intracellular reactive oxygen species (ROS) accumulation (Patel et al., 2007, Kumar et al., 2008). The exact molecular pathways involved in increasing ROS production in cancer cells have not been clearly defined. However, there are a range of factors that are known to increase intracellular ROS production such as the activation of oncogenes, dysfunctional mitochondria, increased metabolism and loss of p53 activity (Irani et al., 1997, Rodrigues et al., 2008, Brandon et al., 2006, Horn and Vousden, 2007).

The increased production of intracellular ROS due to heightened cancer cell metabolism can induce oxidative stress to toxic levels and may facilitate the selective pressure for cell populations that have developed intrinsic molecular mechanisms to evade cell death (Irmak et al., 2003). Evidence for a molecular basis of ROS-mediated cancer cell death evasion has previously been shown in transcription regulation studies with melanoma cells. For example, the down regulation of \textit{c-myc} which controlled the expression of glutathione (GSH) synthesis enzymes depleted intracellular GSH and induced apoptosis-mediated cancer cell death (Benassi et al., 2006).

GSH is a tripeptide that has previously been shown to increase its intracellular concentration in response to oxidative stress (Estrela et al., 1995). GSH functions as a ROS scavenger and restores biomolecules back to their reduced state by neutralising HO• groups (Sies, 1986). The role of GSH in preventing ROS-induced apoptosis has also been shown with cancer cells stimulated by mitogenic growth factors which exhibit increased concentrations of intracellular GSH (Shaw and Chou, 1986). This has resulted in a decrease in cell proliferation to exhibit quiescence (Estrela et al., 1992) by regulating protein kinase C activity and intracellular pH (Terradez et al., 1993).
The high concentrations of GSH in cancer cells is one of the limiting factors that can mitigate effective chemo-pharmaceutical treatment with platinum(II) metallo-drugs (Litterst and Schweitzer, 1988) in a clinical setting. Platinum(II) metallo drugs, like cisplatin, exhibit a high affinity to bind to sulfur donors such as GSH and can be titrated away from other intracellular targets such as DNA bases (Bose et al., 1995, Reedijk, 1999, Kasherman et al., 2009). This mechanism of GSH-mediated cancer cell resistance to metallo-drug pharmaceuticals is an area of investigation for Inorganic Chemists with the aim of developing new compounds that overcome this area of treatment deficiency (Ang et al., 2005) and is a focus of work presented in this thesis.

1.3 The relationship between DNA structure and function

The central dogma of molecular biology model describes a linear pathway from DNA to RNA to protein biomolecules for the transfer of biological information which enables cell function to occur (Crick, 1970). Later work showed that the relationship between the three central dogma biomolecules operated in a non-linear manner and that regulation of each element was critical for cell function and survival (Bustamante et al., 2011). However, the cell’s ability to maintain DNA integrity is paramount for cell survival as DNA is the only biomolecule where biological information is stored within the cell and is therefore an important target for cellular studies (Li and Xie, 2011).

1.3.1 DNA structure organisation

It is often convenient to describe the structural organisation of eukaryote DNA as having between three or four hierarchical levels within the cell. This is an arbitrary division as it can be difficult to distinguish between DNA secondary and tertiary structures (Madan Babu et al., 2008, Banfalvi, 1986). The primary building blocks of DNA are purine (deoxyadenosine 5’-monophosphate and deoxyguanosine 5’-monophosphate) and pyrimidine (deoxycytidine 5’-monophosphate and deoxythymidine 5’-monophosphate) nucleotides. A nucleotide consists
of a nitrogen base covalently bound to the C1 of a ribose sugar which is phosphate esterified to the C5 and covalently linked to the C3 of an adjacent ribose sugar to form a sugar phosphate chain in which only the nitrogen bases differ in these subunits. Furthermore, the formation of purine-pyrimidine H-bond bridges between two strands running 5’ to 3’ in opposite directions makes the polymer chain of nucleotides double stranded.

The secondary and tertiary levels typically describe the three dimensional steric orientation of the nucleotides within the DNA structure. Conventionally, eukaryotic DNA consists of two complementary strands of linear DNA arranged as a double stranded helix with a 35.9° right-handed rotation per base which constitutes B-DNA conformation (Wing et al., 1980). The B-DNA double helix can also exhibit a range of localised structural features such as looped, supercoiled, catenated, knotted and relaxed conformations which adds to the macromolecule’s overall steric complexity (Banfalvi, 1986). Finally, at the quaternary level, clusters of histone proteins associate with the double helix strands to form chromatin structures and ultimately various higher order structures to form chromosomes that compress the DNA and allow it to fit within the cell’s nucleus (Kornberg, 1974, Roque et al., 2008).

Both prokaryote and eukaryote DNA exhibits a variety of homologous structural features at the secondary/tertiary level. In addition, plasmid DNA (pDNA) is a closed circular loop that is approximately 100,000 times smaller than genomic (gDNA). pDNA is capable of self replication and resides as a separate intracellular structure from the host cell’s gDNA which allows for it to be exploited as a tool for gene expression studies in both prokaryotic (Bouma and Lenski, 1988) and eukaryotic transfection research (Iwaki and Umemura, 2011).

pDNA can present a variety of secondary/tertiary structural conformations. The common plasmid conformations are relaxed open circular, nicked circular, supercoiled, linear double-stranded and linear single stranded structures. All of these conformations can exist in both monomer or dimer isoforms and are easily discernible by routine slab gel electrophoresis (Hamilton and Wilker, 2004, Dixit et al., 2010).
The variations in tertiary level conformation seen in pDNA may also be present in localised regions of gDNA. For example, domains of supercoiled loops can sit within B-DNA strands that can span across several tens of kilo-bases (Buongiorno-Nardelli et al., 1982). Further variations in tertiary level conformations that are both localised and sequence-dependant include cruciforms, triplexes, hairpins, tetraplexes and left-handed Z-DNA (Bacolla and Wells, 2004).

1.3.2 The relationship between DNA conformation and function

The relationship between DNA conformation and its functional role relies on all four levels of DNA structure to mediate protein binding for downstream replication and transcription activities (Travers, 1989, Smale, 2009). The first three levels of structural organisation are of particular importance as they only involve the DNA molecule to contribute to its own conformational state. The quaternary level gives an additional layer of architecture that condenses the DNA and also acts to contain irregular conformations which could affect DNA replication and transcription in both prokaryotic (Kayoko et al., 1992) and eukaryotic cells (Zehnbauer and Vogelstein, 1985).

The secondary/tertiary level conformational structures have been shown to affect DNA replication and transcriptional activity (Arneodo et al., 2011). In some cases it has been demonstrated that supercoiling in both prokaryotic and eukaryotic gDNA can disrupt replication and transcription (Ohyama et al., 2005, Koster et al., 2010). This supercoiling effect has also been shown to inhibit transcription with pDNA in bacterial cells (Brahms et al., 1985, Westra et al., 2012).

Modifications to secondary/tertiary DNA structure can also influence high order conformational states and affect replication and transcription activity (Meijsing et al., 2009). Phosphate backbone strand breaks seen in pDNA can also occur in gDNA (Perry et al., 2010). However, these structural features are usually repaired by DNA repair pathway mechanisms.
unless a genetic-based disease is present such as hereditary defects (Caldecott, 2008) or damage that is too extensive for the cell to recover (Roos and Kaina, 2006). The inhibition of H-bond melting through the formation of interstrand crosslinks by exogenously derived compounds, such as platinum-based drugs, distorts DNA conformation (Suchankova et al., 2012) and inhibits DNA replication and transcription activities (Deans and West, 2011).

1.3.3 DNA damage repair mechanisms

DNA damage can occur from a variety of exogenous and endogenous sources which trigger several DNA repair pathways. Sources of endogenous DNA damage can arise from increased ROS which are indicative of cancer cells with increased metabolic activity (Maynard et al., 2009, Wang, 2008) and replication errors in the genome (Budzowska and Kanaar, 2009). Exogenous DNA damage sources can arise from UV, X-ray and gamma-ray irradiation (Rochette and Brash, 2010, Wojewódzka et al., 2009, Kameya et al., 2011), viral DNA damage such as that induced by the Epstein-Barr virus (Gruhne et al., 2009) or platinum(II) based metallo-drug anti-cancer pharmaceuticals which form covalent adducts (Takahara et al., 1996).

There are five cellular pathways involved in DNA repair; nucleotide excision repair (NER), miss match repair (MMR), base excision repair (BER), double-strand break repair and direct repair. Each pathway exhibits different molecular mechanisms to repair cellular DNA. BER, for example, involves the excision and replacement of a single base which may be damaged by a covalently bound adduct (Sharma and Dianov, 2007). Where NER excises a 24 to 30 bp ssDNA molecule which contains a DNA fragment that is structurally distorted due to the adduct formation (Sugasawa et al., 2001).

DNA repair pathways have been proposed as a mechanism by which cancer cells develop metallo-drug resistance. Platinum(II) metallo-drugs, such as cisplatin, form covalent adducts with DNA and induce structural modifications to the double helix (Rabik and Dolan, 2007).
The DNA repair pathway enzymes recognise the platinum(II)-induced modified DNA which forms the basis of cancer cell resistance in the clinic.

Two DNA repair pathways that are strongly implicated in cancer cell resistance to cisplatin treatment are the nucleotide excision repair and mismatch repair pathways. The NER pathway principally involves the ERCC1 protein and is activated by the recognition of a damaged base in the DNA which is then unwound, excised, resynthesised and re-ligated (Rabik and Dolan, 2007). The MMR pathway is initiated by the recognition of DNA damage by a complex of undefined Mut proteins that excise the effected site which is then resynthesised by DNA polymerase (Kunkel and Erie, 2005).

### 1.4 Cisplatin as an anti-cancer agent

Cisplatin (cis-dichlorodiammine-platinum(II)) ([Figure 1.1](#)) was reported to have an effect on inhibiting *E. coli* cell division and subsequently identified as an effective anti-cancer agent that paved the way for a renaissance in metallo-drug development (Rosenberg *et al.*, 1965, Rosenberg *et al.*, 1969). Rosenberg was originally interested in determining whether electric or magnetic fields played a role in cell division. He employed an experimental design that consisted of a culture of *E. coli* cells subjected to an electromagnetic field using a pair of (what was considered inert at the time) platinum electrodes. Analysis of the treated culture showed the *E. coli* cells were 300 times their normal length. Rosenberg later concluded that the effect was not induced by the electric field but rather by the presence of platinum(II) and platinum(IV) compounds ([Figure 1.1](#) a,d) that were generated by the electrolysis and reaction between the ammonium chloride molecules present in the buffer solution with the platinum electrodes (Rosenberg *et al.*, 1965). After further testing on bacterial models, it was revealed that cisplatin stopped cell division at non-toxic concentrations.

From this work, Rosenberg hypothesised that cisplatin could be used at non-toxic concentrations to prevent the uncontrolled division of cancer cells (Rosenberg *et al.*, 1969).
Figure 1.1: Chemical structures of platinum compounds. (a) cis-diammine-platinum(II) (cisplatin), (b) cis-diammine(1,1-cyclobutane dicarboxylato)-platinum(II) (carboplatin), (c) cis-diammine(1,1-cyclobutanedicarboxylato)platinum(II) (oxaliplatin) and (d) cis-diamine-tetrachloride-platinum(IV).
To test this theory, a 10 mg piece of sarcoma-180 (the generic model tumour of the day) was implanted under the skin of a mouse and treated with a non-lethal dose of 8 mg.kg$^{-1}$ cisplatin. Over the preceding 36 days, the tumours mass decreased by over 100 fold and was able to be removed as one piece as it did not metastasise. This was the first evidence that cisplatin was an effective anti-cancer agent.

1.4.1 The molecular basis for the anti-cancer activity of cisplatin

Cisplatin was subjected to clinical trials and was approved for use by the US Food and Drug Administration in 1978 after renal toxicity side effects were overcome through diuresis by aggressive hydration therapy (Jamieson and Lippard, 1999, Kelland, 2007, Hayes et al., 1976). Cisplatin is used to treat many kinds of malignancies including non-small lung cancer, head and neck, oesophageal, ovarian and cervical cancers (Loehrer and Einhorn, 1984). However, the most significant effect was the cure rate of testicular cancer, which is now more than 90% for early diagnosed tumours (Bosl and Motzer, 1997).

Cisplatin is delivered to the patient via intravenous injection at a dose of 50-120 mg.m$^{-2}$ over a 3 to 4 week period (Loehrer and Einhorn, 1984). The compound is neutral in the bloodstream due to the relatively high extracellular chloride concentration (100 mM). Upon entry into the cell, the chloride ligands are hydrolysed (Figure 1.2) due to the low intracellular chloride concentration (~10 mM) and replaced with water molecules, activating the compound and allowing it to interact with cellular targets (Kelland, 2007, Jamieson and Lippard, 1999).

Cisplatin has a limited cellular uptake of up to 1 mM (Gale et al., 1973, Binks and Dobrota, 1990, Hromas et al., 1987, Mann et al., 1990). Furthermore, cisplatin is not inhibited by other analogue metallo-drugs (e.g. carboplatin) nor does it have an optimum pH, suggesting it enters the cell by non-carrier mediated passive diffusion (Andrews et al., 1988). There is evidence that cisplatin also enters the cell by active transport (Gately and Howell, 1993). For
Figure 1.2: The aquation of cisplatin by a two stage hydrolysis reaction. The first stage hydrolysis reaction replaces a chloride ligand with a water molecule giving a $1^+$ net positive charge to the whole compound. The second stage hydrolysis reaction replaces the second chloride ligand with another water molecule which gives the whole compound a $2^+$ net positive charge.
example, the copper transporter-1 (CTR-1) plasma membrane transporter, responsible for copper homeostasis, has been implicated with cisplatin influx into the cell (Ishida et al., 2002, Katano et al., 2002).

Upon entry into the cell, cisplatin has been shown to affect many cellular components (Wang et al., 1996) such as cytoskeleton activity (Kopf-Maier and Muhlhausen, 1992), plasma membrane phospholipids, phosphatidylserines (Speelmans et al., 1996, Speelmans et al., 1997), and protein activity due to cisplatin’s high binding affinity to thiol groups, particularly proteins with many cysteine residues (Aull et al., 1980, Pizzo et al., 1988, Zeng et al., 1997, Aull et al., 1979, Kamal, 1996). However, it is generally accepted that the cells genetic material is the clinically significant target for cisplatin (Jamieson and Lippard, 1999, Kelland, 2007, Kozelka, 2009, Reedijk, 1999). The DNA binding activity of anti-cancer metallo-drugs is a focus of current work presented in this thesis.

The long filamentous growths of *E. coli* cells observed in Rosenberg’s experiment are characteristic of other DNA-damaging agents such as hydroxyurea (Rosenkranz et al., 1966), UV and other ionizing radiation (Witkin, 1967, Adler and Hardigree, 1965). Another result shared by DNA damaging agents is the lysis of *E. coli* cells treated with cisplatin when infected with bacteriophage λ (Reslova, 1971). Past work has shown cisplatin preferentially binds to DNA over other nucleic acids such as mRNA, rRNA, tRNA and protein molecules (Pascoe and Roberts, 1974). Additionally, RNA, DNA and protein synthesis assays using human amnion AV3 cells and Ehrlich ascites tumour cells treated with cisplatin showed the selective inhibition of DNA synthesis compared to RNA and protein synthesis (Harder and Rosenberg, 1970, Howle and Gale, 1970).

Cisplatin preferentially binds to purine bases, in particular guanine residues (Eastman, 1996, Jamieson and Lippard, 1999). Experiments have shown that the binding of cisplatin to DNA is kinetically controlled due to the rate limiting hydrolysis of a chloride ligand forming
aquated $\text{cis-}[\text{Pt(NH}_3)_2\text{Cl(H}_2\text{O)}]^+$, followed by the preferential binding of this to a guanine N7 atom which forms a mono-functional adduct (Bancroft et al., 1990) (Figure 1.3). The current consensus is that a guanine N7 atom is the preferential site where the platinum atom forms a covalent bond to the DNA substrate (Kozelka, 2009). Furthermore, it has also been shown that the guanine N7 atom is thermodynamically favoured over an adenine N7 atom (Lebwohl and Canetta, 1998). Hydrolysis of the second chloride ligand allows for the formation of a bi-functional adducts (Bancroft et al., 1990).

The most favoured bi-functional adduct formations are GG 1,2 intrastrand (60-65%) and AG 1,2 intrastrand (20-25%). However, other less common bi-functional adducts such as GNG 1,3 intrastrand (~2%) and GG interstrand (~2%) can form in addition to monofunctional adducts on guanine bases (~2%) (Kelland, 2007, Kozelka, 2009, Reedijk, 1999). The formation of cisplatin adducts distorts the DNA structure and can unwind and condense the helix length by up to 50% upon platinum saturation (Cohen et al., 1979). The most common bi-functional GG 1,2-intrastrand adduct can bend the helix up to 60° towards the major groove (Den Hartog et al., 1985b, Den Hartog et al., 1985a). Cisplatin’s ability to distort the DNA structure suggests broader consequences for DNA/protein interaction. These DNA/protein interactions would affect gene expression which is critical for cell division and protein production.

Cisplatin’s propensity to inhibit DNA replication and modulate transcription activity has been suggested to be the likely mode of cancer cell cytotoxicity. Inhibition of DNA replication was demonstrated when the SV40 chromosome from green monkey CV-1 cells was treated with cisplatin and produced a significant decrease in DNA synthesis (Ciccarelli et al., 1985). Cisplatin has also been shown to alter DNA transcription activity. The monkey cell line CV-1, transfected with a number of strong and weak promoter genes, was treated with
Figure 1.3: Reaction schematic showing the formation of a covalent adduct on a DNA guanine base by cisplatin. Cisplatin subjected to first stage hydrolysis replaces a chloride ligand with a water molecule which enables the formation of a covalent bond to the N7 position of a guanine base in a DNA sequence (5′-TGC-3′).
cisplatin which resulted in the down-regulation of strong promoter genes and up-regulation of genes with weak promoters (Evans and Gralla, 1992a, Evans and Gralla, 1992b). It was suggested that cisplatin was able to reduce the expression of genes with strong promoters because these generally reside in regions of more accessible chromatin where adduct formations could occur more readily. Further evidence that chromatin inhibits cisplatin binding with DNA by means of steric hindrance was recently shown in linear amplification (LA) experiments (Galea and Murray, 2010).

1.4.2 Cisplatin resistance and side-effects in the clinic

Tumour resistance to cisplatin treatment is one of the major failings of its use in the clinic. Tissues treated with cisplatin can have an inherited resistance profile or develop resistance over a course of treatment. Cisplatin’s high affinity to bind to thiol groups of polypeptides such as glutathione, a tripeptide free radical scavenger highly abundant within the cell, establishes a competitive mechanism and titrates the drug away from more effective targets (Chu, 1994). An example of this mechanism was seen in an ovarian cancer cell line that initially responded to cisplatin treatment, however, secondary cisplatin-resistant tumour growths developed along with a marked increase in glutathione synthesis (Godwin et al., 1992). This increase in glutathione synthesis was attributed to cisplatin resistance.

Limited evidence of the interaction of cisplatin with the cells phospholipid bilayer has been published. Cisplatin has been characterised for preferential binding to negatively charged lipids in low chloride, acidic conditions via electrostatic interactions (Speelmans et al., 1996). NMR studies have suggested a possible mode of cisplatin transport into the cell by the binding of the cisplatin carboxyl group on phosphatidylserines, leading to various degrees of phospholipid bilayer reorganisation that is dependent on the saturation of the lipids’ acyl chains (Jensen and Nerdal, 2008). Cisplatin-bound lipids with unsaturated acyl chains are more likely to move from lamellar phase to isotropic and hexagonal phase configurations,
facilitating cisplatin transport into the cell (Jensen and Nerdal, 2008). It has been suggested that the resistance of lung adenocarcinoma cells to cisplatin is mediated by this mechanism as resistant cells appear to have a higher percentage of saturated phospholipids that facilitates plasma membrane lamellar phase conformation, thus inhibiting cisplatin entry into the cell (Liang and Huang, 2002). Other modes of resistance such as changes in the intracellular concentration of cisplatin mediated through the down-regulation of active transporters such as the plasma membrane localised copper transporter CRT-1 (Ishida et al., 2002, Katano et al., 2002) can decrease drug’s availability within the cell and lead to insufficient DNA binding.

Although cisplatin has been shown to be an important anti-cancer agent, its effectiveness in the clinic has been hampered due to significant side effects. There are a wide range of symptoms from cisplatin treatment including tingling, numbness, paresthesia in distal extremities, difficulty with ambulation due to gait disturbances and proprioception, decreased vibration sense in toes, deep tendon reflexes and manual dexterity difficulties (Boulikas et al., 2007). Additionally, major side effects include nephrotoxicity, neurotoxicity, ototoxicity and gastrointestinal tract toxicity (Boulikas et al., 2007, Kelland, 2007).

During clinical trials undertaken in the 1970’s, nephrotoxicity was a significant issue and was only resolved after hydration therapy was integral (Jamieson and Lippard, 1999, Kelland, 2007, Hayes et al., 1976). As a result, there are some arguments questioning whether cisplatin would be approved for use by regulatory authorities today if it were subjected to current clinical trial standards (Kelland, 2007). Other major side effects include ototoxicity due to cisplatin-induced apoptosis of auditory sensory cells (Devarajan, 2005) and neurotoxicity symptoms that can persist for over 52 months after cisplatin treatment (Al-Sarraf, 1987). The continual development of anti-cancer compounds aims to address resistance and side-effects in addition to improving antitumor activity.
1.5 The application of a rational drug design to reduce resistance and toxic side-effects associated with cisplatin anti-cancer treatment

The basis of rational drug design centres on correlating the biological and clinical data with the molecular structure. Cisplatin, the first generation platinum(II) compound, has been well characterised both in the clinic and laboratory and is considered to be a lead compound for the comparison to novel metallo-drugs. This enables chemists to predict, from first principal investigations, the probability of downstream clinical effectiveness of the novel compound. Although cisplatin proved to be an important anti-cancer drug, it has been plagued with side-effects and resistance issues. To address this, a screen of over 3000 cisplatin analogues was conducted in addition to a number of different compounds which form the basis of the current regime of metallo-drug studies (Weiss and Christian, 1993).

1.5.1 First and second generation cisplatin analogue compounds

Carboplatin (Figure 1.1 b) was developed by Britol-Myers Squibb in collaboration with industry and academic groups (Desoize and Madoulet, 2002) and is the first cisplatin analogue compound to enter the clinic (Lebwohl and Canetta, 1998). In comparison to the chloride leaving groups of cisplatin, carboplatin's cyclobutane ring provides a more stable leaving group and has been credited to a lower nephro- and neuro-toxicity profile in addition to reduced nausea and vomiting (Boulikas et al., 2007). Its similar chemical structure to cisplatin has been accredited for it retaining equivalent anti-cancer activity (Boulikas et al., 2007, Desoize and Madoulet, 2002, Lebwohl and Canetta, 1998). However, this compound is cross-resistant with cisplatin and exhibits a dose limiting myelosupression toxicity profile (Desoize and Madoulet, 2002, Lebwohl and Canetta, 1998). Oxaliplatin (Figure 1.1 c) is currently used to treat colorectal cancer and was originally selected for study due to its low toxicity profile, higher efficacy and minimal cross-resistance with cisplatin (Bleiberg, 1998,
A number of platinum(IV) compounds (Figure 1.1 d) were developed for oral administration (Kelland et al., 1995).

### 1.5.2 Additional metallo-drug designs

Although cisplatin and its first and second generation analogues proved some success in the clinic, none of these compounds possessed a natural affinity to target DNA. Therefore, chemists employed strategies to develop more effective anti-cancer agents such as the incorporation of ligand groups designed to target specific DNA sequences. It has previously been shown that various combinations of pyrrole heterocyclic rings, imidazole, β-alanine chains and aminobutyric acid with L₄ linkers (Figure 1.4) can target specific DNA sequences (Wade et al., 1992). For example, by combining a pyrrole ring with either an imidazole or imidazole ring that targets a GC or CG sequences, respectively. Furthermore, the combination of two pyrrole rings or the combination of β-alanine chain and amino butyric acid with an L₄ linker binds to AT or TA sequences, respectively.

Other DNA intercalation metallo-drugs have been designed to form π-π base stacking which has previously been shown to unwind and lengthen the DNA structure (Liu and Wang, 1975, Keck and Lippard, 1992, Jennette et al., 1976). One such example is [Pt(5,6-dimethyl-phen)(1⁵S,2⁵S-diaminocyclohexane)]Cl₂ (56MESS) (Figure 1.5 c) which incorporates a 1,10-phenanthroline (phen) ligand (Figure 1.5 a) to enable π-π base stacking DNA intercalation. This compound has potential clinical efficacy as it exhibited an IC₅₀ value over 100 fold less than cisplatin in L1210 cell line cytotoxicity assays (Wheate et al., 2007b, Krause-Heuer et al., 2009a). Furthermore, platinum(II) based metallo-intercalators have previously been shown to preferentially intercalate with dsDNA minor groove at G-C rich regions (Jaramillo et al., 2006, Collins et al., 2000).

Another approach to drug design has been to replace the platinum metal of the coordination complex with other metal centres to expand the range of metallo-drugs (ruthenium, zinc, gold,
Figure 1.4: The chemical structure of various ligands used to target metallo-drugs to DNA. Ligand linkers include (a) imidazole, (b) pyrrole, (c) β-alanine and (d) γ-aminobutyric acid (L₄).

Figure 1.5: Ligand and chemical structure for 56MESS. Ligand linkers (a) phen and (b) DACH are covalently bound to a Pt centre to form (c) 56MESS.

Figure 1.6: Chemical structure of [Cu(phen)₂]^{2⁺}.
ligands exhibit DNA exonuclease cleavage in the presence of H$_2$O$_2$ (Marshall et al., 1981) in addition to the phen ligand mediated $\pi$-$\pi$ base stacking (Lu et al., 2003a). The exact mechanism behind the copper(II)-mediated nuclease activity has not been entirely elucidated. However, it is believed that a [Cu(phen)$_2$]$^{2+}$ (Figure 1.6) is reduced to [Cu(phen)$_2$]$^{+}$ which allows for non-covalent binding to DNA in the minor groove then subsequently H$_2$O$_2$ oxidises [Cu(phen)$_2$]$^{+}$ to facilitate an oxidative attack on the deoxyribose backbone to facilitate DNA cleavage (Bales et al., 2002, Pitié et al., 2000).

1.6 The aims of this thesis

The aims of this thesis are to employ molecular biology tools to assess the DNA binding properties to a range of novel and analogue cisplatin metallo-drugs. In the long term, it is hoped that by developing a suite of tools to assess these drugs, it would be possible to screen many novel compounds quickly and efficiently as possible chemotherapeutic agents.

1.6.1 Metallo-drug DNA binding mode and DNA structure modification

The initial aims of this thesis are to characterise the ability of the metallo-drugs to induce modification of DNA structure. The first aim sets out to identify the binding mode of various metallo-drugs with DNA by measuring DNA structure modifications of native plasmid DNA. The second aim was to measure these drug’s ability to induce structure modifications on a linear dsDNA by a novel application of Capillary Electrophoresis in the critical conditions. The third aim is to determine if these drugs were capable of forming interstrand cross links on a linear dsDNA substrate.

1.6.2 Metallo-drug induced inhibition of DNA replication and DNA binding sequence specificity

The second body of work consists of two aims which employ the LA reaction assay and fluorescent detection techniques to characterise metallo-drug activity with a DNA substrate.
The first aim is to measure and compare the metallo-drugs for their ability to disrupt DNA polymerase extension. The second aim elaborates on this to measure and compare the binding sequence of these drugs at base-pair resolution.

1.6.3 Metallo-drug thiol binding preference with GSH

The final body of work consists of two aims which set out to characterise the affinity of test metallo-drugs to preferentially form thiol bonds with glutathione (GSH). The first aim was to identify metallo-drugs that preferentially form thiol bonds with GSH. The second aim was to determine the affinity of metallo-drugs to preferentially bind to GSH sulfur donors over DNA nitrogen donors under competitive reaction conditions.
Chapter 2: Materials and Methods

2.1 General materials

2.1.1 General reagents

Tryptone powder, yeast extract powder, sodium chloride pellets, sodium hydroxide pellets, ampicillin sodium salt, glycerol, Tris.HCl powder, EDTA powder, DNase-free water, 100% DMF, bromophenol blue powder, urea pellets, glacial acetic acid, 40% polyacrylamide (acrylamide: bisacrylamide, 29:1), boric acid powder, ammonium persulfate powder, 100% TEMED, agarose type II-A powder, DTT powder, reduced glutathione (GSH) and 1 M HEPES pH 7.4 buffer were purchased from Amresco (Solon, USA).

Sodium dodecyl sulphate (SDS) powder, sodium acetate solid, potassium acetate solid, molecular grade 100% ethanol, magnesium chloride solid, ammonium acetate solid and NP-40 tergitol, boric acid and 100% DMSO were purchased from Sigma-Aldrich (Castle Hill, Australia).

Solid 6-Carboxyrhodamine-6G-succinimidyl-ester-6 (R6G) and DMSO were purchased from Sigma-Aldrich (Castle Hill, Australia). Amino-allyl dUTP was purchased from Invitrogen (Life Technologies, Mulgrave, Australia). Bicarbonate buffer (pH 8.3), glycine and Tris.HCl were purchased from Amresco, Solon, USA.

Reduced glutathione (GSH) was purchased from Sigma-Aldrich (Castle Hill, Sydney, Australia) and dissolved in DNase-free water to a final concentration of 280 mM prior to experimentation.
2.1.2 Cell culture materials

a) Mammalian cell culture

Mammalian cell lines were incubated in a HERA Cell 150 CO₂ incubator (Thermo Scientific, Scoresby, Australia) set at 37 °C with 5% (v/v) CO₂. Observations of mammalian cell quality and quantity were made using a CKX41 inverted microscope (Olympus, Macquarie Park, Australia). Aseptic cell culture practices were done in a BH-EN Class II biological safety cabinet (Glenaire, Seven Hills, Australia).

Cell culture media materials consisted of Gibco® Advanced Dulbecco’s Modified Eagle Medium (DMEM), Gibco® L-Glutamine, Gibco® Penicillin-Streptomycin, Gibco® Dulbecco’s Phosphate-Buffered Saline (DPBS, no calcium, no magnesium, pH 7.0-7.2) and Gibco® 0.25% (w/v) Trypsin with Phenol Red were purchased from Invitrogen Life Technologies (Mulgrave, Australia).

b) Bacterial cell culture

Liquid broth (LB) media contained 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) sodium chloride and was adjusted to pH 7.5 with sodium hydroxide pellets in MilliQ RO water. The suspended contents were sterilised by autoclaving at 121 °C, 105 kPa for 15 minutes. Bacterial cell cultures were incubated in an OM11 Medium Digital Orbital Mixer/Incubator set at 37 °C, rotating at 250 rpm (Ratek, Boronia, Australia).

Ampicillin sodium salt was dissolved in MilliQ RO water to a stock concentration of 100 mg/mL and sterilised with a 0.45 µm syringe filter. Bacterial cells were stored in glycerol supplemented with LB media to a final concentration of 20% (v/v) for bacterial cell freezer storage at –80 °C.
2.1.3 Genomic DNA and plasmid DNA extraction materials

a) Genomic DNA extraction from mammalian cells

Sodium dodecyl sulphate (SDS) was dissolved in RO water to make a 10% (w/v) working stock. Sodium acetate was dissolved in DNase-free water to a final concentration of 3 M and sterilised by passing though a 0.45 µm filter by syringe. TE 50/20 buffer contained 50 mM Tris.HCl and 20 mM EDTA in RO water with pH adjusted to 8.0 with sodium hydroxide and sterilised with a 0.45 µm filter and syringe.

b) Plasmid DNA extraction from bacterial cells

Solution I contained 50 mM glucose, 25 mM Tris.HCl pH 7.75, 10 mM EDTA and 5 mg/mL egg white lysozyme (Amresco, Solon, USA) in MilliQ RO water. Solution II contained 0.2 M NaOH and 1% (w/v) SDS in MilliQ RO water. Solution III contained 5 M potassium acetate adjusted to pH 4.8 with HCl in MilliQ RO water.

2.1.4 DNA purification and restriction enzyme materials

a) Enzyme-based DNA purification and restriction digest reagents

Pancreatic RNase A (Amresco, Solon, USA) was dissolved in autoclaved MilliQ RO water to a working stock concentration of 10 mg/mL. Proteinase K (Amresco, Solon, USA) was dissolved in autoclaved MilliQ RO water to a working stock concentration of 20 mg/mL. Both enzymes were stored at – 30 °C prior to experimental use. Restriction enzymes PvuII and ScaI and accompanying buffers were purchased from New England Biolabs (Genesearch, Arundel, Australia).

b) DNA purification by Phenol/Chloroform/Isoamyl alcohol (IAA)

Chloroform/IAA (24:1) and saturated phenol (pH 7.9) were purchased from Amresco (Solon, USA). Chemicals were stored at 4 °C prior to experimentation. Before experimentation, one
part chloroform/IAA was combined with one part saturated phenol solution to yield 25:24:1 ratio of phenol/chloroform/IAA.

c) **DNA purification and recovery by ethanol precipitation**

Sodium acetate was dissolved in MilliQ RO water to a final concentration of 3 M and sterilised with a 0.45 µm filter and syringe. Where indicated, 0.1 M magnesium chloride was combined with 3 M sodium acetate solution to improve DNA precipitation yield.

d) **DNA elution buffer for slab gel excision**

DNA elution buffer contained 500 mM ammonium acetate, 0.2% (w/v) SDS, 1 mM EDTA was combined with DNase-free water and stored at room temperature prior to experimentation. Buffer was sterilised by passage though a 0.45 µm filter and syringe.

e) **DNA UV absorbance qualification / quantification**

Micro-litre sampling of DNA for qualification and quantification by UV absorbance was done using an Implen Nanophotometer (Munich, Germany).

2.1.5 **PCR and LA reaction materials**

*Taq* DNA polymerase at 5 U / µL along with PCR buffer and 25 mM MgCl₂ solution were purchased from GenScript (Piscataway, USA). KAPPA2G Hot Start *Taq* DNA polymerase at 5 U / µL along with KAPPA2G Buffer A (supplemented with 1.5 mM MgCl₂) were purchased from Kappa Biosystems (Woburn, USA).

Deoxy nucleotide mixture (25 mM / base) and individual deoxy nucleotide set (100 mM / base) were purchased from Amresco (Solon, USA). Dideoxy nucleotide set of ddA, ddC, ddG and ddT bases (10 mM / base) was purchased from Bio Scientific (Gymea, Australia).

Thermocycling for PCR and LA reactions was performed using a G-Storm GS424 Thermal Cycler (Somerset, UK).
Primers used for PCR and LA reactions were purchased from Invitrogen (Life Technologies, Mulgrave, Australia). Primers were dissolved in DNase-free water to a final concentration of 100 µM and stored at –30 °C as primary stocks. Working stocks for use in reactions were prepared by dilution with DNase-free water to a final concentration of 10 µM for PCR primers and 1 µM for LA primers. Primers used for PCR reactions are shown in Table 2.1.

Table 2.1: List of primers used for PCR and LA reactions. The primer name is the conventional name of the primer referred to in the thesis text, followed by the primer extension direction (FWD = forward, REV = reverse) relative to the template DNA, the length of the primer (bp), the reaction in which the primer was used (either PCR or LA) and the primer sequence.

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<thead>
<tr>
<th>Primer Name</th>
<th>Direction</th>
<th>Length</th>
<th>Reaction</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>pUC/M13 Forward</td>
<td>FWD</td>
<td>24</td>
<td>PCR</td>
<td>5’-d(CGCCAGGGTTTTCAGCTACGAC)-3’</td>
</tr>
<tr>
<td>pUC/M13 Reverse</td>
<td>REV</td>
<td>22</td>
<td>PCR</td>
<td>5’-d(TCACACAGGAACAGCTATGAC)-3’</td>
</tr>
<tr>
<td>RUVBL2_F1</td>
<td>FWD</td>
<td>21</td>
<td>PCR</td>
<td>5’-d(CTCATTTGGAACGGGTTTG)-3’</td>
</tr>
<tr>
<td>RUVBL2_R1</td>
<td>REV</td>
<td>25</td>
<td>PCR</td>
<td>5’-d(CTAACATCAAGGCTCCAAATG)-3’</td>
</tr>
<tr>
<td>RUVBL2_F2_FAM</td>
<td>FWD</td>
<td>21</td>
<td>PCR</td>
<td>FAM-5’-d(CTCATTTGGAACGGGTTTG)-3’</td>
</tr>
<tr>
<td>Primer A 5’FAM-pUC/M13</td>
<td>REV</td>
<td>22</td>
<td>LA</td>
<td>FAM-5’-d(TCACACAGGAACAGCTATGAC)-3’</td>
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<td>(Rev)</td>
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</tr>
<tr>
<td>Primer B 5’FAM-pUC/M13</td>
<td>REV</td>
<td>17</td>
<td>LA</td>
<td>FAM-5’-d(AACAGCTATGACCATGA)-3’</td>
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<tr>
<td>(Rev)</td>
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</tbody>
</table>
2.1.6 Slab gel electrophoresis and imaging materials

a) Slab gel loading buffers

Non-denaturing loading buffer for native slab gels contained 30% (v/v) glycerol and 0.25% (w/v) bromophenol blue in DNase-free water and stored at 4 °C. Denaturing urea gel loading buffer contained 8 M urea, 1 mM Tris.HCl (pH 7.4), 0.25% (w/v) bromophenol blue and 1% (w/v) NP-40 tergitol in DNase-free water and stored at room temperature.

b) Molecular weight ladders

DNA molecular weight 100 bp & 1 kb ladders were purchased from Amresco (Solon, USA). Working stocks of both DNA molecular weight ladders were diluted 18:1:1 with non-denaturing loading buffer, DNase-free water and DNA molecular weight ladder, respectively.

c) DNA stains for UV absorbance detection

Post-electrophoresis staining for DNA detection by UV absorbance in slab gels was done using GelRed (Biotium, Hayward, USA) diluted 1/10,000 in RO water. For slab gels requiring a pre-stained ladder, water was replaced with SYBR-1 (Life Technologies, Mulgrave, Australia).

d) Horizontal native and denaturing agarose gels

TAE buffer consisted of 24.2% (w/v) Tris base, 5.72% (v/v) glacial acetic acid, 50 mM EDTA in RO water. Non-denaturing horizontal slab gels consisted of 1% (w/v) agarose with TAE running buffer. Denaturing urea horizontal slab gel and buffer consisted of 1% (w/v) agarose and 1 M urea with TAE / 1 M urea running buffer. All horizontal slab gel electrophoresis work was carried out using either Owl A3-1 Large Gel or B1 EasyCast Mini Gel Electrophoresis System from Thermo Scientific (Waltham, USA).
e) Vertical native polyacrylamide gels

TBE buffer consisted of 89 mM Tris base, 89 mM boric acid, 20 mM EDTA in RO water. Non-denaturing vertical polyacrylamide gels contained 6% (w/v) polyacrylamide (acrylamide/bisacrylamide, 29:1), 0.05% (w/v) ammonium persulfate and 0.05% (v/v) TEMED with TBE running buffer. Vertical slab gel electrophoresis was carried out using omniPAGE Vertical Electrophoresis System from Fisher Biotech (Wembley, Australia).

f) Slab gel imaging docks

Rhodamine or FAM labelled DNA was measured using UV Molecular Imager Gel Dock XR+ (Biorad, Gladesville, Australia). Fluorescence detection and quantification of labelled DNA bands in slab gels was carried out using a Fuji Film Image Quant™ LAS4000 Fluorescence Imager (GE Life Science, Rydalmere, Australia).

2.1.7 Capillary electrophoresis materials

Sodium borate buffer was prepared from 0.5 M boric acid and titrated to pH 9.20 with 10 M sodium hydroxide and diluted to desired concentrations with MilliQ water. Mobility standards consisted of ~ 2% (v/v) DMSO and 10 g/L oligoacrylates “AA4” standard solution described as a capillary electrophoresis standard with reproducible mobility signature across multiple UV absorbance wavelengths was previously described (Gaborieau et al., 2010). Capillary electrophoresis of DNA was undertaken using the Agilent 7100 CE systems (Agilent Technologies, Waldbronn, Germany). UV transparent polyimide-coated fused silica capillaries with a 50 µm internal diameter were purchased from Polymicro Technologies, (Phoenix, USA).

2.1.8 Test compounds for DNA interaction studies

Cisplatin, carboplatin, oxaliplatin, DCBP, DCRP, DACH and phleomycin were purchased from Sigma-Aldrich (Castle Hill, Sydney, Australia). 56MESS, Cu56MESS, 3478MEEN,
RuP1 and RuP2 were produced by Prof. Janice Aldrich-Wright from the University of Western Sydney, Campbelltown Campus, Sydney, Australia. Table 2.2 summarises all test compounds along with chemical structures and other chemical data.

All compounds were dissolved in 100% (v/v) DMF to a final concentration of 1 mM and stored at –30 °C prior to use. In some experiments (where indicated) cisplatin, carboplatin, oxaliplatin were dissolved in MilliQ RO water to a final concentration of 1 mM and stored at –30 °C prior to use.

**Table 2.2: List of test compounds for DNA interaction studies.** The compound conventional name is listed below, along with the compounds structure, formula weight and atomic composition and either known or proposed DNA interaction mechanism.

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>Chemical Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cisplatin</strong></td>
<td></td>
</tr>
<tr>
<td>300.05 g mol⁻¹</td>
<td></td>
</tr>
<tr>
<td>Cl₂H₆N₆Pt</td>
<td></td>
</tr>
<tr>
<td>Covalent Binder</td>
<td></td>
</tr>
<tr>
<td>cis-diaminedichloro-platinum(II)</td>
<td></td>
</tr>
<tr>
<td><strong>Carboplatin</strong></td>
<td></td>
</tr>
<tr>
<td>371.04 g mol⁻¹</td>
<td></td>
</tr>
<tr>
<td>C₆H₁₂N₂O₄Pt</td>
<td></td>
</tr>
<tr>
<td>Covalent Binder</td>
<td></td>
</tr>
<tr>
<td>cis-diammine(1,1-cyclobutanedicarboxylato)-platinum(II)</td>
<td></td>
</tr>
<tr>
<td><strong>Oxaliplatin</strong></td>
<td></td>
</tr>
<tr>
<td>397.29 g mol⁻¹</td>
<td></td>
</tr>
<tr>
<td>C₈H₁₄N₂O₄Pt</td>
<td></td>
</tr>
<tr>
<td>Covalent Binder</td>
<td></td>
</tr>
<tr>
<td>cis-diammine(1,1-cyclobutanedicarboxylato)-platinum(II)</td>
<td></td>
</tr>
<tr>
<td>Compound</td>
<td>Molecular Weight</td>
</tr>
<tr>
<td>----------</td>
<td>-----------------</td>
</tr>
<tr>
<td>DCBP</td>
<td>422.17 g·mol⁻¹</td>
</tr>
<tr>
<td>DCRP</td>
<td>424.18 g·mol⁻¹</td>
</tr>
<tr>
<td>DACH</td>
<td>380.17 g·mol⁻¹</td>
</tr>
<tr>
<td>Phleomycin</td>
<td>1427.52 g·mol⁻¹</td>
</tr>
</tbody>
</table>

**Chapter 2: Materials and Methods**
<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular Formula</th>
<th>Molecular Weight</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu56MESS</td>
<td>C_{23}H_{34}CuN_{4}</td>
<td>430.09 g/mol</td>
<td>Strand Breaker</td>
</tr>
<tr>
<td>56MESS</td>
<td>C_{23}H_{34}N_{4}Pt</td>
<td>561.62 g/mol</td>
<td>Intercalator</td>
</tr>
<tr>
<td>3478MEEN</td>
<td>C_{21}H_{32}N_{4}Pt</td>
<td>535.58 g/mol</td>
<td>Intercalator</td>
</tr>
<tr>
<td>RuP1</td>
<td>C_{63}H_{60}N_{16}O_{7}Ru</td>
<td>1254.32 g/mol</td>
<td>Groove Binder</td>
</tr>
</tbody>
</table>

Cu56MESS:
\[(\text{5,6-dimethyl-1,10-phenanthroline})(1S,2S-diaminocyclohexane)copper(II)]^{2+}\]

56MESS:
\[(\text{5,6-dimethyl-1,10-phenanthroline})(1S,2S-diaminocyclohexane)platinum(II)]^{2+}\]

3478MEEN:
\[(\text{3,4,7,8-tetramethyl-1,10-phenanthroline})(1S,2S-diaminocyclohexane)platinum(II)]^{2+}\]

RuP1:
\[\Delta \text{-[Ru(phen)_{2}(3-[(methyl-4-p-1H-imidazole-2-carbonyl)-amino]-butyrylamino]-1H-pyrrole-2-carbonyl)-amino]-propionic acid)(PF_{6})_{2}\]
2.1.9 Compound/DNA incubation buffer

An incubation buffer was prepared for the reaction of the drug with DNA. This buffer consisted of 2 mM HEPES pH 7.4 and 10 mM sodium chloride in DNase-free water. The stock buffer was sterilised with a 45 µm syringe filter and stored at room temperature prior to experimental use. Incubation of samples to 37 °C was carried out using an Accublock™ Digital Dry Bath (Labnet, Woodbridge, USA).

2.1.10 Miscellaneous hardware resources

General centrifugation of volumes up to 2 mL were carried out using an Allegra® X-12 centrifuge and high speed microfugation was completed using an Allegra® 22R microfuge (Beckman-Coulter, Gladesville, Australia). Samples were refrigerated to -80 °C using a HERAfreeze HUF T-Series Ultra-Low freezer (Thermo Scientific, Waltham, USA) and samples requiring storage in -30 °C were refrigeration using a Sanyo MDF-U5312 freezer (Panasonic, Wood Dale, USA).
2.1.11 **Miscellaneous software resources**

Plasmid and small oligo analysis was carried out using ApE v1.17 (Copyright © 2003-2008 M. Wayne Davis, University of Utah, US). Analysis of primer sequences for dimers and hairpin loops was performed using Oligo Analyser v1.1.2 (Copyright © 2000-2002 AIV Institute University of Kuopio, Finland). Primer design was carried out using FastPCR v6.0.136 beta (Kalendar *et al.*, 2011). Densitometry and mobility analysis of DNA bands in post-electrophoresis slab gels was carried out using Multi Gague v2.0 (Copyright © 2002 Fujifilm). Data analysis and generation of graphics was carried out using OriginPro v8.5 (Copyright © 2012 Origen Lab Corporation). Chemical structures were drawn using ChemDraw v10.0 (Copyright © 1986-2005 Cambridge Soft). Analysis of fluorescent capillary electrophoresis peak data was carried out using Peak Scanner v1.0 by Applied Biosystems (Mulgrave, Australia).
2.2 General Methods

2.2.1 Bacterial cell culture

*E. coli* DH5α transfected with native pUC19 and suspended in LB media containing 20% (v/v) glycerol was donated by Dr Michelle Moffitt from the University of Western Sydney, Australia. Samples were stored at –80 °C prior to experimentation.

A starter culture was prepared by aliquoting 10 mL of LB media containing 100 µg/mL ampicillin into a sterile tube, this was inoculated with 50 µL of *E. coli* DH5α transfected with native pUC19. The starter culture was incubated for 8 hours at 37 °C, rotating at 250 rpm.

Main cultures were carried out in sterile 500 mL conical flasks that contained 200 mL of LB media with 100 µg/mL ampicillin. These were inoculated with 100 µL of starter culture. Main cultures were incubated for 16 hours at 37 °C, rotating at 250 rpm.

2.2.2 Plasmid extraction from bacterial cells

Plasmid extraction was carried out by rapid alkaline lysis protocol, as previously described (Bimboim and Doly, 1979). Approximately 50 mL of bacterial cell culture was aliquoted into a sterile 50 mL tube. The cells were pelleted by centrifugation for 10 min, 4 °C at 1600 × g and the supernatant was decanted. The tube was briefly inverted to allow the pellet to drip dry.

The pellet was resuspended by vortex with 3.5 mL Solution I and incubated at room temperature for 5 minutes. Next, 7 mL of Solution II was added and mixed by inverting and tapping the tube before being incubated on ice for 10 minutes. Finally, 5.25 mL Solution III was added followed by a brief vortex and incubation on ice for 5 minutes. Cell debris was pelleted by centrifugation for 20 minutes at 1600 × g at 4 °C. The supernatant was transferred to a fresh tube and centrifugation and supernatant collection was repeated at least twice to remove any remaining cell debris. DNA (including native pUC19) contained in the
Supernatant was precipitated and pelleted by the addition of 2 volumes of 100% (v/v) ethanol, incubation at –80 °C for 1 hour and centrifugation at 3200 × g at 4 °C for 16 hours. The supernatant was decanted and the remaining pellet was washed twice with 2-5 mL of 70% (v/v) ethanol, dried and resuspended by vortex with approximately 2 mL DNase-free water.

Precipitated RNA and protein debris were enzymatically degraded in the extracted plasmid DNA by digestion with 500 ng/µL RNase A and 200 µg/ml Proteinase K, for 30 minutes at 37 °C. Residual proteins fragments and other cellular debris were removed by phenol/chloroform/isoamylalcohol (25:24:1) extraction.

2.2.3 Mammalian cell culture

Human embryonic kidney cells (HEK293) were donated by Dr Sabine C. Piller from the University of Western Sydney. Cells were seeded in Gibco® Advanced DMEM supplemented with Gibco® L-Glutamine and Gibco® Penicillin-Streptomycin to a final volume of 5 mL in a T25 flask and incubated at 37 °C, 5% (v/v) CO2 until confluence.

Cell line maintenance was carried out by removal of liquid media, addition of 4 mL Gibco® 0.25% (w/v) Trypsin and 4 mL Gibco® Advanced DMEM. The flask was tapped to encourage cell dislodging and incubated at 37 °C for 2-5 minutes until cells lost adherence, as observed by inverted microscope. Suspended cells were collected in a 15 mL centrifuge tube and pelleted by centrifugation for 5 min, 4 °C at 1600 × g. Supernatant was removed and cells were washed twice in Gibco® Dulbecco’s PBS and then resuspended in 5 mL Gibco® Advanced DMEM supplemented.

Cell counts and viability were quantified using a haemocytometer and subsequent passage was established with a starting cell concentration of approximately 2×10^5 cells/mL in 5 mL culture.
**2.2.4 Genomic DNA extract from mammalian cells**

HEK293 cells were collected and washed twice in Gibco® Dulbecco’s PBS, suspended in TE (50/20) pH 8.0 buffer and the cell concentration was quantified using a haemocytometer. The cells were stored at –30 °C prior to experimental use. Genomic DNA was extracted as previously described (Gilbert and Vance, 2001). The cells were lysed in 0.2% (w/v) SDS and passed through a 23 gauge needle twice. RNA and proteins were removed by digestion with 20 µg/ml RNase and 200 µg/ml Proteinase K and incubation at 37 °C for 3 hours. Genomic DNA samples were further purified by phenol/chloroform/isoamylalcohol extraction and concentrated by ethanol precipitation.

**2.2.5 Concentration of DNA by ethanol precipitation**

DNA was concentrated by ethanol precipitation with the addition of 0.1 volume 3 M NaOAc, then mixed by vortex, followed by the addition of 2 volumes 100% (v/v) ethanol, mixed by inversion and then incubated at –80 °C for 1 hour. DNA was pelleted by centrifugation at 16000 × g for 15 minutes. Pellets were washed twice in 70% (v/v) ethanol, dried and resuspended in DNase-free water. In some experiments, 3 M NaOAc was supplemented with 0.1 M MgCl₂ to improve precipitation yield.

**2.2.6 Restriction digest of pUC19 DNA**

pUC19 was linearised by restriction digest with ScaI or PvuII restriction enzyme. Reactions consisted of 1 U enzyme per 1 µg of DNA in NEBuffer4 in 50 µL total volume then incubated at 37 °C overnight. Confirmation of successful restriction digest was determined by electrophoresis of 15 µg pUC19 DNA by 1% (w/v) agarose horizontal slab gel.

**2.2.7 PCR amplification of pUC19**

PCR amplification of a 135 bp DNA fragment from the pUC19 plasmid DNA template was used to confirm the identity of the isolated plasmid. PCR reactions consisted of a total volume
of 20 µL which contained 50 pg of plasmid DNA template, 4 pmol of each pUC/M13 forward (24mer) and pUC/M13 reverse (22mer) sequencing primers, 1.5 mM MgCl$_2$, 0.2 mM of each dNTP, 1 U Taq DNA Polymerase and PCR buffer. Thermocycling conditions involved initial denaturation at 95 °C for 5 minutes then 25 cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s, then a final extension of 72 °C for 60 s. Size determination of the PCR reaction product was carried out by subjecting an aliquot of the PCR reaction to electrophoresis on a 1% (w/v) agarose gel.

2.2.8 Generation of DNA templates by PCR

Synthetic dsDNA template was generated for CE-CC drug/DNA binding studies. Multiple replicate PCR samples were pooled together and concentrated by ethanol precipitation and quantified/qualified by visualisation on 1% (w/v) agarose gel electrophoresis and nanophotometer. A 20 µL reaction PCR contained 100 ng gDNA, 0.25 µM of both RUVBL2_F1 (21 mer) forward and RUVBL2_R1 (25 mer) reverse primers, 0.2 mM of each dNTP, 1.5 mM MgCl$_2$, 1 U Taq DNA polymerase and PCR buffer. Thermocycling consisted of initial denaturation of 95 °C for 5 minutes, then 30 rounds of denaturing at 95 °C for 60 s, annealing 54.8 °C for 30 s and extension at 72 °C for 60 s, followed by final extension 72 °C for 60 s.

Synthetic 5′FAM-dsDNA template was generated for ICL drug/DNA binding studies by substituting the forward primer with a 5′FAM labelled primer, RUBL2_F2_FAM. PCR thermocycling consisted of 30 rounds of denaturing at 95 °C for 60 s, annealing 56.4 °C for 30 s and extension at 72 °C for 60 s.

2.2.9 Purification of 5′FAM DNA template by slab gel excision

A 6% (w/v) Polyacrylamide slab gel was prepared on a omniPAGE Vertical Electrophoresis System. The 5′FAM-dsDNA template was combined in a 1:5 ratio of non-denaturing loading buffer, loaded into multiple wells (to capacity) and submitted to electrophoresis at 40 V/cm.
The slab gel was visualised under UV light and all bands running at approximately 865 bp were manually excised and collected.

Slab gel pieces were crushed by pipette tip and resuspended in DNA elution buffer to a ratio of 0.5 mL gel to 2 mL buffer. Tubes were incubated overnight at 37 °C in the dark. Gel was pelleted by microfuge centrifugation at 14,000 × g, 10 minutes at room temperature and supernatant collected.

The gel pellet was resuspended in DNA elution buffer, re-pelleted again by microfuge and the supernatant again collected. Supernatant samples were combined, ethanol precipitated (with supplemented 0.1 M MgCl₂) and resuspended in DNase-free water. Samples were quantified by visualisation on 1% (w/v) agarose gel and nanophotometer.

2.2.10 Quantification and qualification of DNA by UV spectrometry

Quantification of dsDNA concentration was performed using an Implen Nanophotometer by measuring UV absorption at 260 nm. Purity of dsDNA and protein contamination was determined by measuring the 260/280 nm ratio with an acceptable value being ~1.8. Contamination of EDTA, phenol and carbohydrates in dsDNA sample was assessed by the UV absorption 260/230 nm ratio with an acceptable value between 2.0-2.2.

2.2.11 Incubation reactions with test compounds and DNA

The type and quantity of DNA in the incubation reactions varied depending on the individual experiments, and amount of DNA used per reaction is summarised in Table 2.3. Test compounds were dissolved in 100% (v/v) DMF and incubated with DNA at final drug concentrations of 0.015, 0.05, 0.15, 0.5, 1.5, 5, 15 and 50 µM and up to 150 µM in some instances to better visualise trends within the data.

Drug/DNA incubation reactions were made up to a final volume of 40 µL with DNase-free water. Phleomycin was incubated in the presence of 25 mM DTT to facilitate strand breaking.
Table 2.3: Summary table showing the DNA type and quantity used for each drug/DNA reaction. Experiments that use DNA are listed with reference to the results section where the DNA being tested was analysed. The type of DNA used in the experiment is listed along with reference to the relevant method Section that describes how the DNA was sourced or generated. The quantity of DNA is described for each compound incubation (a.k.a. damaging) reaction. Entries with “N/A” indicate that DNA was not used in that experiment.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Method Sections</th>
<th>Results Sections</th>
<th>DNA Type</th>
<th>DNA Quantity per Reaction (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Measuring the metallo-drug induced structure modifications of native plasmid DNA</td>
<td>2.2.2</td>
<td>3.2</td>
<td>Native pUC19 from Alkaline Lysis</td>
<td>15</td>
</tr>
<tr>
<td>Detecting metallo-drug induced DNA structure modifications by capillary electrophoresis</td>
<td>2.2.8</td>
<td>4.2</td>
<td>DNA Template from PCR (RUHBL2)</td>
<td>15</td>
</tr>
<tr>
<td>Detecting metallo-drug induced interstrand crosslinks by denaturing slab gel electrophoresis</td>
<td>2.2.9</td>
<td>5.2</td>
<td>5′FAM DNA Template from PCR (RUVBL2)</td>
<td>0.3</td>
</tr>
<tr>
<td>Characterising metallo-drug induced inhibition of DNA replication by Linear Amplification with Rhodamine-labelled dUTP detection</td>
<td>2.2.2 2.2.6</td>
<td>6.2.1</td>
<td>pUC19 with Scal Restriction Digest</td>
<td>1.8</td>
</tr>
<tr>
<td>Characterising the DNA sequence specificity of metallo-drugs by Linear Amplification</td>
<td>2.2.2 2.2.6</td>
<td>6.3.1</td>
<td>pUC19 with PuvII Restriction Digest</td>
<td>4.73</td>
</tr>
<tr>
<td>Investigating the ability of cisplatin analogues and other novel metallo-drugs to form thiol bonds with glutathione</td>
<td>N/A</td>
<td>7.2.1</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Competition assay for metallo-drug binding between GSH and DNA</td>
<td>2.2.9 2.2.13</td>
<td>7.3.1</td>
<td>5′FAM DNA Template from PCR</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Note: DNA quantities above are for drug/DNA reactions. DNA template quantities for subsequent Taq DNA Polymerase experiments are described in sections 2.2.15 to 2.2.16.
Reactions were incubated overnight (~14 hours), at 37 °C in the dark, then terminated by ethanol precipitation and resuspended in 20 µL DNase-free water.

### 2.2.12 Kinetic reactions with test compounds and GSH

Reactions contained 180 mM of GSH and 360 µM of test compound to produce a drug to GSH ratio of 1:500 and drug/DNA incubation buffer to a final volume of 140 µL with DNase-free water. Reactions with phleomycin contained 25 mM DTT to replicate the reaction conditions that would normally facilitate DNA strand breaking.

The formation of thiol-bonds was measured by UV absorbance at 280 nm with an Implen Nanophotometer over a time course extending to 48 hours to ensure reaction completion. Buffer at zero time point was measured in the absence of GSH as a blank for this experiment. Reactions were replicated in triplicate and graphs were analysed by Hill non-linear regression.

### 2.2.13 Competition reactions with test compounds, GSH and 5'FAM DNA template

Reactions were set up as described in Section 2.2.11. DNA and Test compound concentrations were held constant in these reactions. Test compounds consisted of cisplatin (5 µM), oxaliplatin (50 µM), DCBP (1.5 µM), DCRP (15 µM), DACH (1.5 µM) and 3478MEEN (150 µM). GSH was the variable in this reaction and consisted of final concentrations of 0.005, 0.015, 0.05, 0.15, 0.5, 1.5, 5, 15 and 50 mM.

### 2.2.14 Coupling reaction of Rhodamine 6G with Amino-Allyl dUTP

The coupling reaction of Rhodamine-6G with amino-allyl dUTP was followed as described in Henegariu et al. (2000). The 6-Carboxyrhodamine-6G-succinimidyl-ester-6 (R6G) was dissolved in 100% (v/v) DMSO to make a final concentration of 40 mM. Amino-allyl dUTP (AA-dUTP) was dissolved in 0.2 M bicarbonate buffer (pH 8.3) to make a final concentration of 20 mM (Figure 2.1).
The coupling reaction was initiated with the addition of 1 volume 20 mM AA-dUTP, 1 volume 0.2 M bicarbonate buffer (pH 8.3), 1.5 volumes MilliQ RO water and 0.5 volumes 40 mM R6G, respectively. Reaction was mixed well by pipette and allowed to incubate in the dark for 3-4 hours at room temperature. The reaction was terminated with the addition of 2 volumes of 200 mM glycine (pH 8.0), 2 volumes 200 mM Tris.HCl (pH 7.75) and 12 volumes MilliQ RO water, to adjust the final nucleotide concentration to 1 mM. Samples were aliquoted and stored at –30 °C prior to experimentation.

Determination of coupling efficiency was undertaken by running equimolar amounts of unreacted R6G stock against the R6GdUTP reaction product sample and loaded in a 1:5 ratio of non-denaturing loading buffer on a 1% agarose horizontal slab gel. Gels were then imaged with LAS4000 fluorescence imager (GE Healthcare, Rydalmere, Australia) and analysed using Multi Gague v3.0.
Figure 2.1 Schematic diagram of the chemical structures for 5-carboxyrhodamine 6G coupling reaction with AminoAllyl-dUTP to form R6GdUTP.

The reaction schematic depicts the amine reactive 5-carboxyrhodamine 6G (▬) and AAdUTP (▬) with the AminoAllyl linker highlighted (▬) and the post coupling reaction in 0.2 M bicarbonate buffer at pH 8.3, 25 °C (approximate room temperature) in the dark to produce 5-carboxyrhodamine-6G-aminoallyl-dUTP (R6GdUTP).
2.2.15  **Linear amplification reactions with R6GdUTP**

Linear amplification reactions of 20 µL contained 50 ng of drug treated pUC19 (*ScaI* restriction digested) DNA template (control reactions contained untreated DNA), 4 pmol pUC/M13 (22 mer) reverse sequencing primer, 1 U KAPPA2G Hot Start *Taq* DNA Polymerase, KAPPA2G Buffer A (with 1.5 mM MgCl₂), 200 µM per d(A,C,G)TP, 85 µM dTTP and 15 µM R6GdUTP in DNase-free water.

Thermocycling involved an initial denaturing step at 95 °C for 60 s, then 45 cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 60 s. Post reaction cleanup involved ethanol precipitation and the pellet was re-suspended in 5 µL DNase-free water. Samples were then combined in a 1:5 ratio with non-denaturing loading buffer and subjected to electrophoresis on a native 1% (w/v) agarose slab gel at 5 V/cm with duplicate 15 µL loading per run. Gels were then imaged with LAS4000 fluorescence imager and fluorescence data was generated using Multi Gauge v3.0.

2.2.16  **Linear amplification reactions with 5’FAM labelled primers**

Linear amplification reactions of 20 µL contained 480 ng of drug treated pUC19 (*PuvII* restriction digested) DNA template (control reactions contained untreated DNA), 2 pmol of either a 5’FAM-pUC/M13(Rev) Primer A (22 mer) or 5’FAM-pUC/M13(Rev) Primer B (17 mer), 1 U *Taq* DNA Polymerase, 6.7 mM MgCl₂, 50 µM per dNTP and PCR buffer in DNase-free water. Sequencing reactions using the Sanger chain termination method were supplemented with 1 mM/base ddATP, 0.5 mM/base ddCTP, 0.5 mM/base ddGTP or 1.75 mM/base ddTTP and 480 µg of untreated pUC19 (*PuvII* restriction digested) DNA template.

Thermocycling involved an initial denaturing step at 95 °C for 60 s, then 20 cycles of 95 °C for 45 s, 55 °C for 60 s and 72 °C for 60 s. Ethanol precipitation was applied for post reaction cleanup including two rounds of pellet washes with 70% (v/v) ethanol. The pellet was allowed to dry after the final wash. Samples were submitted to University of New South
Wales Ramaciotti Centre for Gene Functional Analysis (Sydney, Australia) where 5′FAM-labelled ssDNA fragments were resuspended in HiDi formamide (Applied Biosystems) and spiked with LIZ500 size standard marker (Applied Biosystems), denatured at 95 °C for 5 minutes and then injected into an AB3750 capillary electrophoresis for separation.

2.2.17 Electrophoresis of DNA by horizontal agarose slab gel

The materials used for horizontal agarose gel electrophoresis are described in Section 2.1.6. DNA samples and either a 100 bp or 1 kb DNA molecular weight ladder were combined in a 1:5 ratio with non-denaturing loading buffer. The slab gel contained 1% (w/v) agarose and TAE buffer (pH 8.0) was cast using Owl A3-1 Large Gel or B1 EasyCast Mini Gel Electrophoresis System. The casted slab gel was then submerged in TAE Buffer (pH 8.0) and the DNA sample was electrophorised at 5 V/cm. Electrophoresis was terminated when the bromophenol blue indicator front migrated to approximately 1 cm from the end of the gel. After electrophoresis, the slab gel was stained with 1 x GelRed for 30 minutes, briefly rinsed with MilliQ RO water and imaged with UV Molecular Imager Gel Dock XR+. DNA bands were quantified by UV or fluorescence absorbance and subsequent data was normalised and measured against the zero control condition.

2.2.18 Denaturing urea / agarose slab gel electrophoresis

Denaturing agarose slab gels containing urea were prepared as described in (Hegedus et al., 2009). The dsDNA controls were not subjected to heat denaturation prior to electrophoresis. A 1% agarose / TAE / 1 M urea slab gel was cast and transferred to an electrophoresis tank filled with TAE / 1 M urea running buffer. Samples were loaded along with a SYBR1-labelled 100 bp ladder and separated at 2.5 V/cm for 7.5 hours in a 4 °C cold room with the lights off. Slab gels were rinsed with double distilled water and imaged by LAS4000 fluorescence imager. Fluorescence absorbance data was generated with Multi Gague v3.0 and analysed with OriginPro v8.0. DNA bands were quantified by UV or fluorescence absorbance
and subsequent data was normalised and measured against the zero control condition. Denaturing slab gel data where the zero control conditions did not exhibit complete dsDNA melting were recalculated to by subtracting the undenatured (dsDNA) fluorescent value from all other conditions.

2.2.19 Free solution capillary electrophoresis

The materials used for horizontal agarose gel electrophoresis are described in Section 2.1.7. Sodium borate buffer (25 mM) was prepared from 0.5 M boric acid, titrated to pH 9.20 with 10 M sodium hydroxide and diluted to 25 mM. The buffer was sonicated 5 min before use and filtered through 0.22 micron polytetrafluoroethylene filter. A 10 g/L AA4 standard solution was prepared by dissolving it in water followed by sonication for 1 min.

Capillary electrophoresis of DNA was completed using the Agilent 7100 CE systems (Agilent Technologies, Germany). The diode array detector was set to detect UV absorbances at 260 nm for DNA and 200 nm for the AA4MQ standard and DMSO electro-osmotic flow (eof) standard. UV transparent polyimide-coated fused silica capillaries with a 50 µm internal diameter and cut to a total length of either 100 cm (91.5 cm effective length) or 64.5 cm (56 cm effective length) were purchased from Polymicro Technologies, Phoenix, USA.

The capillary temperature was thermostatted at 25 ºC, separation voltage was set at maximum (30,000 V) and injections were hydrodynamic with pressure at 30 mbar for 10 s on the vial containing the sample at the capillary end. The capillary was pre-treated by flushing for 10 minutes with 1 M NaOH and then for 5 minutes with 0.1 M NaOH. The capillary was then conditioned with water and sodium borate buffer (concentration varied depended on experimental conditions) for 5 min prior to sample injection.

All reaction samples were subjected to duplicate capillary electrophoresis injections with UV absorbance measurements over 25 minutes. CE separation optimisation of pUC19 plasmid DNA in the critical conditions was carried out in the presence of sodium borate buffer.
concentrations of 25, 50, 300 and 500 mM. CE of PCR generated dsDNA template treated with test metallo-drugs, ethidium bromide and Hoechst33258 was undertaken in 25 mM sodium borate to facilitate critical condition separation.

Analysis of electropherogram data was carried out using OriginPro v8.5. To account for electro-osmotic drift between individual CE runs, time (min) was converted to mobility (m².V⁻¹.s⁻¹) using the equation below. The DMSO electro-osmotic flow ($eof$, min), length from the capillary end to UV detector window ($\ell_1$, cm), length of the entire capillary ($\ell_0$, cm) and the number of volts applied during electrophoresis ($V$) were used in the equation below.

$$ Mobility = \ell_1 \cdot \ell_0 \cdot \frac{1}{V} \cdot \left( \frac{1}{eof} - \frac{1}{Time} \right) \cdot \frac{1}{60} \quad (2.1) $$
Chapter 3: Measuring the metallo-drug induced structure modifications of native plasmid DNA

3.1 Introduction

Native plasmid DNA exists in a variety of conformational states when extracted from *E. coli* cells by alkaline lysis (Bimboim and Doly, 1979, Silva *et al.*, 2012). Furthermore, plasmid DNA has routinely been employed to characterise metallo-drug induced DNA structure modifications. Cisplatin adducts are known to induce localised DNA bending and unwinding as measured by gel electrophoresis using closed circular supercoiled plasmid DNA (Stehlikova *et al.*, 2002, Keck and Lippard, 1992). This approach has also been used to characterise DNA structural modifications induced by novel platinum(II) metallo-intercalators (Krause-Heuer *et al.*, 2009a). Furthermore, DNA structure studies with ruthenium(II) based metallo-drugs have considered the structural modification of native plasmid conformations by DNA cleavage (Otero *et al.*, 2007).

The first approach taken to differentiate and compare several classes of metallo-drugs for DNA binding and structure modification was to assess their efficacy at modifying a variety of DNA conformational states. Work presented in this chapter focuses on the aims outlined in Section 1.6.1 which characterise test metallo-drug’s DNA binding mode and affinity to modify DNA structure. Specifically, the detection of metallo-drug induced variations in plasmid structure was confirmed by monitoring DNA mobility changes with slab gel electrophoresis.
3.2 Results

This study characterises broad changes to native pUC19 DNA structure by measuring changes in plasmid mobility upon treatment with several classes of metallo-drugs. Native pUC19 plasmid DNA typically consists of three bands when subjected to horizontal native slab gel electrophoresis that correspond to nicked, linear and supercoiled plasmid conformations. Metallo-drug induced DNA structure modifications were monitored for changes in DNA mobility which is indicative of relaxation or condensation of the double helix structure.

3.2.1 Purification of pUC19 template DNA

*E. coli* DH5α transfected with native pUC19 was incubated overnight at 37 °C in 50 mL of LB media until the culture reached confluence (Section 2.2.1). Native pUC19 was isolated by alkaline lysis (Section 2.2.2), concentrated by ethanol precipitation (Section 2.2.5) and the absorption of the solution was qualified/quantified by UV nanophotometer (Section 2.2.10).

Native pUC19 was extracted from 50 mL cultures of *E. coli* DH5α with an average yield of 1175 µg. Electrophoresis of native pUC19 using horizontal gel electrophoresis with a 1 kb ladder ($r^2 = 0.991$) that yielded three distinct bands with average apparent molecular weights of 1856, 2551 and 2929 bp ([Figure 3.1 a, Table A1](#)). The native conformation of these three bands was determined by comparing their apparent molecular weight and relative migration pattern to previously published observations and it was concluded to the plasmid conformations present were supercoiled, linear and nicked, respectively (Hamilton and Wilker, 2004, Dixit *et al.*, 2010).

Densitometry was performed on these three bands by analysis of the area under the line which revealed that native pUC19 consisted of approximately 39% supercoiled, 33% linear and 28% nicked conformations ([Figure 3.1 b, Table A2](#)). Further confirmation of the identity of the pDNA as pUC19 was performed by restriction digest with ScaI which yielded one band with
an apparent molecular weight of 2740 bp, as determined by horizontal gel electrophoresis (Figure 3.1 c) against a 100 bp ladder ($r^2 = 0.997$). Finally, the 139 bp naked multiple cloning region of pUC19 was amplified by PCR and subjected to horizontal gel electrophoresis (Figure 3.1 d) against a 100 bp ladder ($r^2 = 0.997$) which yielded a DNA product with the expected molecular weight of approximately 141 bp.

3.2.2 Analysis of native horizontal slab gels for metallo-drug induced changes in plasmid DNA mobility

This section analyses DNA band migration of the three native pUC19 conformations treated with metallo-drugs on native agarose horizontal slab gel. For these treated samples, the apparent molecular weight of the supercoiled, linear and nicked pUC19 conformations was measured against a 1 kb ladder and plotted on semi-log graph against the metallo-drug treatment concentration gradient. The quality of the DNA band intensity and apparent changes in DNA molecular weight over metallo-drug treatment gradient for all three pUC19 conformations is discussed. Drug/DNA incubation reactions were performed in triplicate as described in Section 2.2.11. Each reaction contained 15 µg native pUC19 and the metallo-drug being tested. Control reactions consisted of water and 5% DMF solvent. Phleomycin reaction controls consisted of a water control and a 25 mM DTT with 5% DMF solvent control.

DNA pellets were resuspended to a final concentration of 500 ng/µL in DNase-free water. Triplicate 5 µg DNA sampling from all reactions were run on horizontal agarose electrophoresis against a 1 kb ladder. Gels were post-stained with gel red and imaged by UV fluorescence. Typically, the gel electrophoresis presented three bands for the untreated plasmid. Drug treatments of these samples changed the appearance of these bands. All gels were analysed for changes in apparent molecular weight against replicate 1 kb ladders using Multiplex v8.0 and graphed using OriginPro v8.5 (Section 2.1.11).
Figure 3.1: Confirmation and characterisation of native pUC19. (a) 1 kb ladder (lane 1) running with native pUC19 nicked, linear and supercoiled conformations (lane 2). (b) Average composition native pUC19 containing the three plasmid conformations expressed as a percentage. (c) 100 bp ladder (lane 1) running with pUC19 restriction digested with ScaI (lane 2). (d) A 100 bp ladder (lane 1) running with a PCR product of pUC19 naked multiple cloning region (lane 2).
a) Control reactions

Untreated native pUC19 was used as a positive control for all experiments. DMF controls were included in all experiments to account for solvent-induced DNA migration changes as all compounds were dissolved in this solvent. The supercoiled, linear and nicked conformations treated with DMF yielded apparent molecular weights of 1868 ± 89 bp, 2570 ± 104 bp and 2957 ± 131 bp, respectively (Table A1). These results are consistent with untreated water controls above.

b) Covalent binding compounds

Native agarose slab gels and semi-log scatter plots of the band mobility, represented as apparent molecular weight measurements are shown in Figure 3.2 with pUC19 treated with cisplatin (a), oxaliplatin (b), DCBP (c), DCRP (d) and DACH (e). DNA band resolution and intensity appears to decrease at higher treatment concentrations. This is particularly evident for cisplatin and DACH treatments greater than 0.15 µM and to a lesser extent for oxaliplatin and DCRP treatments greater than 0.5 µM.

The overall trend seen with the covalent binders is that the two higher molecular weight bands converge into a single band as the metallo-drug concentration increases. The two higher molecular weight bands are thought to represent nicked and linear plasmid conformations of pUC19. Typically, the change in migration of these two bands occurs in tandem. That is, the nicked and linear plasmid DNA bands merge towards each other with a similar profile and the merged band occurs at the mid-point between the two bands in the untreated sample.

The nicked plasmid band resolution reached zero detection with cisplatin and DACH treatments greater than 1.5 µM and band apparent molecular weights decreased by 113 ± 46 bp and 68.5 ± 4 bp, respectively. To a lesser extent, the nicked plasmid DNA band intensity reached zero detection with oxaliplatin, DCBP and DCRP treatments greater than 15 µM (Figure 3.2 b-d, lanes 11 & 11'). The apparent molecular weight of nicked plasmid DNA
bands treated with oxaliplatin, DCBP and DCRP decreased by $107 \pm 85 \text{ bp}$, $256 \pm 127 \text{ bp}$ and $66 \pm 23 \text{ bp}$, respectively.

The linear plasmid DNA bands of native pUC19 treated with covalent binder compounds increased in apparent molecular weight and band intensity was detectable across all treatment conditions. Treatments with DACH (Figure 3.2 e) yielded the biggest increase of $322 \pm 77 \text{ bp}$. Treatments with cisplatin, oxaliplatin DCBP and DCRP (Figure 3.2 a-d) also yielded apparent molecular weight increases of $151 \pm 80 \text{ bp}$, $137 \pm 67 \text{ bp}$, $86 \pm 48 \text{ bp}$ and $160 \pm 16 \text{ bp}$, respectively.

The supercoiled plasmid DNA bands were detected in all covalent binder metallo-drug treatment conditions except cisplatin and DACH treatments greater than 15 µM (Figure 3.2 a&e, lanes 11 & 11'). There was a minimal increase in supercoiled plasmid DNA band apparent molecular weight in all conditions with DACH showing the largest increase of $139 \pm 93 \text{ bp}$. The supercoiled plasmid DNA treated with DCBP at 50 µM showed a band shift at $1810 \pm 11 \text{ bp}$ tending towards a possible convergence with the linear monomer band at $2634 \pm 28 \text{ bp}$ if higher metallo-drug treatments were tested.

c) Strand breaking compounds

With respect to the phleomycin treated sample (Figure 3.3 a), the nicked plasmid conformation DNA band decreased in apparent molecular weight by $264 \pm 12 \text{ bp}$. Detection ceased with treatments greater than 15 µM as it converged with the linear monomer to form one detectable band. The linear plasmid DNA band was detectable in all phleomycin treatment conditions and showed an increase in apparent molecular weight of $159 \pm 20 \text{ bp}$. The supercoiled plasmid DNA band was not detectable with phleomycin treatments greater than 1.5 µM. The supercoiled plasmid conformation DNA band also showed a slight increase in apparent molecular weight by $19 \pm 3 \text{ bp}$. With respect to Cu56MESS (Figure 3.3 b), minimal increases in apparent molecular weight with the nicked conformation increasing of
40 ± 13 bp, linear conformation of 31 ± 8 bp and supercoiled conformation of 28 ± 4 bp were observed.

d) **Intercalating compounds**

The results of the pUC19 treated with 56MESS (a) and 3478MEEN (b) are shown in Figure 3.4. DNA bands for all treatment conditions were detectable. Each of the three conformations of pUC19 displayed an increase in apparent molecular weight for each metallo-intercalator compound tested. Plasmid conformations treated with 56MESS showed the greatest increase in apparent molecular weight with the nicked conformation increasing by 1307 ± 109 bp, the supercoiled conformation increasing by 1024 ± 15 bp and the linear conformation increasing by 1003 ± 79 bp. Treatments with 3478MEEN showed increases in apparent molecular weights for supercoiled conformation of 523 ± 37 bp, linear conformation of 480 ± 99 bp and nicked conformation of 317 ± 119 bp.

e) **Groove binding compounds**

Figure 3.5 shows agarose slab gels and translated semi-log scatter plots with apparent molecular weight measurements for the three plasmid conformations of pUC19 treated with RuP1 (a) and RuP2 (b). The apparent molecular weight of all pUC19 conformations increased with both groove binder metallo-drug treatments. The DNA bands on native agarose slab gels were detectable for all treatment conditions except supercoiled and linear plasmid conformations treated with RuP2 above 15 µM.

Treatments with RuP2 produced marginally larger increases in each plasmid conformation’s apparent molecular weight when compared to RuP1. The nicked conformation for RuP2 treatment increased by 360 ± 119 bp compared to RuP1 treatment which increased by 266 ± 38 bp. The linear and supercoiled plasmid conformations showed the same trend with RuP2 treatment increasing the apparent molecular weight by 295 ± 10 bp and 207 ± 80 bp compared to RuP1 treatment with an increase of 280 ± 13 bp and 196 ± 17 bp, respectively.
Chapter 3: Metallo-drug induced plasmid structure modifications

- **Cisplatin**
  - Samples a) through e) show gel electrophoresis results with different concentrations of Cisplatin under various conditions.

- **Oxaliplatin**
  - Similar gel patterns and concentration gradients as Cisplatin.

- **DCBP**
  - Gel patterns with concentration gradients.

- **DCRP**
  - Gel patterns with concentration gradients.

- **DACH**
  - Gel patterns with concentration gradients.

The graphs on the right side illustrate the base pair (bp) versus concentration measurements for each compound, showing the effects of concentration on the plasmid structure modifications.
Figure 3.2: Images of agarose horizontal slab gels and apparent molecular weight semi-log scatter plots with linear regressions for the three plasmid conformations of pUC19 treated with covalent binding metallo-drugs. Shown in this figure are native pUC19 samples treated with (a) cisplatin, (b) oxaliplatin, (c) DCBP, (d) DCRP and (e) DACH at 0.015, 0.05, 0.15, 0.5, 1.5, 5, 15 and 50 µM (lanes 5→12 & 5′→12′) that were run on 1% native agarose horizontal slab gels. Each slab gel shows duplicate loading (inverted directions) of all samples, a 1 kb ladder (lanes 2 & 2′) for apparent molecular weight measurements and water (lanes 1 & 1′) and DMF controls (lanes 4 & 4′) that show three DNA bands that correspond to the three native pUC19 conformations. Also shown are semi-log plots of the apparent molecular weight measurements for covalent binder metallo-drug treated nicked (▬), linear (▬) and supercoiled (▬) plasmid conformations with linear regression lines of best fit.
Figure 3.3: Images of agarose horizontal slab gels and apparent molecular weight semi-log scatter plots with linear regressions for the three plasmid conformations of pUC19 treated with DNA strand breaker compounds. Shown in this figure are native pUC19 samples treated with the DNA strand breaker (a) phleomycin and (b) Cu56MESS. Other details are described in the legend of Figure 3.2.
Figure 3.4: Images of agarose horizontal slab gels and apparent molecular weight semi-log scatter plots with linear regressions for the three plasmid conformations of pUC19 treated with metallo-intercalator drugs. Shown in this figure are native pUC19 samples treated with (a) 56MESS and (b) 3478MEEN. Other details are described in the legend of Figure 3.2.
Figure 3.5: Images of agarose horizontal slab gels and apparent molecular weight semi-log scatter plots with linear regressions for the three plasmid conformations of pUC19 treated with metallo-drug DNA groove binders. Shown in this figure are native pUC19 samples treated with (a) RuP1 and (b) RuP2. Other details are described in the legend of Figure 3.2.
3.2.3 Analysis of linear regression gradients to summarise the effect of metallo-drug/DNA interactions on plasmid conformations

This section analyses test metallo-drugs for the indication of structural changes to the three plasmid conformations of pUC19. Metallo-drug induced changes in the apparent molecular weight of the three plasmid conformations were determined by linear regression. By determining positive or negative changes in apparent molecular weight as a result of increasing drug concentration, it is possible to characterise expansion or condensation, respectively, of native plasmid structure.

Linear fit gradients were applied to data sets resulting from change in migration due to an increasing drug treatment, these are summarised in Table 3.1. This table summarises the linear fit gradients (bp/µM) with standard deviation (StdDev), linear regression coefficient ($r^2$) and any terminal data points (µM) for all test compounds. The analysis was performed for the nicked, linear and supercoiled plasmid conformations. Data presented as a vertical bar graph (Figure 3.6) compares all three plasmid conformations with the various test compound treatments summarised in Table 3.1.

56MESS treatments gave rise to the greatest positive gradients for each of the three plasmid conformations yielding $23.47 \pm 4.711$ for nicked conformation, $14.42 \pm 4.321$ for linear conformation and $18.76 \pm 1.529$ for the supercoiled conformation. The nicked conformation treated with the covalent binder or strand breaking compounds induced a negative gradient, where the greatest change was observed for phleomycin treatment at $-15.37 \pm 2.893$. All other metallo-drug treatments induced positive gradients for the linear and supercoiled plasmid conformations.

The covalent binder metallo-drug treatments induced a distinct gradient pattern where nicked plasmid conformation gradients were all negative and linear and supercoiled conformations had positive gradients. DCBP treatment induced the greatest negative nicked conformation
gradient of -9.17 ± 2.341. However, DACH treatment induced the most positive gradient values for linear conformation at 4.58 ± 0.699 and supercoiled conformation at 10.50 ± 0.502.

The DNA strand breaker phleomycin induced the same gradient trend pattern as the covalent binder metallo-drugs. However, phleomycin exhibited a greater change in gradient with nicked and supercoiled conformations at -15.37 ± 2.893 and 1.00 ± 5.201, respectively. The linear conformation yielded a gradient of 3.03 ± 0.853 which is similar to any covalent binder induced gradient change. On the other hand, the strand breaker Cu56MESS induced the most minimal gradient increases in the whole data set with 0.40 ± 0.253, 0.61 ± 0.144 and 0.32 ± 0.517 for nicked, linear and supercoiled conformations, respectively.

All metallo-intercalators induced positive gradients for all plasmid DNA conformations. As stated previously, 56MESS yielded the greatest positive increase in the whole data set. The DNA groove binder metallo-drugs induced positive gradient increases for all plasmid conformations. RuP2 induced the largest change in gradients for the groove binders with 9.66 ± 4.557, 20.07 ± 4.875 and 6.29 ± 3.766 for nicked, linear and supercoiled conformations, respectively. These values are not as large as those seen with metallo-intercalator treatments but are larger than the covalent binder treatments.

Detection of bands corresponding to nicked plasmid conformations could not be detected when treated with any covalent binder metallo-drugs 15 µM. This was also the case for supercoiled plasmid conformation treated with cisplatin above 15 µM, DACH above 5 µM and phleomycin above 5 µM. However, the DNA bands for all three plasmid conformations treated with all other metallo-drugs were resolvable.
Table 3.1: Summary listing the linear regression gradients and discontinuous data points for the three native pUC19 conformations treated with several categories of metallo-drugs. The linear regression gradients are expressed as a fraction of apparent molecular weight (bp) over compound concentration (µM) with their standard deviation (StdDev) and determinant coefficient ($r^2$). The last data point (µM) identifies the final compound concentration where the DNA band on slab gel was detected.

<table>
<thead>
<tr>
<th>DNA Interaction</th>
<th>Compounds</th>
<th>Nicked</th>
<th>Linear</th>
<th>Supercoiled</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Linear Fit Gradient ($\text{bp/µM}$)</td>
<td>StdDev (±)</td>
<td>$r^2$</td>
</tr>
<tr>
<td>Covalent Binders</td>
<td>Cisplatin</td>
<td>-6.053</td>
<td>7.936</td>
<td>0.950</td>
</tr>
<tr>
<td></td>
<td>Oxaliplatin</td>
<td>-6.415</td>
<td>0.773</td>
<td>0.920</td>
</tr>
<tr>
<td></td>
<td>DCBP</td>
<td>-9.178</td>
<td>2.341</td>
<td>0.719</td>
</tr>
<tr>
<td></td>
<td>DCRP</td>
<td>-4.746</td>
<td>1.388</td>
<td>0.661</td>
</tr>
<tr>
<td></td>
<td>DACH</td>
<td>-6.044</td>
<td>1.047</td>
<td>0.913</td>
</tr>
<tr>
<td>Strand Breaker</td>
<td>Phleomycin</td>
<td>-15.378</td>
<td>2.893</td>
<td>0.825</td>
</tr>
<tr>
<td></td>
<td>Cu56MESS</td>
<td>0.407</td>
<td>0.257</td>
<td>0.264</td>
</tr>
<tr>
<td>Metallo-</td>
<td>56MESS</td>
<td>23.479</td>
<td>4.711</td>
<td>0.780</td>
</tr>
<tr>
<td>intercalators</td>
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<td>5.354</td>
<td>0.409</td>
<td>0.961</td>
</tr>
<tr>
<td>Groove Binders</td>
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<td>4.939</td>
<td>0.070</td>
<td>0.999</td>
</tr>
<tr>
<td></td>
<td>RuP2</td>
<td>9.666</td>
<td>4.557</td>
<td>0.391</td>
</tr>
</tbody>
</table>
Figure 3.6: Vertical bar graph comparing the linear regression gradients of the three conformations of native pUC19 treated with several classes of metallo-drugs. Linear regression gradients of nicked (■), linear (▲) and supercoiled (▼) native pUC19 DNA conformations treated with several classes of metallo-drugs are expressed as a fraction of apparent molecular weight (bp) over compound concentration (µM). Linear regression gradients with negative or positive values indicate a collapse or expansion in DNA helical structure, respectively.
3.3 Discussion

3.3.1 Confirmation and characterisation of native pUC19 DNA

In our hands, native pUC19 was resolved into three conformational states by slab agarose gel electrophoresis (Figure 3.1 a). These were determined to be nicked circular, linear and supercoiled conformations based on relative band migration order and apparent molecular weights by comparison to previously published data (Hamilton and Wilker, 2004, Dixit et al., 2010). Densitometry analysis of the three plasmid conformation populations showed roughly 1/3 distribution with the supercoiled conformation showing a slightly higher representation (Figure 3.2 b) which differs from typical extractions of pUC series plasmids by alkaline lysis where supercoiled plasmid would typically represent between 70 to 95% of the total plasmid extract (Clemson and Kelly, 2003). This decrease in supercoiled plasmid recovery could be attributed to shearing forces encountered during the alkaline lysis procedure (Levy et al., 1999) or an artefact of E. coli metabolism (Silva et al., 2012) which contributed to the increased linear and nicked conformation populations.

Confirmation of the linear pUC19 plasmid band was established by restriction digest with Scal which collapsed all circular plasmid conformations into a single band where the molecular weight was determined against a size standard on agarose slab gel post electrophoresis (Figure 3.1 b). Additional confirmation of pUC19 plasmid DNA was carried out by PCR of the multiple cloning region naked backbone where the molecular weight of the amplified product was also measured against a size standard on agarose slab gel post electrophoresis (Figure 3.1 d). Both restriction digest and PCR molecular weight measurements were compared to published data on the pUC series vector construction (Yanisch-Perron et al., 1985) and NCBI database (Accession No: M77789) with positive confirmation with measured and expected molecular weights on both accounts.
3.3.2 Justification of data analysis methodology

Initial apparent molecular weight measurements of native pUC19 DNA treated with various metallo-drugs and subjected to electrophoresis on agarose slab gels showed unique patterns of DNA band retardation depended on the metallo-drug category and plasmid DNA conformation. For example, the DNA covalent binder metallo-drug cisplatin appeared to converge all three plasmid conformations into one predominant band with an apparent molecular weight similar to the linear conformation (Figure 3.2 a). On the other hand, the metallo-intercalator 56MESS increased the apparent molecular weight of all three pUC19 conformations in a dose-dependent manner whilst retaining separation of the three plasmid conformations (Figure 3.4 a).

Previous cisplatin analogue metallo-drug studies have established a qualitative relationship between DNA conformation and DNA mobility retardation on agarose slab gel electrophoresis in relation to the unwinding of the supercoiled plasmid conformations to reflect the nicked plasmid conformation apparent molecular weight (Baruah et al., 2002). DNA mobility retardation on agarose slab gel electrophoresis has also been associated with actual increases in DNA template apparent molecular weight due to the accumulation of metallo-drugs through π-π base stacking in a dose-dependent manner in studies with aluminium(III) (Rajendran et al., 2008) and copper(II) (Chowdhury et al., 2009) metallo-intercalators. Furthermore, a previous ruthenium(II)-based metallo-drug study with pUC19 plasmid took a quantitative approach where an increase in nicked conformational populations occurred at the expense of other conformational forms due to DNA cleaving (Patel et al., 2011).

An analysis rationale was adopted based on the observations made in our studies and those methodologies used in the literature. In this analysis, metallo-drugs were categorised into classes based on characteristic change to patterns of plasmid DNA band mobility. This was achieved by measuring the apparent molecular weight of all plasmid conformations across the treatment concentration gradient. A linear regression analysis was applied to this data to determine the
straight line gradient for each plasmid conformation (Figure 3.6). It was anticipated that this approach would differentiate metallo-drugs into classes that reflect their DNA binding mode.

3.3.3 Association of metallo-drug DNA binding modality and DNA mobility retardation patterns measured on agarose slab gel electrophoresis

Observations of agarose slab gels with native pUC19 treated with covalent binder metallo-drugs cisplatin (Figure 3.2 a) and DACH (Figure 3.2 c) showed an increase in apparent molecular weight of the supercoiled plasmid conformation coupled with the decrease and eventual convergence of the nicked plasmid conformation. These trends observed in the cisplatin and DACH treated plasmid have previously been reported and attributed to the metallo-drug induced unwinding of negatively supercoiled plasmid DNA which retards DNA migration to reflect nicked conformation migration on agarose slab gel in a dose dependent manner (Kasparkova et al., 2010). Furthermore, this trend of supercoiled and nicked conformation DNA band retardation was also observed with native pUC19 DNA treated with the covalent binder oxaliplatin, although both bands did not achieve convergence in our hands (Figure 3.2 b), yet the retardation mechanism was also previously attributed to unwinding of negatively supercoiled plasmid DNA (Kasparkova et al., 2010).

The incomplete convergence of the three plasmid DNA bands was also observed with DCBP (Figure 3.2 c) and DCRP (Figure 3.2 d) covalent binder metallo-drug treatments which was akin to the oxaliplatin treatment (Figure 3.2 b). In particular, the supercoiled form treated with DCBP was clearly retarding towards the linear and nicked conformations more rapidly than DCRP treatment. However, for both DCRP and DCBP treatment cases the nicked and linear plasmid conformations achieved convergence before the supercoiled conformation converged with the linear conformation. These trends have not been previously reported.

This pattern of more rapid convergence between nicked and linear plasmid conformations was also observed with the other tested covalent binder metallo-drugs cisplatin (Figure 3.2 a),
oxaliplatin (Figure 3.2 b), and DACH (Figure 3.2 e). This suggests that either or both the nicked and linear conformations are more sensitive to metallo-drug structure modifications than the supercoiled conformation. In particular, the linear regression gradient analysis showed that the nicked conformation appears to be the most accurately sensitive plasmid conformation to covalent binder metallo-drug damage in both magnitude and uniquely the apparent molecular weight decreased in value producing negative gradients (Figure 3.6).

The negative gradient of the treated nicked plasmid can be explained as a tightening of the dsDNA helix structure due to increased metallo-drug accumulation. This was previously observed in atomic force microscopy experiments where cisplatin treatment caused a lambda dsDNA template to adopt a more condensed conformation (Hou et al., 2009). Furthermore, the negative gradient values measured for the nicked plasmid conformation induced by all tested covalent binder metallo-drugs due to tightening of dsDNA structure is precisely the inverse mechanism described for the positive gradient values associated with the unwinding of the negatively supercoiled plasmid conformation (Figure 3.6).

Two possible mechanisms could explain why covalent binder metallo-drugs seem to exhibit higher affinity to bind with the nicked plasmid conformation over the other tested pUC19 plasmid conformations. Firstly, the nicked conformation is the most relaxed conformation according to the relationship between DNA band running order on agarose slab gel and helix relaxation and therefore the most easily accessible DNA template for metallo-drug binding. The relationship between DNA template accessibility and steric hindrance for cisplatin analogue metallo-drug binding has previously been established in experiments where chromatin-induced DNA condensation inhibited metallo-drug binding in linear amplification experiments (Galea and Murray, 2010). Secondly, the phosphate backbone breaks on the nicked plasmid conformation exhibit a covalently free negatively charged oxygen ligand which has previously been shown to preferentially increase cisplatin binding to DNA molecules as cisplatin assumes positive charges when in an aquated state (Campbell and Miller, 2008). This observation can
explain why bleomycin administered in combination with cisplatin has improved pharmacological outcomes for cancer patients (Evans et al., 1982). Bleomycin induces single strand breaks on a dsDNA template which renders DNA conformational changes analogous to the nicked plasmid conformation and exposes free phosphate groups for increased cisplatin binding (Hecht, 1999). Unfortunately, at the time of the experiment it was not thought to pursue combinational treatment of the plasmid.

The bleomycin analogue phleomycin is a DNA single strand breaker compound (Sleigh, 1976) which was also tested with native pUC19 DNA. Observations were made on agarose slab gels to monitor for DNA band retardation (Figure 3.3). The linear regression gradient measurements for all three plasmid conformations treated with phleomycin showed the same pattern of DNA band retardation as the covalent binder metallo-drugs which suggested both groups of compounds induce similar DNA conformation modifications (Figure 3.6).

However, closer inspection of agarose slab gels with the supercoiled plasmid conformation treated with phleomycin (Figure 3.3) showed no evidence of band retardation like any of the tested covalent binder metallo-drugs (Figure 3.2 a-e). Rather, the supercoiled conformation population appeared to have decreased in abundance due to phleomycin treatment in a dose-dependent manner and a smear of cleaved DNA molecules is apparent at higher treatment concentrations. On the other hand, the nicked and linear conformations do appear to converge, as observed with the covalent binder metallo-drug treatments, although, their abundance seems to also decrease at higher treatment concentrations as well (Figure 3.3).

Phleomycin is known to bind to DNA prior to inducing single strand breaks in the presence of a reducing agent like DTT (Stern et al., 1974, Sleigh, 1976). The binding of phleomycin to DNA may explain why the linear and nicked conformations retard and begin to converge similar to what was observed with the covalent binder metallo-drug treatments. Finally, the apparent DNA smearing pattern on agarose slab gels observed with phleomycin treatment has previously been attributed to DNA cleaving in experiments with bleomycin treated mouse gDNA on agarose slab
gels (Kuwano et al., 2001). Furthermore, this pattern of plasmid DNA modification is consistent with other DNA strand breaker compounds, like certain ruthenium(II)-based metallo-drugs, which reduce supercoiled conformation populations and increase the nicked conformational populations due to DNA cleaving (Patel et al., 2011).

The strand breaker Cu56MESS, an analogue to 56MESS, was tested with native pUC19 and analysed for differential DNA band retardation patterns. Previous DNA binding experiments with Cu56MESS showed it exhibited strand breaking properties in reactions that contained H₂O₂ as a reducing agent (Krause-Heuer et al., 2012). However, this work took an approach to characterise the DNA binding activity in parallel treatment conditions for all metallo-drugs to assess the biological activity of the drugs alone in the absence of other confounding factors.

Analysis of agarose slab gels of native pUC19 treated with Cu56MESS showed little to no change in DNA band retardation (Figure 3.3b) which was also reflected in the linear regression gradient measurements which indicate that Cu56MESS is essentially non-reactive with DNA (Figure 3.6). Qualitatively, evidence of Cu56MESS interaction with the supercoiled conformation is apparent as a decrease in band intensity between 0.5 µM and 15 µM which suggests that Cu56MESS could have competitively inhibited the intercalation of the SYBR DNA stain. This effect on DNA band resolution has previously been shown in other metallo-drugs studies where ethidium bromide was out-competed for DNA binding sites which resulted in decreases in densitometry measurements (Rajendran et al., 2008).

The supercoiled conformation band intensity increased at Cu56MESS treatment concentrations above 15 µM. This change in supercoiled conformation DNA band intensity could suggest that amount of Cu56MESS interaction with the supercoiled DNA is related to the metallo-drug concentration. However, we cannot offer an explanation about what mechanism could be at play at this time.
Overall, analysis of the native pUC19 DNA treated with the covalent binder metallo-drugs (Figure 3.2 a-e) showed that three plasmid conformations converge to occupy a final apparent molecular weight in-between the linear and nicked conformations of the DMF control sample. The linear regression gradient analysis revealed that the linear plasmid conformation increased apparent molecular weight in a dose-dependent manner (Figure 3.6) which may reflect a net increase in DNA molecular weight due to increasing metallo-drug accumulation. Measurable increases in linear DNA apparent molecular weight due to increasing metallo-drug accumulation has previously been shown in experiments where restriction digested linear plasmid DNA was treated with cisplatin and other analogue compounds. It was then subjected to agarose slab gel electrophoresis where DNA bands showed slight increases in apparent molecular weight in a dose-dependent manner (Cheng et al., 2005).

In addition, analysis of native pUC19 treated with the metallo-intercalator 56MESS revealed a DNA band retardation pattern where the three conformations retained their separation profile, as with the DMF control, and increased apparent molecular weight in a dose-dependent manner (Figure 3.4 a). The dose-dependent increase in all three pUC19 conformations apparent molecular weight was also reflected in the linear regression gradient measurements (Figure 3.6). This pattern of native pUC19 band retardation due to 56MESS treatment (Figure 3.4 a) differs from other cisplatin analogue covalent binders previously shown (Figure 3.2 a-e) as no convergence between the three plasmid conformations was observed.

The pattern of pUC19 band retardation induced by 56MESS, where the three plasmid conformations do not converge, suggests that this metallo-intercalator does not alter DNA conformation like the other cisplatin analogue covalent binder metallo-drugs. The dose-dependent increase in DNA band retardation induced by 56MESS can be explained as increased drug accumulation in the DNA structure which contributed to a net increase in the DNA molecules molecular weight without changing the native DNA conformation. The difference in plasmid DNA band retardation induced by 56MESS in comparison with other cisplatin analogue...
covalent binders suggested that there is a different mechanism for DNA binding between these two classes of compounds.

This conclusion contradicts previously published data which attributed 56MESS-induced DNA band retardation of pSP73 to the unwinding of negatively supercoiled DNA (Krause-Heuer et al., 2009a). Alternatively, other studies with aluminium(III) (Rajendran et al., 2008) and copper(II) (Chowdhury et al., 2009) metallo-intercalators have shown that π-π base stacking accumulation of these metallo-drugs in the DNA structure increased its apparent molecular weight in a dose-dependent manner. It is believed that the current data which considered three DNA conformation states provides a stronger argument for the DNA binding mechanism of 56MESS to follow a model where π-π base stacking is capable of retaining DNA conformation while in tandem it increases the apparent molecular weight of all three plasmid conformations.

Inspections of agarose slab gels showing native pUC19 treated with the metallo-intercalator 3478MEEN revealed a DNA band retardation pattern where the three plasmid conformations appeared to be migrating towards convergence and also concurrently increasing in apparent molecular weight (Figure 3.4 b). The migration distance between the supercoiled conformation and the nicked and linear conformations were clearly decreasing in comparison with the DMF control which was similar to the trends observed with the covalent binder metallo-drugs (Figure 3.2). However, linear regression gradient measurements showed that the nicked and linear bands increased in apparent molecular weight (Figure 3.6) which contradict covalent binder metallo-drug trends (Figure 3.2) and is more in line with trends observed with the metallo-intercalator 56MESS (Figure 3.4 a).

These observations suggest that 3478MEEN assumed two DNA binding mechanisms that are similar to the cisplatin analogue metallo-drug covalent binders and the metallo-intercalator 56MESS. Previous circular dichroism spectra experiments concluded that 3478MEEN binds to DNA by π-π base stacking intercalation (Brodie et al., 2004). However, an explanation for the unexpected covalent binder metallo-drug like DNA binding mechanism of 3478MEEN could be
attributed to its tetramethyl ligands which are in a different orientation to the bimethyl ligands on 56MESS.

Work with two ruthenium(II) based metallo-drugs was also carried out to compare how non-platinum based compounds affected the conformations of native pUC19. Observations of native pUC19 treated with RuP1 (Figure 3.5 a) and RuP2 (Figure 3.5 b) both showed retention and retardation profiles of the three plasmid conformations where the apparent molecular weight increased in a dose-dependent manner. This trend, where all three plasmid conformations increased apparent molecular weight due to RuP1 and RuP2 treatment, was also demonstrated in the linear regression gradient measurements (Figure 3.6).

The dose-dependent retardation of the three pUC19 conformations induced by both ruthenium(II) compounds was similar to what was observed with the 56MESS treated DNA (Figure 3.4 a) where π-π base stacking intercalation contributed to net increases in DNA molecular weight due to metallo-drug accumulation. Previous linear dichroism experiments showed spectra where DNA underwent π-π transitions due to RuP1 treatment (Orkey et al., 2012) which agree with the interpretation of present data that both ruthenium(II) metallo-drugs intercalate with native pUC19 and increase net molecular weight while retaining DNA conformation.

Closer inspection of the agarose slab gels showing native pUC19 treated with both ruthenium(II) compounds (Figure 3.5) along with the linear regression gradient measurements (Figure 3.6) revealed that RuP2 induced a greater increase in DNA retardation for all three plasmid conformations than RuP1. An explanation for this plasmid retardation profile most likely relates to RuP2 having a longer polyamide tail than its RuP1 analogue. The polyamide tail of RuP1 was previously associated with DNA helix minor groove binding (Orkey et al., 2012), therefore, it would be reasonable to assume that a longer polyamide tail would contribute to increased binding of RuP2 which would reflect the increases in native pUC19 apparent molecular weight.
In summary, this work aimed to develop a methodology that could differentiate and compare several classes of metallo-drugs for DNA binding by analysing structural modifications using three native plasmid conformations on agarose slab gel. This approach combined previous methodologies that focused on measuring metallo-drug induced supercoiled conformational changes (Keck and Lippard, 1992), and expanded this work to consider the retardation profile of all three pUC19 conformations. Furthermore, a novel analysis methodology was adopted that measured the linear regression apparent molecular weight gradient of the three plasmid conformations to differentiate the binding mode each class of metallo-drugs adopts for DNA.

Evidence was provided to show that the linear regression gradient analysis was capable of differentiating covalent binder metallo-drugs from other modes of DNA binding. However, quantitative comparisons of the linear regression gradient measurements between the three plasmid conformations was not appropriate as data points across all treatment concentrations were either not detectable or distinguishable on agarose slab gel which was summarised in Table 3.1. The reasons for the loss of DNA band resolution on agarose slab gel may be related to metallo-drug activity on the test DNA through convergence of the three plasmid bands, degradation of the plasmid bands or inhibition of SYBR binding to metallo-drug occupied plasmid DNA.

However, consideration of the plasmid mobility profiles suggests that certain metallo-drugs do not strictly behave as intended by their design. This conclusion was determined by combining the linear regression gradient analysis coupled with agarose slab gel observations and previously published literature. Future work using plasmid DNA for assessing metallo-drug binding modalities should consider the effect that these compounds have on all three plasmid conformations.
Chapter 4: Detecting metallo-drug induced DNA structure modifications by capillary electrophoresis

4.1 Introduction

Recently, free solution capillary electrophoresis (CE), has been employed to characterise differences in structure of anionic bio-molecules such as gellan gum (Taylor et al., 2012), human serum albumin dosed with cisplatin and other analogue platinum(II) metallo-drugs (Timerbaev et al., 2004), and protein induced structural changes on 12-20 bp oligo DNA molecules (Araya et al., 2007). The diffusion dynamics of larger DNA molecules by free solution CE has been extensively researched (Stellwagen et al., 1997, Stellwagen et al., 2003, Stellwagen and Stellwagen, 2002, Stellwagen and Stellwagen, 2003). This work established the conditions for capillary electrophoresis in the critical conditions (CE-CC) where the diffusion of DNA molecules larger than ~400 bp becomes uniform and, unlike slab gel electrophoresis, is independent of molecular weight but still dependent on DNA structural modifications (Lu et al., 2006, Lu et al., 2003b). However, to our knowledge no metallo-drug work has previously been published with a DNA template greater than 400 bp where CE-CC separation facilitated the detection of modified DNA structures.

The dsDNA linear template chosen for this study corresponds to the promoter element for the RUVBL2 gene which was significantly up-regulated in a gene expression study with human foreskin fibroblast cell line (FFbw002) treated with cisplatin (Galea and Murray, 2008). This will form the basis for future research, beyond the scope of this thesis, on the effect that metallo-drugs like cisplatin have on gene expression through transcription studies.
The work presented in this chapter relates to the aims outlined in Section 1.6.1 which focus on comparing test metallo-drugs for their efficacy to modify the structure of a linear dsDNA template. This section of work established the CE-CC conditions necessary for the free solution separation of DNA molecules with different structures. The optimal drug to nucleotide ($n_b$) ratio was determined with cisplatin as it is a well known covalent binder metallo-drug which induces structural modifications to DNA molecules (Bancroft et al., 1990, Hou et al., 2009). Finally, a comparative analysis was carried out with a range of metallo-drugs which represent a variety of DNA binding modes that modify DNA structure.

### 4.2 Results

This section of work assesses several categories of metallo-drugs for their ability to induce structural distortions to a PCR-generated linear dsDNA template using capillary electrophoresis. Capillary electrophoresis in the critical conditions (CE-CC) was used to detect metallo-drug induced structure modifications to the dsDNA template. CE-CC is particularly well suited to study metallo-drug induced structure modifications to a DNA substrate as this technique separates DNA by structure rather than molecular weight. This methodology characterised the effectiveness of several metallo-drugs for expanding or condensing dsDNA structures.

Data is presented in this chapter that describes the optimisation of the buffer conditions for CE-CC separation of DNA structures using untreated pUC19. Comparisons between native plasmid DNA and synthetic linear dsDNA were made to select the most appropriate template for metallo-drug/DNA studies. The drug/DNA incubation conditions were optimised to determine the appropriate drug to nucleotide ratio ($n_b$) to observe changes to dsDNA template structure. Finally, comparisons were made between several categories of metallo-drugs to assess their ability to induce the expansion or condensation of the DNA structure.
4.2.1 Synthesis of linear dsDNA template for metallo-drug studies

HEK293 cell line was established and maintained as described in Section 2.2.3. Genomic DNA (gDNA) was extracted as described in Section 2.2.4. gDNA product was concentrated by ethanol precipitation (Section 2.2.5) and quantified/qualified by UV nanophotometer (Section 2.2.10).

Multiple PCR reactions were performed out of the isolated gDNA from HEK293 cells using RUVBL2_F1 (21 mer) forward and RUVBL2_R1 (25 mer) reverse primers (Section 2.2.8) to generate sufficient quantities of dsDNA template for the drug/DNA studies. All PCR reactions were combined. The DNA was concentrated by ethanol precipitation (Section 2.2.5) and quantified/qualified by UV nanophotometer (Section 2.2.10). Confirmation of successful DNA template production was performed by analysis of a 5 µL aliquot of PCR product on horizontal 1% agarose slab gel against a 100 bp ladder (Section 2.2.17) and imaged by UV fluorescence.

A linear dsDNA template was amplified by PCR using primers specific for the promoter element of the RUVBL2 gene located at 19q13.33 on the human genome (Section 2.2.8). A typical PCR reaction would yield 1.4 µg of dsDNA template. Template quality had a typical $260\text{nm} / 280\text{nm}$ ratio of 1.8 and $260\text{nm} / 230\text{nm}$ ratio of 2.1. Analysis by agarose gel electrophoresis (Figure 4.1) with a 100 bp ladder (lane 1, $r^2 = 0.998$) yielded three products (lane 2) running at approximately 1617 bp, 959 bp and 176 bp corresponding to a non-specific PCR product, specific PCR product and primer smear, respectively. Densitometry analysis revealed the total reaction yield which consisted of approximately 85% specific PCR product and 15% non-specific PCR product.
Figure 4.1: Slab gel electrophoresis separation of dsDNA PCR product used for drug/DNA complex studies. Lane 1 shows the separation of the 100 bp ladder and lane 2 shows PCR product separation which yields a predominant band below 1000 bp, a faint non-specific band above 1500 bp and a primer smear around 100 bp.
4.2.2 Establishing control conditions for the separation of DNA by CE in the critical conditions

Optimisation of sodium borate buffer concentration for DNA CE separation in critical conditions was performed using native pUC19 prepared as previously described, dissolved in DNase-free water to a final concentration of 15 ng/µL. Sodium borate concentrations chosen to optimise the running conditions were 25 mM, 50 mM, 300 mM and 500 mM. All reaction samples were subjected to duplicate capillary electrophoresis injections at 30,000 V with detection by UV absorbance measurements at 260 nm for up to 60 minutes (Section 2.2.19), these data were analysed using OriginPro v8.5. This section describes the optimisation of the buffer conditions for subsequent metallo-drug/DNA studies by CE-CC. An optimal sodium borate buffer concentration was determined for DNA structure separation using native plasmid DNA pUC19 which naturally exhibits multiple structures as previously shown in native slab agarose gel electrophoresis (Figure 3.1 a).

a) Sodium borate buffer optimisation to establish CE-CC

CE separation electropherograms of native pUC19 in higher concentrations of sodium borate buffer showed that the DNA sample resolved into three peaks (Figure 4.2 a). The 500 mM sodium borate condition showed a large peak at 16.14 minutes and two smaller peaks resolved at 17.89 minutes and 18.15 minutes. The large peak within the 300 mM sodium borate condition resolved in 12.77 minutes and two smaller peaks resolved in 13.83 minutes and 14.02 minutes, respectively.

CE separation electropherograms of native pUC19 in lower sodium borate measuring UV absorbance at 260 nm showed a range of peaks that increased in intensity and distribution at lower concentrations (Figure 4.2 a). The effect of DMSO on DNA mobility was considered as this was an internal eof standard for further mobility calculations. The 50 mM sodium borate condition showed a range of peaks that resolved between 8.58 and 11.76 minutes. The
25 mM sodium borate condition showed a larger range of peaks that resolved between 6.69 and 12.46 minutes.

Sodium borate buffer concentration effect on DMSO migration was the same as trends observed with DNA CE migration (Figure 4.2 b). High sodium borate buffer concentrations at 500 mM showed a slow DMSO peak which ran at 16.22 minutes. However, when the sodium borate buffer was reduced to 50 mM, the DMSO peak emerged much quicker at 3.38 minutes.

b) Characterisation of electrophoretic peaks for synthetic linear dsDNA template

The CE-CC electropherogram of pUC19 showed a complex peak profile that is too difficult to interpret DNA mobility changes for the current purposes of this study. Therefore, an alternative DNA substrate was applied for drug/DNA studies. Further work used a PCR synthesised dsDNA substrate which was described in the previous section.

Four peaks were identified in the untreated DNA samples dissolved in water on the electropherogram graphs and these were designated peaks A to D with increasing mobilities between 3.63 and 3.84×10⁻⁸ m².V⁻¹.s⁻¹ (Figure 4.3 & Table B1). Comparisons of peak mobilities between the water control and the DMF control shows the DMF treated DNA peaks had an increase in mobility by up to 1.56%. DNA treated with DMF showed increasing mobilities for peaks A to D between 3.65 and 3.90×10⁻⁸ m².V⁻¹.s⁻¹, respectively (Figure 4.3 & Table B1).

Although the DMF control peak mobilities increased compared to water controls, the differences between each peak interval did not show a significant change. This suggests the peak order was not affected by DMF treatment. The mobility distance between peaks A to B, B to C and C to D for water or DMF treatments were within the margin of error.

c) Optimisation of drug to nucleotide ratio for linear dsDNA mobility studies
Linear dsDNA template that was treated with cisplatin at concentrations of 0.015 µM ($r_b = 0.000012$), 0.05 µM ($r_b = 0.000041$) and 0.15 µM ($r_b = 0.000123$), were separated by CE (Figure 4.3) and the resultant mobilities were converted to a ratio relative to the mobility of the water treated control (Figure 4.4, Table B1). Relative to the DMF control, a clear increase in mobility peak ratios was observed with cisplatin treatments of 0.015 µM. This treatment increased the mobility ratios for peaks A, B, C and D to $1.058 \pm 0.003$, $1.060 \pm 0.004$, $1.058 \pm 0.003$, $1.065 \pm 0.013$, respectively. Higher concentrations of cisplatin treatment produced an increase in peak mobilities relative to the DMF control in a dose-dependent manner. Peak A showed peak mobility of $1.058 \pm 0.003$, $1.069 \pm 0.003$ and $1.072 \pm 0.005$ corresponding to cisplatin treatments of 0.015 µM, 0.05 µM and 0.15 µM, respectively (Figure 4.4). Peaks B, C and D showed increases in peak mobility ratios from $1.060 \pm 0.004$, $1.058 \pm 0.003$, $1.065 \pm 0.013$ with 0.015 µM cisplatin treatment to $1.071 \pm 0.001$, $1.074 \pm 0.004$, $1.091 \pm 0.003$ with 0.05 µM cisplatin treatment, respectively (Figure 4.4).

However, peaks B, C and D of the 0.05 µM to 0.15 µM cisplatin treated samples showed a slight decrease in peak mobility ratios relative to the DMF control (Figure 4.4). Although, the difference between each peak’s mobility was not significant as all of these peaks fall within each other’s standard error. Between cisplatin treatments 0.05 µM and 0.15 µM, peaks B, C and D decreased from $1.071 \pm 0.001$ to $1.068 \pm 0.004$, $1.074 \pm 0.004$ to $1.066 \pm 0.006$ and $1.091 \pm 0.003$ to $1.083 \pm 0.008$, respectively (Figure 4.4).

4.2.3 Free solution capillary electrophoresis of linear dsDNA treated with several classes of metallo-drugs

Drug/DNA incubation reactions were performed in triplicate as described in Section 2.2.11. Each reaction contained 15 µg dsDNA PCR product, 1 × drug/DNA incubation buffer and test compounds cisplatin, oxaliplatin, DCBP, DCRP, DACH, phleomycin, Cu56MESS, 56MESS,
3478MEEN, RuP1 and RuP2 at final concentrations of 0.015, 0.05, 0.5, 1.5, 5, 15 and 50 µM. Phleomycin was supplemented with 25 mM DTT to facilitate DNA strand breaking. Control reactions consisted of water and 5% DMF solvent. Phleomycin reaction controls consisted of water, 25 mM DTT, 5% DMF, and 25 mM DTT with 5% DMF.

This section compares the effect that selected metallo-drugs have on linear dsDNA mobility. In each case, the linear dsDNA template was treated with test compounds at a common concentration of 0.015 µM prior to separation by CE-CC. Peak mobilities were determined from electropherograms (Figure 4.5), these were converted to a mobility ratio relative to water controls (Figure 4.6, Table B2).

a) Control reactions

DNA mobility controls utilised in this experiment consisted of a 5% DMF treatment to the DNA as this solvent was used to solubilise the test compounds, in addition to a DTT control which was used since this was added to phleomycin treatments to facilitate redox reactions to strand break dsDNA (Sleigh, 1976). DMF control treatments induced slight increases in the mobility ratios (relative to water controls) of peaks A to D with values up to 1.024. DTT control treatments also increased the mobility of DNA peaks A to D with values up to 1.020. Furthermore, treatment with both DMF and DTT induced larger DNA peak mobility ratios for peaks A to D with a maximum value of 1.036.

b) Covalent binders

Cisplatin was the only covalent binder to increase DNA peak mobility relative to the DMF control. Electropherograms (Figure 4.5 a) showed that the four DNA peaks increased with a value between 3.80 and 4.02×10⁻⁸ m².V⁻¹.s⁻¹. DNA peaks A to D mobility ratios showed cisplatin induced a maximum increase of 1.049 relative to DMF control ratios (Figure 4.6 a).
Figure 4.2: Electropherograms showing the optimisation of sodium borate buffer concentration to establish CE-CC separation using native plasmid DNA. (a) Electropherogram traces tracking the migration at UV absorbance 260 nm of native plasmid pUC19 DNA (dotted squares) by CE with sodium borate buffer concentrations of 25 mM (●), 50 mM (■), 300 mM (▲) and 500 mM (▲). (b) Electropherogram peak profile of DMSO (dotted squares) at UV absorbance 200 nm when subjected to CE in sodium borate buffer at 50 mM (■) and 500 mM (▲).
Figure 4.3: Optimisation of metallo-drug treatment concentration using linear dsDNA template subjected to CE-CC. Electropherogram traces show the CE-CC separation by mobility ($m^2 V^{-1} s^{-1}$) of linear dsDNA PCR template treated with water control (▬), 5% DMF control (▬), and cisplatin at 0.015 µM (▬), 0.05 µM (▬) and 0.15 µM (▬). Linear dsDNA PCR template resolved into 4 peaks that were labelled A, B, C and D. All DNA products were monitored by UV absorbance at 260 nm (offset y-axis)
Figure 4.4: A bar chart showing changes in peak mobility due to cisplatin treatment.

This data is presented as a function of the ratio between DNA treatments relative to water control. Four DNA peaks (A, B, C and D) were monitored for changes in mobility presented as a ratio relative to water control against 5% DMF (█) and cisplatin treatments with 0.015 (█), 0.05 (█) and 0.15 (█) µM. Peak mobility was converted to a ratio of DNA treatment to water control which allowed for a more direct comparison between treatments.
All other DNA covalent binding compounds – oxaliplatin, DCBP, DCRP and DACH – induced decreases in DNA peak mobility relative to the DMF control. DCBP showed slightly less reduction in mobility for DNA peaks A to D, relative to DMF, with a maximum ratio 1.011. In most cases oxaliplatin, DCRP and DACH showed similar DNA peak mobility ratios.

c) *Strand breakers*

Phleomycin induced decreases in DNA peak mobility relative to DMF and DTT combination controls (Figure 4.5 b). DNA peaks A to D treated with phleomycin marginally decreased mobility with values between $3.72 \times 10^{-8}$ m$^2$.V$^{-1}$.s$^{-1}$. DMF and DTT control increased peak mobilities for peaks A to D with values between $3.75 \times 10^{-8}$ m$^2$.V$^{-1}$.s$^{-1}$. Cu56MESS was the least effective at decreasing DNA peaks A to D mobility with values between $3.69 \times 10^{-8}$ m$^2$.V$^{-1}$.s$^{-1}$ relative to the DMF control, although, this was within the margin of error. Treatment with Cu56MESS also showed slightly reduced peak mobility relative to the DMF control with a maximum value of 1.020 for DNA peaks A to D.

d) *Metallo-intercalators*

Treatments with 56MESS and 3478MEEN induced similar and significant decreases in DNA peak mobilities that were either equal to or lower than both water and DMF controls. 56MESS showed slightly lower mobility values down to 0.992 for DNA peaks A to D (Figure 4.5 c, Figure 4.6 c). However, 3478MEEN induced slightly higher DNA peak mobilities relative to the DMF control.

e) *Groove binders*

Both RuP1 and RuP2 treatments induced increases in DNA peak mobility (Figure 4.5 d, Figure 4.6 d). RuP2 induced higher mobility with the values of DNA peaks A to D between $3.73 \times 10^{-8}$ m$^2$.V$^{-1}$.s$^{-1}$ compared to RuP1 with mobility values between 3.73 and
Conversely DNA peaks C and D show RuP1 induced higher mobilities of 3.93 and $4.02 \times 10^{-8} \text{ m}^2 \text{V}^{-1} \text{s}^{-1}$ compared to RuP2 with values of 3.87 and $3.98 \times 10^{-8} \text{ m}^2 \text{V}^{-1} \text{s}^{-1}$. 


Figure 4.5: Example electropherogram showing the mobility separation of dsDNA treated with DNA interacting compounds and their associated controls. DNA was monitored by UV absorbance at 260 nm (offset y-axis) where 4 peaks assigned A, B, C and D were observed and mobility (x-axis) recorded. Image (a) shows separation of dsDNA template treated with covalent binding compounds cisplatin (▬), oxaliplatin (▬), DCRP (▬), DCBP (▬) and DACH (▬). Image (b) shows separation of dsDNA template treated with 25 mM DTT control (▬), DMF & DTT control (▬) and the strand breakers phleomycin (▬) and Cu56MESS (▬). Image (c) shows separation of dsDNA template treated with the metallo-intercalators 56MESS (▬) and 3478MEEN (▬). Image (d) shows separation of dsDNA template treated with the metallo groove binder compounds RuP1 (▬) and RuP2 (▬). All traces are shown with water control (▬) and DMF control (▬).
Figure 4.6: Bar graph showing changes to DNA peak mobility due to treatment as a function of the ratio between DNA covalent binding compounds to water control. The average ratio (y-axis) and standard deviation of the four DNA peaks (A, B, C and D, x-axis) are presented in vertical bar graphs above. Image (a) shows mobility ratios of dsDNA template peaks of DMF control (■), cisplatin (▲), oxaliplatin (▲), DABP (▲), DCRP (▲) and DACH (▲). Image (b) shows ratios of strand breaker with DMF control (■), DTT control (■), DMF and DTT control (■), phleomycin (■) and Cu56MESS (■). Image (c) shows ratios of DMF control (■), 56MESS and 3478MEEN. Image (d) shows ratios of DMF control (■), RuP1 (■) and RuP2 (■).
4.3 Discussion

4.3.1 Optimisation of experimental protocols that differentially separate a variety of DNA molecule structures by free solution CE-CC

Sodium borate buffer (pH 9.2) was chosen for free solution CE separation of DNA molecules as it was previously reported to increase the zeta potential along a fused silica capillary wall and prevent the absorption of other similar anionic bio-molecules with repeating subunits such as gellan gum (Taylor et al., 2012). Previous studies that separated DNA molecules by free solution CE used TAE (pH 8.0) and TBE buffers (pH 8.3) (Araya et al., 2007, Stellwagen and Stellwagen, 2002, Stellwagen et al., 1997) as an analogous convention to slab gel based electrophoresis separations. We argue that the use of complex buffers such as TAE and TBE are not necessary and that the advantage of using a simpler sodium borate buffer was that it decreases the likelihood of complex interactions with the buffer and thereby giving more accurate mobility profiles.

Optimisation of sodium borate buffer concentration was initially performed to ensure that counter-ion penetration of the DNA molecule generated sufficient friction (Stellwagen et al., 2003) to differentiate the widest possible variety of DNA structures. Native pUC19 was chosen as the DNA template to verify that the CE-CC was established as it has a molecular weight greater than 400 bp and it is known to exist in a variety of conformational states (Hamilton and Wilker, 2004, Dixit et al., 2010). Furthermore, the multiple conformations of pUC19 were resolved as distinct bands by agarose slab gel electrophoresis as previously discussed (Section 3.3.1).

Electropherograms showing the free solution separation of native pUC19 by CE at 500 mM sodium borate revealed three distinct peaks which decreased in migration time and differentiated into multiple peaks as the sodium borate buffer concentration was reduced to 25 mM (Figure 4.2 a). The migration pattern of the three native pUC19 peaks appears to be
analogous to what was observed in previous agarose slab gels which were defined as supercoiled, linear and nicked conformations according to running order and apparent molecular weight (Figure 3.1, Section 3.3.1). Furthermore, the migration order of the three pUC19 peaks roughly and the peak size agrees with previously published CE electropherogram data showing the native conformations of pUC19 (Schmidt et al., 1999).

The relationship between decreasing migration time of DNA products subjected to free solution CE and decreasing buffer concentration was observed with our native pUC19 electropherogram data (Figure 4.2 a). This relationship between migration time and buffer concentration was observed with the DMSO organic solvent electropherogram data which served as the electroosmotic flow (EOF) for mobility calculations (Figure 4.2 b). Both trends were in agreement with published data that showed a decrease in migration time of linear pUC19 subjected to free solution CE in decreasing concentrations of TAE and TBE buffers (Stellwagen and Stellwagen, 2002).

The increased number of DNA peaks observed with free solution CE electropherogram traces of native pUC19 in 25 mM sodium borate buffer (Figure 4.2 a) is a result of the increased separation and level of detection of plasmid sub-populations with small structure differences produced as an artefact of E. coli metabolism (Silva et al., 2012). These data indicated that the ability to resolve DNA structure differences was improved by optimising the sodium borate buffer concentration. Interestingly, our observation that 25 mM sodium borate (pH 9.2) achieved the best peak resolution of DNA structure differentiation was more basic in pH and about half the buffer concentration than in other studies which used 40 mM Tris acetate (pH 8.0) and 45 mM Tris borate (pH 8.3) buffers (Araya et al., 2007, Stellwagen and Stellwagen, 2002, Stellwagen et al., 1997).
4.3.2 Characterisation of a linear dsDNA template for metallo-drug binding studies

The free solution CE-CC of native pUC19 with 25 mM sodium borate buffer (pH 9.2) produced electropherograms that are too complex to use for metallo-drug DNA binding studies (Figure 4.2 a). Therefore, a PCR generated linear dsDNA template was used which corresponded to an 879 bp section of the RUVBL2 gene promoter element located on human chromosome 19q13.33. Successful generation of the linear dsDNA template was verified by molecular weight measurement on agarose slab gel electrophoresis relative to appropriate size standards (Figure 4.1 a).

A non-specific PCR product with an apparent molecular weight of 1617 bp was detected on the agarose slab gel (Figure 4.1) which made up 16% of the PCR population (Figure 4.1) according to densitometry analysis. PCR optimisation to eliminate the non-specific PCR product was not undertaken as CE-CC enables uniform mobility of any DNA molecules greater than 400 bp (Stellwagen et al., 1997) and further sequence specificity studies were not going to be pursued in this study. Interestingly, other compound-DNA studies used small DNA templates such 12-20 bp (Araya et al., 2007) or 199 bp (Lu et al., 2006) which would appear to defy the constraints of CE-CC DNA structure based separation which reportedly requires DNA templates greater than 400 bp to achieve uniform free solution mobility separation independent of molecular weight (Stellwagen et al., 2003, Stellwagen et al., 1997).

Electropherograms of an untreated water control dsDNA sample subjected to free solution CE-CC with 25 mM sodium borate buffer (pH 9.2) produced four peaks (Figure 4.3) with mobilities ranging from $3.29 \times 10^{-8} \pm 4.12 \times 10^{-9}$ m$^2$.V$^{-1}$.s$^{-1}$ to $3.84 \times 10^{-8} \pm 8.56 \times 10^{-11}$ m$^2$.V$^{-1}$.s$^{-1}$ (Table B1). These free solution CE-CC linear dsDNA template mobility measurements are consistent with other reported mobility values for DNA molecules greater than 400 bp subjected to free solution CE-CC in similar buffer conditions (Stellwagen and Stellwagen, 2002, Stellwagen et al., 1997). An explanation for the four peaks observed with the PCR
generated linear dsDNA template could be a result of the ethanol precipitation protocol following the drug/DNA incubation reactions (Section 2.2.11) which is known to modify DNA conformation (Ivanov et al., 1973) or the application of an electric field during CE separation induced DNA bending (Lu et al., 2003b).

Electropherogram traces of the linear dsDNA template treated with 5% DMF solvent control revealed four peaks (Figure 4.3) with mobilities between $3.65 \times 10^{-8} \pm 5.41 \times 10^{-11} \text{ m}^2 \cdot \text{V}^{-1} \cdot \text{s}^{-1}$ to $3.90 \times 10^{-8} \pm 8.56 \times 10^{-11} \text{ m}^2 \cdot \text{V}^{-1} \cdot \text{s}^{-1}$ (Table B1). The DMF solvent control increased the linear dsDNA mobility of all four peaks by up to $101.6 \pm 0.4\%$ in comparison with the water control (Figure 4.4). This solvent-dependent increase in DNA mobility contradicts previously reported trends (Lu et al., 2006), although, our methodology differs from the published data as unbound solvent is removed from the DNA sample prior to CE injection in the current data.

4.3.3 Determination of optimal drug to nucleotide ratios to detect metallo-drug induced DNA structure modifications by CE-CC

The well known metallo-drug cisplatin forms covalent bonds with DNA and induces conformational changes to the helical structure (Takahara et al., 1996, Jamieson and Lippard, 1999). Cisplatin is often used as a positive control to establish the validity of experimental protocols because of its well defined characteristics for covalent DNA binding (Alonso et al., 2006). This section determined the optimal drug to nucleotide ratio ($r_b$) to detect metallo-drug induced DNA structure modifications which followed from the optimisation and characterisation of the linear dsDNA template for structure-based free solution CE-CC separation.

Electropherogram traces of free solution CE-CC separation of the linear dsDNA template treated with cisplatin at $0.015 \mu\text{M}$ ($r_b = 0.000012$) $0.05 \mu\text{M}$ ($r_b = 0.000041$) $0.15 \mu\text{M}$ ($r_b = 0.000123$) revealed a dose dependent mobility increase of all four DNA peaks relative to the
water control ranging from 105.8 ± 0.3% to 109.1 ± 0.3% (Figure 4.3, Table B1). However, taking into account that the 5% DMF solvent control causes an increase in DNA mobility, the effective cisplatin-induced increase in DNA mobility was reduced to between 104.5 ± 0.2% and 107.5 ± 0.4% (Figure 4.4). This data suggested that low concentrations of cisplatin condensed the linear dsDNA structure which reduced counter-ion friction on the DNA molecule and resulted in an increase in DNA mobility.

The effect of counter-ion penetration to induce friction for DNA diffusion along a capillary in an electric field has previously been described (Stellwagen et al., 2003). This is the first time cisplatin has been described inducing this behaviour to a DNA molecule greater than 400 bp in free solution CE-CC conditions and that relatively small concentrations of cisplatin are needed to observe this effect. Furthermore, our conclusion that cisplatin induces condensation of DNA structure is consistent with atomic force microscopy experiments where cisplatin at similar concentrations was also shown to induce structure condensation with DNA molecules greater than 400 bp (Liu et al., 2010, Hou et al., 2009).

### 4.3.4 Comparative analysis of various metallo-drugs for their efficacy to induce DNA structure modifications

Inspection of electropherograms traces showing the free solution CE-CC separation of the linear dsDNA template treated with cisplatin and other analogue covalent binder metallo-drugs at 0.015 µM (rₘ = 0.00012) (Figure 4.5 a) showed clear peak mobility changes relative to the 5% DMF control (Figure 4.6 a). Cisplatin induced a similar trend as previously described with the plasmid mobility work where mobility of all four dsDNA template peaks with an effective increase of 102.8 ± 0.4% when considering the 5% DMF control (Table B2). However, in contrast all other cisplatin analogue covalent binder metallo-drugs decreased all four dsDNA template peak mobilities relative to the 5% DMF control with oxaliplatin and DACH showing the greatest maximum effective decreases of 97.7 ± 0.6% and 97.8 ± 1.8%, respectively.
The decrease in the linear dsDNA template peak mobility induced by the cisplatin analogue covalent binder metallo-drugs relative to the DMF solvent control suggests that DNA treatment with these compounds increased counter-ion friction as a result of DNA structure relaxation which retarded DNA diffusion along the capillary when an electric field was applied. This conclusion that cisplatin analogue covalent binder metallo-drugs relax DNA structure contradicts previous conclusions in a study where λ DNA treated with cisplatin or DACH at a drug to nucleotide ratio (r_b) from approximately 0.5 to 5 induced condensation and eventual globulation of DNA structure when observed with atomic force microscopy (Hou et al., 2009).

However, we argue that these high r_b values which induced globulation of large DNA templates (Hou et al., 2009) are too high to be relevant in a cellular context. For example, the MCF-7 cell line displayed a phenotype response to cisplatin treatment after 24 hours incubation with a measured r_b value of 0.00001 (Reile et al., 1992) which is of similar magnitude as that used in our study. Furthermore, any influences from mono-functionally bound metallo-drugs affecting DNA mobility in free solution CE-CC can be discarded as the low r_b value used in our study meant that the DNA phosphate backbone charge was the primary influencing factor on its mobility and that any change in mobility had to be a reflection of change in counter-ion friction on the DNA structure rather than the metallo-drug.

Inspection of electropherogram traces showing the free solution CE-CC separation of the linear dsDNA template treated with DNA metallo-intercalators 56MESS and 3478MEEN at 0.015 µM (r_b = 0.00012) (Figure 4.5 c) showed clear peak mobility decreases relative to the 5% DMF control (Figure 4.6 c) such as that which was observed with the cisplatin analogue covalent binder metallo-drugs. 56MESS induced the greatest decrease in linear dsDNA template mobility relative to the 5% DMF control with an effective reduction of 96.9 ± 0.8 % (Figure 4.6 c).
Previous studies characterised 56MESS’s effect on DNA conformation to be analogous to the influence that cisplatin has on supercoiled plasmid DNA conformation (Krause-Heuer et al., 2009a). However, the data presented here contradicts this claim and instead suggests the reported π-π intercalation binding mode of 56MESS to DNA (Krause-Heuer et al., 2009a) induced a decrease in DNA mobility at cellular relevant drug to nucleotide concentrations. This increased the counter-ion friction as a result of a relaxation (or expansion) of the DNA structure to accommodate metallo-drug base stacking.

Electropherogram traces showing the free solution CE-CC separation of the linear dsDNA template treated with ruthenium(II) groove binders at 0.015 µM (τ₀ = 0.00012) showed slight increases in mobility relative to the 5% DMF control (Figure 4.5 d) that contrasted with the other platinum(II) metallo-drugs tested (Figure 4.6 d). RuP1 showed the greatest increase in DNA peak mobility at 102.4 ± 1.2%. However, RuP2 treatment appeared to induce the greatest increase in DNA peaks A and B with highest mobility at 100.8 ± 0.2% relative to the 5% DMF control.

The latter observation with RuP2 being the more influential treatment for increased DNA mobility was more in line with plasmid mobility studies previously discussed. Previously it was reported that the primary mode of DNA binding for RuP1 was by groove binding (Orkey et al., 2012). The increase in DNA mobility induced by both RuP1 and RuP2 suggests that the groove binding mode of both compounds contributed to a reduction in counter-ion friction as a result of metallo-drug induced condensation of the DNA structure.

The strand breaker phleomycin was submitted to free solution CE-CC DNA mobility studies and initial observations of electropherogram traces (Figure 4.5 b) showed a slight decrease in DNA mobility down to 99.0 ± 0.6% (Table B2) relative to the DMF and DTT control (Figure 4.6 b). Phleomycin has previously been reported to both bind and cleave the phosphate backbone of DNA (Sleigh, 1976, Stern et al., 1974) and resulting into relaxation of plasmid
DNA structure. Furthermore, this CE-CC data indicate that the decrease in DNA mobility brought about by increased counter-ion penetration was a result of phleomycin-induced strand-breaking on the DNA structure. In contrast, the strand breaker Cu56MESS induced almost negligible mobility change in dsDNA template mobility relative to the 5% DMF control with the largest recorded peak difference of 99.4 ± 0.5 % (Figure 4.6 b).

In summary, work presented here described a methodology that confirmed optimal conditions were established for DNA structure differentiation by free solution CE-CC. Data showed that the CE-CC conditions established for this work were sensitive enough to detect changes in DNA conformation at low drug to nucleotide ratios (\( r_b \)) that are relevant to platinum(II) metallo-drug doses that induce phenotypic responses at the cellular level. Furthermore, most DNA mobility data generated from a variety of metallo-drugs was able to be reconciled with the literature or previous studies presented in this body of work.
Chapter 5: Detecting metallo-drug induced interstrand crosslinks by denaturing slab gel electrophoresis

5.1 Introduction

A unique characteristic which differentiates cisplatin analogues from other metallo-drug compounds is the formation of covalent inter-strand crosslink’s (ICLs) between the two DNA strands. Cisplatin and other bi-functional analogues such as oxaliplatin and carboplatin are capable of forming ICLs through covalent binding with DNA (Kasparkova et al., 2010, Woynarowski et al., 1998, Flchtlnger-Schepman et al., 1995). The significance of metallo-drug ICL formation means that normal H-bond melting induced by DNA binding proteins does not separate DNA strands and prevents transcription and replication from occurring which ultimately leads to cell death by apoptosis (Todd and Lippard, 2009). Therefore, it is standard practice for any newly synthesised metallo-drugs that is designed for covalent DNA binding to be analysed for their ability to inhibit dsDNA strand separation though the formation of ICLs in comparison with the known ICL capability of cisplatin (Mlcouskova et al., 2012, Halámiková et al., 2008).

Work presented in this chapter focuses on the aims outlined in Section 1.6.1. This section of work begins by describing the application of updated methodologies to improve the data generation for ICL analysis. This included an application of a newly developed denaturing slab gel protocol and the introduction of fluorescently labelled DNA templates for improved quantification. Finally, a comparative analysis was carried out with a range of metallo-drugs with a variety of DNA binding modes that were assessed for their capability to form ICLs.
5.2 Results

This chapter characterises the ability of several metallo-drugs to induce cross-links between opposing strands of linear dsDNA using denaturing slab gel electrophoresis. The dsDNA linear template chosen for this study corresponds to the promoter element for the RUVBL2 gene which was significantly up-regulated in a gene expression study with human foreskin fibroblast cell line (FFbw002) treated with cisplatin (Galea and Murray, 2008). This will form the basis for future research, beyond the scope of this thesis, on the effect that metallo-drugs like cisplatin have on gene expression through transcription studies.

However, the important property of this dsDNA fragment is the presence of adduct sites that may give rise to inter-strand cross-links, since these will prevent the separation of the dsDNA template during electrophoresis under denaturing conditions. The dsDNA template was 865 bp in length and composed of 20% adenine and thymine bases and 29% guanine and cytosine bases. As cisplatin is well characterised for preferential binding to guanine bases, it was assumed this template would be appropriate to facilitate cisplatin-induced interstrand crosslinks which would subsequently serve as a positive control for additional compounds.

The 5’FAM-labelled dsDNA template was incubated with several categories of metallo-drugs over a drug to nucleotide ratio ($n_b$) that ranged from 0.000619 to 6.19. Treated DNA was subjected to slab gel electrophoresis under denaturing conditions using a novel urea method (Hegedus et al., 2009). Detection of ssDNA was carried out using the LAS4000 fluorescent gel imager. Measurement of ssDNA band fluorescence intensity was carried out by densitometry analysis to measure band intensity which was normalised by subtracting background fluorescence.

The ssDNA band intensity data was expressed as a percentage of change from a negative control of 100% melted DNA (as this is the native conformation of dsDNA under denaturing conditions) and plotted on semi-log graphs. From these graphs, the treatment concentrations...
that induced 50% and 5% ssDNA reduction were calculated, then subsequently converted to $r_b$ values to simplify data into a single integer.

### 5.2.1 Generation and characterisation of FAM-labelled dsDNA template for drug/DNA studies

Generation of a fluorescently labelled dsDNA template was performed by PCR amplification of a genomic DNA sequence coding for the promoter element of the RUVBL2 gene. The forward primer was fluorescently labelled with a 5’FAM modification, and multiple replicate reactions were pooled together, concentrated by ethanol precipitation and subjected to purification by slab gel excision (Section 2.2.9).

Genomic DNA (gDNA) was extracted from HEK293 cells as described previously. Multiple PCR’s were performed using 5’FAM-labelled RUVBL2_F2_FAM (21 mer) forward and non-labelled RUVBL2_R1 (25 mer) reverse primers to generate a 5’FAM-labelled dsDNA product for drug/DNA studies. All PCR reactions were combined, and DNA was concentrated by ethanol precipitation and resuspended in DNase-free water to a final volume between 100 and 200 µL, depending on ease of pellet re-suspension (Section 2.2.5). The 5’FAM-labelled DNA products were purified as described in Section 2.2.9, concentrated by ethanol precipitation (Section 2.2.5) and quantified/qualified by UV nanophotometer (Section 2.2.10).

This DNA fragment was later treated with the metallo-drug of interest for drug/DNA crosslink studies. Quantity and quality analysis was assessed by UV absorbance measurements and horizontal slab gel electrophoresis. A typical single PCR reaction would yield greater than 300 ng of 5’FAM labelled dsDNA with $\frac{260\text{nm}}{280\text{nm}}$ ratio of 1.9 and $\frac{260\text{nm}}{230\text{nm}}$ ratio of 1.4 following post reaction purification. Analysis of PCR product by agarose gel electrophoresis and fluorescent imaging (Figure 5.1 a) with a SYBR labelled 100 bp ladder (lane 1, $r^2 = 0.998$) shows an expected 5’FAM labelled dsDNA product at 865 bp and a 5’FAM labelled primer derived smear at 238 bp. Post purification analysis by denaturing
agarose gel electrophoresis and fluorescent imaging (Figure 5.1 b) with a SYBR labelled 100 bp ladder (lane 1, $r^2 = 0.999$) showed a denatured 5′FAM labelled ssDNA product at 676 bp (lane 2) and its parent dsDNA isoform at 820 bp (lane 3).

5.2.2 **Characterisation of the ability of various metallo-drugs to crosslink DNA**

Each reaction contained 300 ng 5′FAM-labelled dsDNA PCR product, 1 × drug/DNA incubation buffer (defined in Section 2.1.9) and test compounds cisplatin, oxaliplatin, DCBP, DCRP, DACH, phleomycin, 56MESS, Cu56MESS, 3478MEEN, RuP1 and RuP2 at final concentrations of 0.015, 0.05, 0.5, 1.5, 5, 15 and 50 µM. Phleomycin was supplemented with 25 mM DTT to facilitate strand breaking. Control reactions consisted of water and 5% DMF solvent. Phleomycin reaction controls consisted of water and 25 mM DTT with 5% DMF. Drug/DNA incubation reactions were performed in triplicate as described in Section 2.2.11.

Reactions were terminated by ethanol precipitation and the DNA pellet was resuspended in 40 µL of denaturing urea gel loading buffer (Section 2.1.6a). The DNA was heat denatured at 95 °C for 10 minutes and the 40 µL samples along with 300 ng of non-denatured 5′FAM DNA and 10 µL of SYBR-labelled 100 bp ladder were subjected to urea-based denaturing 1% agarose horizontal slab gel electrophoresis as described in Section 2.2.18. Slab gels were imaged using the LAS4000 fluorescent gel dock and ssDNA band densitometry was analysed using Multiplex v8.0 and data analysed using OriginPro v8.5 (Section 2.1.11).

_a) Covalent binders_

The results of the covalent binding metallo-drugs cisplatin, oxaliplatin, DCBP, DCRP and DACH showed that these compounds induce similar DNA migration patterns (Figure 5.2). The overall trend observed as the drug concentration increased was a progressive decrease in the intensity of the ssDNA conformation and a corresponding increase of cross-linked dsDNA conformation. Furthermore, at high treatment doses the migration of the dsDNA band
Figure 5.1: Images of two slab gels comparing before and after purification of 5′FAM-dsDNA template for downstream drug/DNA studies. Image (a) shows agarose slab gel electrophoresis separation of SYBR labelled 100 bp ladder (lane 1) and 5′FAM labelled PCR product (lane 2). Image (b) shows a denaturing agarose slab gel electrophoresis of SYBR labelled 1 kb ladder (lane 1) and purified 5′FAM labelled denatured ssDNA template (lane 2) and its non-denatured parent dsDNA isoform (lane 3). Note the 2 kb size standard was omitted due to cropping of image (a).
appeared to decrease indicating an increase in DNA molecular weight due to drug accumulation.

The semi-log graphs (Figure 5.2) show a sharp initial decrease in the amount of ssDNA present for all covalent binder compounds between 0 µM and 0.015 µM. The DCRP treatment showed the most pronounced decrease of the covalent binders with a ssDNA retention of 69.99 ± 3.74% at 0.015 µM treatment indicating that approximately 30% of the DNA was cross-linked. DACH showed the smallest decrease in ssDNA retention at 86.43 ± 11.76% after 0.015 µM of treatment.

After the initial decrease, all compounds induce a steady decrease in ssDNA retention between 0.015 µM and 0.15 µM. DACH and cisplatin showed the greatest decrease in ssDNA retention after 0.015 µM and 0.15 µM of treatment with ssDNA percentages from 86.43 ± 11.76% to 65.55 ± 21.30% and 75.48 ± 5.95% to 54.97 ± 2.26%, respectively. Oxaliplatin and DCBP showed the smallest decrease in ssDNA retention after 0.015 µM and 0.15 µM of treatment with ssDNA percentages from 80.07 ± 3.85% to 70.56 ± 6.40% and 71.81 ± 3.77% to 62.07 ± 2.64%, respectively.

A sharp decline of ssDNA was observed between 0.15 µM and 0.5 µM treatments with DACH and cisplatin. DACH treatment decreased ssDNA retention from 65.55 ± 21.30% to 26.06 ± 7.85% and cisplatin decreased by 22.32% from 54.97 ± 2.26% to 32.66 ± 4.08% between 0.15 µM and 0.5 µM of treatment. This contrasts with DCRP treatment decreased ssDNA retention from 58.45 ± 3.09% to 57.22 ± 10.92% between 0.15 µM and 0.5 µM of treatment.

Complete loss of the ssDNA band occurred for each compound indicating fully cross linked DNA. Cisplatin, DCRP and DACH showed no detectable ssDNA band intensity at treatment concentrations over 15 µM. No discernable ssDNA band was detected for oxaliplatin and DCBP at treatments that greater than 50 µM.
b) Strand breaker

It is expected that any DNA strand breaker compound would not form ICLs with a DNA product. Therefore, no higher order DNA structures than ssDNA would be observed on agarose slab gels. However, it is expected that strand breaker compounds that cleave the DNA phosphate backbone and subjected to denaturing slab gel electrophoresis conditions will produce a degraded smear of smaller DNA molecules.

Phleomycin was shown to induce a progressive depletion of ssDNA species in a dose dependant manner (Figure 5.3 a). Furthermore, the decline in ssDNA band retention was accompanied by an increased smear of degraded DNA molecules. Initially, phleomycin induced minimal loss of ssDNA species to 96.22 ± 10.58% for doses up to 0.5 µM. However, ssDNA species declined to 46.92 ± 14.05% for doses up to 1.5 µM. A complete loss of ssDNA detection occurred with phleomycin treatments up to 50 µM. On the other hand, Cu56MESS showed no ICL activity or degraded smear of DNA molecules (Figure 5.3 b).

c) Metallo-intercalators

3478MEEN was the only tested metallo-intercalator that induced ICLs at high treatment concentrations (Figure 5.4 b). 3478MEEN induced a steady decline in ssDNA species to 64.41 ± 26.87 % up to 15 µM of treatment. No ssDNA bands were detectable with 3478MEEN treatments up to 150 µM. 56MESS showed no ICL activity.

d) Groove binders

No ICL activity was detected for the groove binder compounds RuP1 and RuP2 (Figure 5.5). No higher order dsDNA species or degraded DNA molecule smears were observed.
Chapter 5: Detecting metallo-drugs that form ICLs on a DNA substrate
Figure 5.2: Fluorescent image of denaturing slab gel and semi-log plot of ssDNA densitometric quantification of 5′FAM DNA treated with covalent binder compounds cisplatin, oxaliplatin, DCBP, DCRP and DACH. Denaturing slab gel images show the electrophoretic migration of 5′FAM dsDNA (lane 1), its denatured ssDNA isoform (lane 2), SYBR labelled 100 bp ladder (lane 3), empty space (lane 4), denatured 5′FAM-dsDNA 5% DMF control (lane 5), and denatured 5′FAM DNA treated with (a) cisplatin, (b) oxaliplatin, (c) DCBP, (d) DCRP and (e) DACH at 0.015, 0.05, 0.15, 0.5, 1.5, 5, 15, 50 and 150 µM (lanes 6 to 14). Accompanying each slab gel is a semi-log plot depicting densitometric quantification of ssDNA bands presented as percentage function (y-axis) over compound concentration gradient expressed in µM (x-axis). Graphs are marked for 50% and 5% ssDNA retention.
Figure 5.3: Fluorescent image of denaturing slab gel and semi-log plot of ssDNA densitometric quantification of 5’FAM DNA treated with strand breakers phleomycin and Cu56MESS. Denaturing slab gel images show the electrophoretic migration of 5’FAM dsDNA treated with (a) phleomycin or (b) Cu56MESS. Other details are described in the legend of Figure 5.2.
Figure 5.4: Fluorescent image of denaturing slab gel and semi-log plot of ssDNA densitometric quantification of 5’FAM DNA treated with metallo-intercalator compounds 56MESS and 3478MEEN. Denaturing slab gel images show the electrophoretic migration of 5’FAM dsDNA treated with (a) 56MESS or (b) 3478MEEN. Other details are described in the legend of Figure 5.2.
Figure 5.5: Fluorescent image of denaturing slab gel and semi-log plot of ssDNA densitometric quantification of 5’FAM DNA treated with metallo-intercalator compounds RuP1 and RuP2. Denaturing slab gel images show the electrophoretic migration of 5’FAM dsDNA treated with (a) RuP1 or (b) RuP2. Other details are described in the legend of Figure 5.2.
5.2.3 Analysis of compounds that induce ssDNA modifications

Analysis of compounds that formed interstrand crosslinks to dsDNA was done by observing denaturing slab gels for higher order DNA structures and quantitatively comparing densitometric data for decreases in ssDNA. The presence of higher order DNA structures indicates that compounds induce ICL branching with multiple DNA strands to create higher molecular weight products. Quantifying ssDNA bands allows for comparison of different compounds’ effectiveness at inducing secondary level modifications. Table 5.1 summarises quantitative methods employed to analyse the effectiveness of the test compounds for modifying DNA secondary structure. Quantitative analysis for compound concentrations that induce ssDNA band loss to 50% and 5% was recorded.

Qualitatively, dsDNA bands were observed in experiments using the covalent binders cisplatin, oxaliplatin, DCBP, DCRP and DACH as well as the metallointercalator 3478MEEN. It was observed that the strand breaker phleomycin caused degradation of ssDNA bands. The other compounds 56MESS, Cu56MESS, RuP1 and RuP2 induced no observable change in ssDNA band integrity.

The concentration for compounds that induced ssDNA band loss to 50% and 5% was determined from the semi-log graphs in Section 5.2.2. The most effective compound for reducing ssDNA to 50% was cisplatin treatment at 0.197 µM, the least effective was 3478MEEN at 20.0 µM (Figure 5.6). The most effective compounds for reducing ssDNA to 5% was DACH treatment at 4.6 µM and the least effective was 3478MEEN at 46.5 µM (Figure 5.6).
Table 5.1: Summary table showing the $r_b$ (drug to nucleotide) ratio that retains 50% and 5% of the 5’FAM-ssDNA isoform for each compound and commentary of DNA integrity for dsDNA and ssDNA isoforms. Concentration where 50% and 5% ssDNA retention was determined by a straight line plot that tracks the fluorescent emission for dsDNA and ssDNA isoforms. Concentrations are converted into moles and incorporated into a ratio with the number of nucleotides present in the experimental system. Commentary on DNA integrity is provided about (a) the presence of the dsDNA isoform across the compound concentration gradient treatment and (b) the presence of a ssDNA degradation smear below the DNA isoforms’ apparent molecular weight. The number of nucleotides in each reaction was $9.69 \times 10^{-10}$.

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<tr>
<th>DNA Interaction</th>
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<th>Concentration (µM)</th>
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<th>$r_b$(drug: nucleotide)</th>
<th>DNA Integrity</th>
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<td>5% ssDNA</td>
<td>50% ssDNA</td>
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<tr>
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<tr>
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<tr>
<td></td>
<td>RuP2</td>
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Figure 5.6: Column graph showing the $r_b$ (drug to nucleotide) ratio that retains 50% and 5% of the 5’FAM-ssDNA isoform for each compound that yielded a detectable change. Drug to nucleotide ratios ($r_b$) where (a) 50% and (b) 5% ssDNA retention was determined by a straight line plot that tracks the fluorescent emission for dsDNA and ssDNA isoforms.
5.3 Discussion

5.3.1 Updated methodologies to improve the data generation for ICL analysis

Traditionally, sodium hydroxide based denaturing agarose slab gel electrophoresis has been used to induce H-bond melting and separation of DNA molecules, followed by a post-electrophoresis staining (Suchánková et al., 2009, Vrâna et al., 1996, Malina et al., 2011). These experiments can be problematic as sodium hydroxide can hydrolyse polysaccharides, allowing the marker dye and the gel matrix float out of the cast plate during electrophoresis. Furthermore, inaccuracies in downstream densitometry analysis could occur as the presence of sodium hydroxide and platinum DNA adducts can inhibit ethidium bromide from binding with DNA (Alonso et al., 2006).

This issue was addressed by pre-labelling the DNA by incorporation of a 5’FAM labelled primer to generate an 865 bp dsDNA product by PCR. This product has a 59% GC content and corresponded to the promoter element of the RUVBL2 gene. This approach enabled a ratio of one fluorescence unit to one DNA molecule which is a similar to a previous approach that used hazardous ³²P labelled primers (Woynarowski et al., 1998). Furthermore, this method eliminates the need for labelling with (for example) ethidium bromide which has previously been shown to be out competed for DNA binding sites by increased metallo-drug uptake and poor ssDNA uptake which resulted in decreases in densitometry measurements (Rajendran et al., 2008).

This section of work applies a recently developed urea-based agarose slab gel electrophoresis method to facilitate H-bond melting of the 5’FAM dsDNA template (Hegedus et al., 2009). Comparisons between the native dsDNA and denatured ssDNA isoforms on denaturing agarose slab gel showed a clear displacement between the two bands apparent molecular weight (Figure 5.1 b). Furthermore, the apparent molecular weight of the ssDNA was not half the apparent molecular weight of the dsDNA as intuitively expected, however, this may be
explained by possible H-bonding between the urea and free bases which could contribute to artificial increases in apparent molecular weight (Hong et al., 2004).

5.3.2 Comparative analysis of various metallo-drugs for their efficacy to induce DNA structure modifications

Conversion of DNA template treated with covalent binder metallo-drugs from ssDNA to dsDNA-like isoforms in a dose-dependent manner was clearly observed on denaturing agarose slab gels (Figure 5.2 a-e). This trend has previously been well reported with cisplatin and attributed to the covalent ICLs formed between the dsDNA strands that prevent their separation under H-bond melting conditions (Mlcouskova et al., 2012, Halámiková et al., 2008). Furthermore, closer inspection of the denaturing agarose slab gels at high cisplatin treatment concentrations also revealed faint DNA bands at higher molecular weights (Figure 5.2 a) which have previously been attributed to cisplatin ICL polymerised DNA branch structures which form the basis of DNA structure globulation when observed with atomic force (Hou et al., 2009) and fluorescent (Katsuda et al., 2009) microscopy.

Comparisons of ICL reactivity with covalent binder metallo-drugs was carried out by measuring the drug to nucleotide ratio ($r_b$) where the ssDNA isoform constituted 50% and 5% of total DNA structures (Table 5.1). Data presented here showed that cisplatin and DACH exhibited almost the same ICL reactivity (Figure 5.6) which is consistent with previously published ICL quantification values (Kasparkova et al., 2010). Furthermore, comparisons for ICL reactivity showed that oxaliplatin was less reactive than cisplatin (Figure 5.6 a&b) which is also in agreement with published literature that attributed this trend to oxaliplatin’s lower DNA binding kinetics (Woynarowski et al., 1998).

Data for the covalent binder metallo-drug DCRP showed that its ICL reactivity profile was almost the same as oxaliplatin (Figure 5.6 a&b). Furthermore, the DCRP and DCBP structural analogues showed the least ICL reactivity out of all covalent binder metallo-drugs.
tested. Differences between the DCRP and DCBP ICL reactivity profiles may be related to DCBP possessing a covalent bridge between the two pyridine ligand non-leaving groups which may increase steric hindrance and prevent the formation of a covalent bond.

The DNA strand breaker phleomycin was also included in this work as a positive control as it served to validate the expected DNA smear, which should be readily observed on denaturing slab gels when H-bonds are melted on a DNA template with multiple phosphate backbone breaks (Sleigh, 1976, Stern et al., 1974). Observations of denaturing agarose slab gels showing the 5′FAM dsDNA template treated with phleomycin showed a pattern where the ssDNA template degraded into a smear of smaller DNA molecules in a dose-dependent manner (Figure 5.3). Unfortunately, the strand breaker Cu56MESS did not produce any evidence of DNA cleaving due to the absence of a reducing agent (Krause-Heuer et al., 2012).

The groove binders RuP1 and RuP2 both did not modify ssDNA isoform populations (Figure 5.5 a&b, Figure 5.6 a&b) as they do not form ICLs (Orkey et al., 2012). Data presented here also showed that the metallo-intercalator 56MESS did not induce ICLs (Figure 5.4 a&c, Figure 5.6 a&b) which followed previous characterisation as a non-covalent DNA π-π base stacking compound (Krause-Heuer et al., 2009b, Wheate et al., 2007a).

Surprisingly, 3478MEEN did induce ICLs (Figure 5.4 b, Figure 5.6 a&b). This contradicts previous conclusions about the DNA binding mode of 3478MEEN as a metallo-intercalator incapable of forming covalent bonds with DNA templates (Krause-Heuer et al., 2009a). This data also supports previous observations made with plasmid mobility studies where it was concluded that 3478MEEN assumed two DNA binding mechanisms that are similar to the cisplatin analogue metallo-drug covalent binders and the metallo-intercalator 56MESS (Section 3.3.3).

In summary, this section of work applied updated molecular biology methodologies to measure, compare and verify selected metallo-drugs of different DNA binding modes for their
capability to form ICL. These updated molecular biology methods included the use of a urea-based denaturing agarose slab gel and 5′FAM labelled dsDNA template. To our knowledge, this combination of molecular biology techniques has not previously been published. Furthermore, metallo-drug ICL profiles of cisplatin, DACH and oxaliplatin data was concordant with results previously published in the literature, contributing to the validation of the ICL data produced from other compounds in this study. Finally, data was presented demonstrating that 3478MEEN designed for π-π base stacking intercalation also forms ICL covalent bonds with DNA.
Chapter 6: Characterisation of metallo-drugs that inhibit DNA replication by DNA polymerase

6.1 Introduction

The Linear Amplification (LA) reaction is a modification of the Sanger DNA sequencing technology (Sanger et al., 1977) developed in the 1970’s. DNA sequencing involves the use of dideoxynucleotides in which the 3’OH of the sugar is chemically modified to inhibit further strand extension by a DNA polymerase. The LA reaction has previously been employed to characterise DNA damage at the sub-gene and single nucleotide level (Ponti et al., 1991, Fox et al., 1998).

The application of LA reactions for metallo-drug sequence specificity studies also involves the inhibition of a DNA polymerase. However, the mechanism of the inhibition relies on the metallo-drug being bound to the DNA template which sterically blocks DNA polymerase extension. Sanger sequencing reactions are run concurrently with metallo-drug damage reactions to identify the position of the metallo-drug bound to the DNA template at base-pair resolution (Ponti et al., 1991, Temple et al., 2002). Furthermore, this method has previously been successfully applied to determine the sequence binding specificity of cisplatin (Murray et al., 1992) and other analogue compounds (Galea and Murray, 2010, Temple et al., 2002, Moumita and Murray, 2011). At the basic level, this type of methodology can be used to quantify decreases in full length DNA product extensions as a measure of metallo-drug induced damage across a broad region of DNA (Zhang and Poirier, 1997). Since the extension
products are run on a low resolving power agarose gel, it is not designed to resolve individual sites of adduct formation.

Previously, metallo-drug sequence specificity studies typically employed polyacrylamide slab gel electrophoresis and $^{32}$P radiolabelled techniques to determine the base binding preference for compounds of interest such as cisplatin and other analogues (Murray et al., 1997). However, advances in automated DNA sequencing technology coupled with laser induced fluorescent detection (CE-LIF) has dramatically improved the read length, precision and sensitivity of DNA fragment analysis in comparison to traditional slab gel electrophoresis (Kambara, 2010, Dolník, 1999). Recent experiments with the Applied Biosystems AB 3730 automated sequencer were shown to be sensitive enough to discriminate between ssDNA fragments with modified 3’- terminal nucleotides (Nguyen and Murray, 2012) or to quantify the sequence binding specificity of cisplatin with conserved DNA sequences such as telomere repeats from a plasmid DNA construct (Moumita and Murray, 2011).

In this chapter, two approaches were taken in performing the LA reaction for metallo-drug characterisation. The first approach compared test metallo-drugs’ effectiveness at inhibiting Taq DNA polymerase using the LA method with the incorporation of R6GdUTP nucleotides to facilitate fluorescent detection of the ssDNA products. However, the application of this protocol for base pair resolution detection of metallo-drug DNA binding sites was problematic and will be discussed later in Section 6.4.1. The second approach employed the LA method with a fluorescently labelled primer which proved to be a more robust and accurate method (Moumita and Murray, 2011) for base pair resolution detection of metallo-drug binding sites which will be discussed further in Section 6.4.2.
6.2 Characterising metallo-drug induced inhibition of DNA replication by Linear Amplification with Rhodamine-labelled dUTP detection

6.2.1 Results

This first approach focused on characterising the effectiveness of metallo-drugs at inhibiting full extension ssDNA LA products and Rhodamine-labelled dUTP was incorporated into the newly synthesised DNA for fluorescent detection. Covalent binders cisplatin, oxaliplatin, DCRP, DCBP and DACH, the strand breakers phleomycin and Cu56MESS, the DNA metallo-intercalators 56MESS and 3478MEEN and DNA groove binders RuP1 and RuP2 were incubated at \( r_b \) values ranging from 0.0001 (0.015 \( \mu \)M) to 0.3290 (50 \( \mu \)M) with a linearised pUC19 (ScaI) template. Products of these LA reactions were run on agarose slab gels using densitometry analysis to measure the intensity of the full extension ssDNA products.

6.2.1.1 Preparation of a DNA template for LA reactions

The methodology used to isolate the pUC19 template from \( E. coli \) DH5\( \alpha \) by alkaline lysis and its confirmation by \( PvuII \) restriction digest and PCR was previously described in Section 2.2.6. Drug/DNA incubation reactions were performed in triplicate as described in Section 2.2.11. Each reaction contained 1.8 \( \mu \)g of linear pUC19, incubation buffer and test metallo-drug. Reactions with phleomycin were supplemented with 25 mM DTT to facilitate strand breaking (Sleigh, 1976, Stern \( et \) al., 1974). Negative control reactions were prepared with either DNase-free water or 5% DMF solvent substituted for the experimental compound. Phleomycin negative control reactions consisted of DNase-free water or 5% DMF solvent supplemented with 25 mM DTT. All reactions were terminated by ethanol precipitation (Section 2.2.5) and the DNA pellet was resuspended in DNase-free water to a final concentration of 15 ng/\( \mu \)L.
Figure 6.1: A simple graphic of pUC19 plasmid showing the ScaI position in relation to the primer annealing site and the LA full extension ssDNA product. The figure shows the full 997 bp extension ssDNA LA product (▬) from a pUC19 template with a ScaI restriction digest site and a pUC/M13 22 mer reverse primer (▬).
6.2.1.2 Slab gel and semi-log densitometry plots of R6G-labelled ssDNA LA full extension products from linear pUC19 treated with metallo-drugs

Synthesis of carboxy-rhodamine-6G-dUTP (R6GdUTP) nucleotides for incorporation into, and subsequent visualisation of, ssDNA LA products was carried out as described in Section 2.2.14. LA reactions with the treated pUC19 DNA template (along with water and DMF treated control samples) and R6GdUTP nucleotides were carried out in triplicate as described in Section 2.2.15. A diagram showing the pUC19 construct, Scal restriction site, primer annealing site and ssDNA full extension product is shown in Figure 6.1. The R6GdUTP-labelled ssDNA LA products were subjected to native 1% agarose gel electrophoresis (Section 2.2.17), imaged with LAS4000 fluorescent gel dock and ssDNA band densitometry measurements were undertaken using Multiplex v8.0 and data graphed by OriginPro v8.5 (Section 2.1.11). Data was plotted on semi-log line graphs over the treatment concentration gradient for metallo-drug performance comparison.

The full extension product was 997 bases in length which is equivalent to approximately 498 bp as measured against a 100 bp dsDNA ladder. As expected, the full extension of the water or DMF control samples LA products measured against a 100 bp ladder ($r^2 = 0.989 \pm 0.009$) on agarose slab gel yielded a single band running at 419 ± 20 bp or 413 ± 18 bp, respectively.

a) Covalent Binders

In the first instance, a comparison of the LA products generated from the DNA template damaged by the covalent binder metallo-drugs were visualised on a native 1% agarose slab gel (Figure 6.2). Densitometry measurements of the full extension products were performed and converted to a percentage value relative to the undamaged 5% DMF control that exhibited maximal extension.

Comparative analysis of these data showed that DACH adducts were the most efficient at inhibiting the Taq DNA polymerase with concentrations of 0.05 µM of higher resulting in no
extension product (Figure 6.2 e, lanes 4 & 4'). Cisplatin (Figure 6.2 a, lanes 8 & 8') and oxaliplatin (Figure 6.2 b, lanes 8 & 8') were less effective than DACH at inhibiting the polymerase since concentrations greater than 1.5 μM were required to inhibit full product extension. Furthermore, the cisplatin gels (Figure 6.2 a) show smears of DNA extension products with lower molecular weight than the full extension product. The intensity of these products was generally inversely proportional to the full extension product, i.e. as the partial extension products increase in densitometry value the full length extension products decreases, this trend is more apparent whilst looking across samples treated with an increasing drug concentration.

A closer inspection of the gels using semi-log plots of the densitometry measurements show that the decrease in percentage of the full extension product intensity quantitatively reflected what was observed on native slab gels and show an eventual decline to near zero for all covalent binder compound treatments. DACH was the most effective at decreasing ssDNA full extension bands to 6.65 ± 2.87% at 0.05 μM of treatment and finally 0 % at 15 μM (Figure 6.2 e). Cisplatin and oxaliplatin were the least effect at inhibiting ssDNA full extension with bands decreasing in quantity to 3.84 ± 1.83% and 8.28 ± 3.58%, respectively, at 15 μM treatment (Figure 6.2 a&b).

b) Strand Breaker

Inspection of native agarose slab gels (column 1) revealed that the full extension ssDNA product was not detectable with phleomycin treatments greater than 0.5 μM (Figure 6.3 a, lanes 7 & 7'). Native slab gel also revealed phleomycin induced smears with lower molecular weight than the full extension ssDNA product that appear to increase in densitometry value as the full extension band decreases with increasing compound treatment. As expected, densitometry plots quantitatively reflect what was observed on native slab gels and show an eventual decline to near zero with phleomycin treatment. Treatment with phleomycin at 0.5
µM decreased full extension ssDNA to 77.43 ± 8.32%. At 1.5 µM, full extension of ssDNA decreased to 2.71 ± 1.89% then zero detection with treatments greater than 5 µM.

LA reaction products derived from the treatment of the template DNA with the strand breaker Cu56MESS (Figure 6.3 b) showed a decrease but no complete loss of full extension ssDNA bands. Cu56MESS (Figure 6.3 b) induced a maximum decrease in ssDNA band intensity to 51.42 ± 1.8% at 5 µM, but then increased to 64.48 ± 3.72% at 50 µM treatment.

c) Metallo-intercalators

Inspections of native slab gels reveal complete loss of the full extension ssDNA product with 3478MEEN treatment greater than 15 µM (Figure 6.4 b). However, 56MESS (Figure 6.4 a) treatment showed a decrease but no complete loss of full extension ssDNA bands.

Densitometry semi-log plots of these data quantitatively reflect what was observed on native slab gels. Treatments with 3478MEEN (Figure 6.4 b) at 15 µM decreased full extension ssDNA to 22.4 ± 0.23% and at higher concentrations no product was observed. The lowest percentage decrease of the full extension ssDNA product observed for 56MESS (Figure 6.4 a) was 4.87 ± 1.4% at a relatively high treatment concentration of 50 µM.

d) Groove Binder

Inspections of native slab gels revealed both compounds minimally decreased, but never completely inhibited the ssDNA full extension product. The full extension bands for RuP2 treatment appear to have a higher densitometry value (Figure 6.5 b) relative to the DMF control bands. This is due to a possible error in the DMF sample preparation and this was not corrected for in the initial analysis. Furthermore, the 1.5 µM RuP2 treatment appears to be an outlier relative to the DMF control and other data points. RuP1 (Figure 6.5 a) appears to increase ssDNA full extension with the maximum extension recorded at 161.01 ± 16.34 % at
0.05 µM and minimum recorded value of 79.73 ± 5.22 %. These densitometry variations are possibly due to operational error.
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a) [Graph showing compound concentration vs. % full extension for cisplatin]

b) [Graph showing compound concentration vs. % full extension for oxaliplatin]

c) [Graph showing compound concentration vs. % full extension for DCBP]

d) [Graph showing compound concentration vs. % full extension for DCRP]

e) [Graph showing compound concentration vs. % full extension for DACH]
Figure 6.2: Slab gel and semi-log densitometry plots of R6G-labelled ssDNA LA products from linear pUC19 (ScaI) DNA template treated with covalent binder compounds. Linear pUC19 (ScaI) template was treated with covalent binder compounds (a) cisplatin, (b) oxaliplatin, (c) DCBP, (d) DCRP and (e) DACH at 0.015, 0.05, 0.15, 0.5, 1.5, 5, 15 and 50 µM (lanes 5-11 & 5′-11′) along with solvent DMF control (lane 3) and water control (lane 1) then subjected to LA reactions containing R6GdUTP for fluorescent detection. R6G-labelled ssDNA products were subjected to agarose gel electrophoresis along with SYBR-labelled 100 bp ladder (lane 2). Full extension R6G-labelled ssDNA products were analysed by fluorescent densitometry and plotted on semi-log straight line graphs.
Figure 6.3: Slab gel and semi-log densitometry plots of R6G-labelled ssDNA LA products from linear pUC19 (ScaI) DNA template treated with strand breaker compounds. Linear pUC19 (ScaI) template was treated with the strand breaker compounds (a) phleomycin and (b) Cu56MESS then subjected to LA reactions containing R6GdUTP for fluorescent detection. Other details are described in the legend of Figure 6.2.
Figure 6.4: Slab gel and semi-log densitometry plots of R6G-labelled ssDNA LA products from linear pUC19 (ScaI) DNA template treated with metallo-intercalator compounds. Linear pUC19 (ScaI) template was treated with metallo-intercalator compounds (a) 56MESS and (b) 3478MEEN then subjected to LA reactions containing R6GdUTP for fluorescent detection. Other details are described in the legend of Figure 6.2.
Figure 6.5: Slab gel and semi-log densitometry plots of R6G-labelled ssDNA LA products from linear pUC19 (Scal) DNA template treated with groove binder compounds. Linear pUC19 (Scal) template was treated with metallo-intercalator compounds (a) RuP1 and (b) RuP2 then subjected to LA reactions containing R6GdUTP for fluorescent detection. Other details are described in the legend of Figure 6.2.
6.2.1.3 Analysis of metallo-drugs that induced decreases in R6G-labelled ssDNA LA full extension synthesis to 50% and 5%

The semi-log plots of the densitometry data presented in the previous section represented trends of the metallo-drugs ability to inhibit the Taq DNA polymerase to fully extend the treated template. These graphs allowed for quantitative interpretation of the data to determine the metallo-drug treatment concentrations at which full extension product intensity was decreased to 50% and 5%. This data was then converted to drug to nucleotide ratios (r_b) where each metallo-drug can be compared.

The data presented in Table 6.1 shows the most effective metallo-drug to inhibit the Taq DNA polymerase and decrease extension to 50% was DACH at a concentration of 0.04 µM. The most effective metallo-drug to decrease ssDNA full extension to 5% was again DACH at 0.07 µM. Cu56MESS, RuP1 and RuP2 did not decrease the ssDNA full extension products to 50% within the concentration range applied, relative to that of the lead cisplatin compound, therefore no calculation was possible.

The data described in Table 6.1 was converted into r_b values and plotted on a vertical bar graph for comparison of metallo-drug performance for ssDNA full extension inhibition to 50% (Figure 6.6 a) and 5% (Figure 6.6 b). The least effective metallo-drug to reduce ssDNA full extension to 50% was cisplatin at r_b 0.0061 while the most effective was DACH at r_b 0.0002. The least effective metallo-drug to reduce ssDNA full extension to 5% was 56MESS at r_b 0.318 while the most effective was again DACH at r_b 0.0004. Cu56MESS, RuP1 and RuP2 did not decrease ssDNA full extension products to 50% or 5%, therefore no data was presented.

This protocol did facilitate the detection of the overall ability of the metallo-drugs to inhibit the DNA polymerase as measured by a reduction in intensity of the full length extension product. In addition, these data provided a guide for the appropriate drug to nucleotide ratios.
(rₖ) that were used in subsequent experiments. Furthermore, to the best of our knowledge, the incorporation of a Rhodamine 6G labelled nucleotide into an LA product to detect metallo-drug induced DNA damage has not previously been done. In addition, this method is advantageous as it is quick and low cost to detect drug/DNA binding though DNA polymerase inhibition within the constraints of the body-labelling setup.
Table 6.1: A summary of LA inhibition by drug treatment of template DNA. The drug treatment concentrations that reduce the synthesis of full extension ssDNA products to 50% and 5% in LA reactions were determined from semi-log densitometry plots (column 2) and compound amounts in reactions were determined (column 3). These compound concentrations were converted to drug to nucleotide ratios ($r_b$, column 4) for comparison of compound effectiveness at inhibiting ssDNA synthesis.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Compound Concentration (µM)</th>
<th>Compound Amount (moles)</th>
<th>Drug to Nucleotide Ratio ($r_b$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50% ssDNA</td>
<td>5% ssDNA</td>
<td>50% ssDNA</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>0.929</td>
<td>4.866</td>
<td>3.7196E-11</td>
</tr>
<tr>
<td>Oxaliplatin</td>
<td>0.093</td>
<td>7.921</td>
<td>3.7588E-12</td>
</tr>
<tr>
<td>DCBP</td>
<td>0.296</td>
<td>0.496</td>
<td>1.1842E-11</td>
</tr>
<tr>
<td>DCRP</td>
<td>0.245</td>
<td>0.499</td>
<td>9.8144E-12</td>
</tr>
<tr>
<td>DACH</td>
<td>0.035</td>
<td>0.064</td>
<td>1.4068E-12</td>
</tr>
<tr>
<td>Phleomycin</td>
<td>0.766</td>
<td>1.468</td>
<td>3.0646E-11</td>
</tr>
<tr>
<td>Cu56MESS</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>ND</td>
</tr>
<tr>
<td>56MESS</td>
<td>0.376</td>
<td>48.297</td>
<td>1.5064E-11</td>
</tr>
<tr>
<td>3478MEEN</td>
<td>0.067</td>
<td>37.086</td>
<td>2.6912E-12</td>
</tr>
<tr>
<td>RuP1</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>ND</td>
</tr>
<tr>
<td>RuP2</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND = indicate the compound concentration for 50% and 5% ssDNA total ssDNA synthesis was not detectable.
Figure 6.6: Graphs showing drug to nucleotide ratios ($r_b$) of compounds that induced a reduction of ssDNA synthesis to 50% and 5%. LA reactions with linear pUC19 treated with a range of DNA interacting compounds over a concentration gradient induced a reduction in ssDNA synthesis. The $r_b$ values that induced a reduction in ssDNA synthesis to (a) 50% and (b) 5% were determined from densitometry analysis and graphed to compare the effectiveness of different compounds to induce reductions in ssDNA synthesis. Metallo-drugs with a red cross (×) indicate that reduction in ssDNA synthesis to 50% and 5% was not detectable within the experimental parameters.
6.3 Characterising the DNA sequence specificity of metallo-drugs by Linear Amplification

6.3.1 Results

Previously in Section 6.2, the Linear Amplification (LA) method was employed to broadly measure *Taq* DNA polymerase inhibition of ssDNA synthesis by a selection of metallo-drugs. Recent developments in fluorescent-based fragment analysis and various experimental protocols have lead to their application to characterise the metallo-drug DNA binding sequence specificity of DNA damaging agents at base pair resolution (Galea and Murray, 2010, Moumita and Murray, 2011). This Section of work employed a similar approach to compare the sequence specificity with a selection of metallo-drugs which exhibit a variety of DNA binding modes.

All metallo-drug reaction peaks and dideoxy sequencing peaks were aligned by the LIZ500 size standard. The LIZ500 size standard served as an internal control and facilitated the assignment of a common reference number to all dideoxy sequencing and metallo-drug reaction peaks. This approach enabled the alignment of metallo-drug reaction peaks to the DNA template sequence. Downstream analyses of the site of adduct formation considered the base pair identity of the site of DNA replication termination and an analysis of the adjacent base at the site of termination.

6.3.1.1 Preparation of DNA template for LA reactions

These LA experiments used pUC19 linearised by *PvuII* restriction enzyme digest as the DNA template. The methodology used to isolate pUC19 from *E. coli* DH5α by alkaline lysis and its confirmation by restriction digest and PCR are similar to those previously described. Restriction digest of pUC19 with *PvuII* restriction enzyme was analysed with 1% agarose slab gel electrophoresis. As expected, this gave rise to two bands with apparent molecular weights
of 2364 bp and 322 bp when compared to a 1 kb ladder (Figure 6.8). The smaller of these two fragments was used as the template DNA for subsequent amplification reactions.

### 6.3.1.2 Linear amplification dideoxy sequencing

LA and dideoxy sequencing reactions were performed using either Primer A (5′FAM-pUC/M13, Rev, 22 mer) or Primer B (5′FAM-pUC/M13, Rev, 17 mer) as described in Section 2.2.16. A diagram showing the pUC19 construct, PvuII restriction site, Primer A and B annealing sites and full extension LA ssDNA product are shown in Figure 6.7.

Samples were submitted to The Ramaciotti Centre for Gene Function Analysis at the University of New South Wales (Sydney, Australia) where they were spiked with a LIZ500 size standard marker and subjected to AB3750 capillary electrophoresis. Fluorescent electropherograms were analysed using PeakScanner v1.0 and data graphed with OriginPro v8.5 (Section 2.1.11).

It was found that 480 ng of linear pUC19 (PvuII) and 2 pmol of primer created optimal conditions for all linear amplification applications. Both the DNA template and primer quantity were considered during optimisation of the LA reaction which is shown as supplementary data in Appendix B. Dideoxy nucleotide concentrations for dideoxy-based sequencing by linear amplification with 1 mM/base ddATP, 0.5 mM/base ddCTP, 0.5 mM/base ddGTP and 1.75 mM/base ddTTP were chosen as they were they gave rise to the best electropherogram traces (supplementary data for sequencing optimisation in Appendix B).

Dideoxy sequencing experiments were performed using either Primer A (5′FAM-pUC/M13, Rev, 22 mer) (Figure 6.9 a) and Primer B (5′FAM-pUC/M13, Rev, 17 mer) (Figure 6.9 b). Both primers amplify the same region of pUC19, although, Primer B produced a ssDNA product 5 bp shorter than Primer A as shown in Figure 6.7. The rational for using two primers with a 5 bp displacement will be addressed in Section 6.4.2.
As expected, the results of the dideoxy sequencing with either primer matched that of the published sequence of pUC19 on the NCBI database (Accession No: M77789). Excess FAM-labelled primer artefacts obscured the distinction of individual peaks at smaller fragment sizes. However, as expected electropherograms from both Primer A and Primer B showed full extension to the position of 376 bp which corresponded to the \textit{PvuII} restriction site. The LIZ500 ssDNA molecular weight size standards of 50, 75, 100, 139, 150 and 160 bp were detected with both primers’ electropherogram traces with reproducible mobility. Importantly, these data were used to accurately assign relative fragment sizes according to the LIZ500 size standards.

The electropherogram traces from the LA data were proven to have high levels of background peaks evident in the DMF controls. These were problematic and to some degree indeterminate and disregarded. These problems may have been due to imperfect optimisation of the LA reaction mixture, over-drying of the DNA during cleanup or due to technical issues with the fragment analysis apparatus. Subsequently, a higher proportion of noise was incorporated into the data analysis than was preferable. Despite this, the overall trends of the control compounds are consistent with the published literature. Furthermore, whilst conclusions are drawn for the novel test compounds these data and conclusions should be considered provisional and these require further duplication in the future. It is not possible to repeat these data within the constraints of this candidature.
Figure 6.7: A simple graphic of pUC19 plasmid showing the PvuII restriction site and the two 5’FAM labelled primer annealing sites for LA full extension reactions. The figure shows the full extension ssDNA LA products (▬) from a pUC19 template with a PvuII restriction digest and 5’FAM labelled primers (a) Primer A (▬) and (b) Primer B (▬) which yield full extension ssDNA products of 246 bp and 241 bp, respectively.

Figure 6.8: Image of agarose slab gels showing the electrophoresis separation of linear pUC19 (PvuII). Image shows the 1 kb ladder (lane 1) and pUC19 fragments resulting from a restriction enzyme digest with PvuII yielding two bands at 2364 bp and 322 bp (lane 2).
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b)
Figure 6.9: Electropherograms showing dideoxy sequencing of linear pUC19 (PvuII) using 5’FAM-pUC/M13(Rev) Primer A (22 mer) and Primer B (17 mer). Electropherogram traces of sequencing reactions for the nucleotides ddA (-), ddC (-), ddG (-) and ddT (-) along with the LIZ500 size standard alignment (-) using the 5’FAM labelled (a) Primer B (17 mer) and (b) Primer A (22 mer) primers. Labelled x-axis shows the parent strand sequence identity (row 1), peak position relative to the numbering of the pUC19 sequence published taken from NCBI (row 2) and fragment size according to the LIZ500 marker (row 3).
6.3.1.3 Identifying a bias in the base selectivity of metallo-drug DNA binding sites

Negative control reactions were prepared in which either DNase-free water or 5% DMF solvent was substituted for the experimental compound. Phleomycin negative control reactions consisted of water or 5% DMF solvent supplemented with 25 mM DTT. The reactions were terminated by ethanol precipitation (Section 2.2.5) and the DNA pellet was resuspended in DNase-free water to produce a final concentration of 100 ng/µL. The drug/DNA incubation reactions were performed in triplicate.

Optimisation of final drug concentration is discussed in Section 6.4.2 and supplementary material in Appendix D. The optimal conditions of the DNA damage reaction were determined empirically to give a LA trace with an even size distribution of peaks. Over damage of the template reduces the extension products resulting from larger fragments, whilst under damage gives rise to more full length extension and weak peak intensities overall. Electropherogram peaks identified for all tested metallo-drugs along with their LIZ500 alignment positions are shown as supplementary data in Appendix E (Figure E1). Example electropherogram trace for DCBP is shown in Figure 6.10. Note that some of these traces contain high background peaks in the control reaction and need to be repeated, despite this the data was still processed and correlates with published literature.

The LIZ500 was included in both the dideoxy sequencing traces and the LA of the damage samples. Whilst the running position (fragment size) of the LIZ500 is inaccurate due to its different sequence composition, it still produced a useful common internal reference to match the position of peaks in different electropherograms. Electropherogram peaks identified as drug-induced DNA damage sites were aligned by matching the LIZ500 alignment positions of the DNA damage peaks to the nearest dideoxy sequencing peak. The damage peak alignments are shown as supplementary data in Appendix E for Primer B (17 mer) (Table E1) and for Primer A (22 mer) (Table E2). Analysis of the DNA damage peaks by both primers was done
by comparing the number of damage sites induced by each test metallo-drug (Figure 6.11 a) and the composition for each base expressed as a percentage (Figure 6.11 b).

The quality of some replicate LA traces was not as good as expected based on the initial experiments performed with the cisplatin treated samples. The experiments were repeated in triplicate with two different primers. It was unclear what factors had changed leading to the noisy traces shown in Figure E1. Of these, the results of the covalent binder metallo-drugs provided the best quality data. Clearly more work must be performed to optimise these experiments. Preliminary analysis of the control compounds was, however, concordant with previously published trends as discussed in Section 6.4.2.

\[ a) \text{ Covalent Binders} \]

Oxaliplatin induced the highest number of DNA damage sites with an average of 62 ± 6 peaks while DACH induced the lowest number of DNA damage sites with an average of 32 ± 2.5. Cisplatin induced an average of 43 ± 3.5 DNA damage sites. DCRP and DCBP induced an average of 40 ± 5.5 and 39 ± 0.5 peaks, respectively.

All covalent binders showed preference for inducing DNA damage corresponding to purine bases. Cisplatin showed the greatest binding preference to guanine bases at 53.4 ± 5.57% while DCRP showed the least binding preference to guanine bases at 38.4 ± 6.05%. DACH showed the greatest binding preference to adenine bases at 37.83 ± 3.35% while DCRP showed the least binding preference to adenine bases at 29.18 ± 8.59%.

There were also some minimal DNA damage sites corresponding to pyrimidine bases. DCRP showed the highest damage sites corresponding to both cytosine at 17.32 ± 6.21% and thymine at 15.10 ± 8.43%. DACH induced the least damage sites for cytosine at 9.84 ± 3.96% while cisplatin induced the least damage sites for thymine at 6.72 ± 4.15%. These relatively high values may be a manifestation of the high levels of background damage in the LA data.
**b) Strand Breaker**

Phleomycin induced an average of 52 ± 18.5 DNA damage sites. Phleomycin showed higher binding preference to purine bases with guanine damage the highest at 33.10 ± 0.24% and adenosine at 26.32 ± 5.11%. To a lesser extent, phleomycin also induced damage to pyrimidine bases with thymine the highest at 22.12 ± 2.12% and cytosine at 18.46 ± 2.75%.

Cu56MESS induced an average of 45 ± 6 peaks DNA damage sites. Cu56MESS showed a binding preference for guanine bases at 32.35 ± 0.98% and adenine bases at 24.96 ± 4.45%. Cu56MESS showed a higher binding preference for cytosine bases at 23.91 ± 4.30% than phleomycin. Cu56MESS binding preference for thymine bases was measured at 18.78 ± 0.83%.

c) **Metallo-intercalators**

3478MEEN induced the highest number of DNA damage sites with an average of 61 ± 14.5 peaks. 56MESS induced an average of 57 ± 18 DNA damage sites. Metallo-intercalator base binding preference was more evenly distributed amongst the four bases, with a slightly higher preference for guanines. 3478MEEN showed the highest binding preference for guanines at 34.23 ± 4.90%, followed by 56MESS at 31.33 ± 2.00%. 56MESS showed the highest binding preference for adenine bases at 26.26 ± 5.74%, followed by 3478MEEN at 23.96 ± 0.04%.

Metallo-intercalator binding preference for pyrimidine bases was of similar magnitude to purine bases. Cytosine base binding preference between 3478MEEN and 56MESS was similar at 23.12 ± 3.55% and 21.49 ± 4.15%, respectively. Binding preference for thymine bases was also detected with 56MESS showing the highest preference at 20.92 ± 0.41%, followed by 3478MEEN at 18.70 ± 1.30%.
d) **Groove Binders**

The groove binders induced similar levels of DNA damage sites with an average of 57 ± 14 for RuP1 and 57 ± 18 for RuP2. The base binding preference for both compounds was very evenly distributed with RuP1 showing the greatest binding preference for guanine and thymine bases at 29.41 ± 5.47% and 25.01 ± 1.75%, respectively. RuP2 showed the greatest binding preference for adenine and cytosine bases at 28.10 ± 0.10% and 26.77 ± 1.44%.
Figure 6.10: Example electropherogram showing DCBP induced ssDNA truncation peaks overlayed with DMF control trace from a LA reaction using 5’FAM-pUC/M13(Rev) Primer B (17 mer). Electropherogram traces of cisplatin induced ssDNA truncation peaks (▬) and DMF solvent control background peaks (▬) along with the LIZ500 size standard alignment (▬) using the 5’FAM labelled 17 mer primer. Truncation peaks have been labelled with the corresponding base according to the known pUC19 sequence. The x-axis (units in base pairs, Bp) identifies selected significant peaks and their associated nucleotides and the y-axis represents fluorescence intensity (in Absorption units, Au).
Figure 6.11: Vertical bar graphs showing the number of peaks identify of the base at which damage occurred for all tested compounds using both Primer A (22 mer) and Primer B (17 mer). A bar graph showing the percentage (%) of sites where ssDNA synthesis terminated for all test compounds (a). A bar graph showing the base selectivity of ssDNA synthesis terminated for all test compounds (b). Values are expressed as a percentage of total damage sites with error bars representing standard deviation.
6.3.1.4 Base binding bias of test metallo-drugs homology with cisplatin

This section compares the one base DNA binding specificity of analogue metallo-drugs to cisplatin. Comparing the number of DNA binding sites at a fixed treatment concentration provides a basis to identify the relative frequency of damage of each metallo-drug compared to cisplatin. Furthermore, an analysis of the specific bases where DNA replication was inhibited compared to the well known purine binding specificity of cisplatin can help elucidate the DNA binding mechanisms of individual metallo-drugs.

a) Frequency of DNA binding sites compared to cisplatin

Three covalent binders were the only metallo-drugs found to have lower DNA binding sites than cisplatin (Figure 6.12 a). DACH showed the lowest number of binding sites at 74%, followed by DCBP at 91% and then DCRP at 94%.

All other tested compounds showed increases in the frequency of DNA binding compared to cisplatin. Oxaliplatin showed the biggest increase to 147% with the other test covalent binder carboplatin only increasing by 122%. Metallo-intercalator 3478MEEN displayed the second highest increase, increasing to 144% with the other analogue 56MESS increasing by 137%, respectively. The groove binder RuP2 showed the third highest increase to 137% while RuP1 increased to 136%. The strand breaker phleomycin showed a moderate increase in DNA binding, increasing to 124%.

b) Base binding preference compared to cisplatin

Cisplatin is well known for binding to purine bases, with high a preference to guanine residues. By comparing the binding preference between cisplatin and other metallo-drugs, it is possible to elucidate a relationship between metallo-drug structure and base binding preference. The base binding comparison between cisplatin and other tested metallo-drugs is
show in Figure 6.12 the binding value of cisplatin for adenine (b), cytosine (c), guanine (d) and thymine (e) is arbitrarily set to 100% for each condition.

Cisplatin showed the highest binding preference for guanine bases out of all tested metallo-drugs (Figure 6.12 d). Both ruthenium groove binders showed the lowest binding preference compared to cisplatin with RuP2 at 46% and RuP1 at 56%. The metallo-intercalators showed a slightly higher guanine binding preference with 56MESS at 59% and 3478MEEN at 65% while the strand breaker phleomycin showed 62% homology. All covalent binders showed the closest homology to cisplatin with DCBP the closest at 97%, then oxaliplatin at 85%, carboplatin at 79%, DACH at 76% and DCRP at 71%.

All covalent binder metallo-drugs showed a higher affinity than cisplatin for binding to adenine bases with DACH the highest at 129%, followed by carboplatin at 119%, then DCBP at 102%, DCRP at 100% and oxaliplatin at 100% (Figure 6.12 b). The ruthenium groove binder RuP1 showed the least homology with 70% while its analogue RuP2 showed the closest homology at 95%. The metallo-intercalator 56MESS showed a closer homology with cisplatin at 90%, followed by 3478MEEN at 81%. The strand breakers phleomycin and Cu56MESS showed a binding homology with cisplatin of 90% and 85%, respectively.

For cytosine bases (Figure 6.12 c), covalent binders showed similar homology to cisplatin with carboplatin and DCRP showing increases of 101% and 162%, respectively, while covalent binders DACH, oxaliplatin and DCBP showed decreases of 92%, 98% and 98%, respectively. The ruthenium groove binders RuP2 and RuP1 showed the highest increases of 253% and 237%, respectively. All metallo-intercalators showed increases with 3478MEEN at 219%, then 56MESS at 202%. Both strands breakers Cu56MESS and phleomycin showed an increase in binding to cytosine bases compared to cisplatin of 225%, and 174%, respectively.

For thymine bases (Figure 6.12 e), all covalent binders showed the closest homology to cisplatin with DCBP the lowest at 87%, followed by oxaliplatin, DCRP, carboplatin and
DACH, which all showed an increase in homology of 355%, 233%, 205% and 186%, respectively. RuP1 increased to 543% and RuP2 increased to 423%. All metallo-intercalators showed an increase with 56MESS the highest at 444%, followed by 3478MEEN at 406%. Phleomycin and Cu56MESS showed an increase in binding to thymine bases compared to cisplatin at 444% and 403%, respectively.
Figure 6.12: Comparison of the number of metallo-drug DNA binding sites and the mono-functional base specificity composition in comparison to cisplatin. Percentage differences in metallo-drug damage site frequency were compared to cisplatin (a). Comparison of base binding specificity for all tested metallo-drugs to cisplatin are expressed as a percentage for adenine (b), cytosine (c), guanine (d) and thymine (e). Values are expressed as a percentage with standard deviation error bars.
6.3.1.5 Two base sequence specificity analysis of metallo-drug DNA binding site

Damage base site composition, along with 3’ adjacent base, were compared to better elucidate the DNA binding sequence specificity of the tested metallo-drugs with both Primers A and B (Figure 6.13).

a) Covalent binders

The two base analysis of the damage sites due to metallo-drug covalent binders showed clear ssDNA truncation clustering around A-x and G-x bases (Figure 6.13a). The highest G-x truncation base combination was GG with DCBP displaying the largest preference out of all tested covalent binder metallo-drugs at this site at 20%. Oxaliplatin showed the least preference at 14%. The lowest G-x truncation base combination was a GT combination with DACH at 4%. The highest A-x truncation base combination was AG with DCRP at 15% while oxaliplatin showed the least preference at 9%. The AC base combination with DCRP showed the least binding preference out of all A-x base combinations at 3% while DACH showed the highest at 6%.

b) Strand breaker

The two base analysis of phleomycin damage sites did not show clear clustering of ssDNA truncation sites around any base-x combination (Figure 6.13b). The highest ssDNA truncation base combination for phleomycin was AA at 11% while the lowest ssDNA truncation combination was CA at 2%. Cu56MESS showed a low GG binding preference at 6% and with TA base combination at 1%.

c) Metallo-intercalators

The two base analysis of 56MESS and 3478MEEN damage sites did not show clear clustering of ssDNA truncation sites around any base-x combination (Figure 6.13c). The highest
ssDNA truncation preference was recorded for 3478MEEN at GG base combination with 14%. The highest TA base combination was with 3478MEEN at 4%.

d) *Groove binder*

The two base analysis of the RuP1 and RuP2 damage sites did not show clear clustering of ssDNA truncation sites around any base-x combination (Figure 6.13 d). The highest ssDNA termination base combination was GA with RuP1 at 11% while RuP2 showed 5%. The lowest ssDNA termination base combination was AT at 0% for RuP1 and 1% for RuP2.
Figure 6.13: Two base composition of the sites of damage for all tested compounds using both Primer A (22 mer) and Primer B (17 mer).

Vertical bar graphs show the two base compositions of sites where ssDNA synthesis terminated for all test compounds, expressed as a percentage with standard deviation error bars. Metallo-drugs are separated into DNA binding mode for (a) covalent binders, (b) strand breakers, (c) metallo-intercalators and (d) groove binders.
6.3.1.6 Two base sequence specificity comparison of metallo-drug base binding homology with cisplatin

This section compares the two base DNA binding specificity of tested metallo-drugs with cisplatin. Cisplatin is the lead metallo-drug and forms a benchmark of comparison for any newly developed analogue. By comparing metallo-drug induced ssDNA truncation homology with cisplatin, conclusions can be drawn about the potential pharmacological activity of new analogue compounds (Figure 6.14). For this comparison, the data previously presented in Figure 6.13 has been presented again although in this instance that data has been normalised with respect to the cisplatin data. The data is intentionally repeated to some degree to facilitate the additional analysis.

a) Covalent binders

The greatest increase in non-cisplatin ssDNA truncation homology was with the TG base combination and DCRP at 105% where DCBP showed the same homology with cisplatin at 100%. The greatest decrease in non-cisplatin ssDNA truncation homology was the GG base combination with oxaliplatin at 93% while DCBP showed 101%. There were no clear differences in cisplatin ssDNA truncation two base homology with any other covalent binder metallo-drug (Figure 6.14 a).

b) Strand breaker

Clear differences were seen with cisplatin ssDNA truncation homology and phleomycin (Figure 6.14 b). The greatest increase in non-cisplatin ssDNA truncation homology was with TT at 105% and TG at 105% two base combinations. The greatest decrease in non-cisplatin ssDNA truncation homology was with the GG two base combination at 88%. Cu56MESS showed some increase binding preference to T-x and C-x sequences in comparison to cisplatin, but showed similar binding preferences to phleomycin.
c) **Metallo-intercalator**

Clear differences exist in cisplatin ssDNA truncation homology around C-x and T-x base combinations with the DNA metallo-intercalators 56MESS and 3478MEEN (Figure 6.14 c). The greatest increase in non-cisplatin ssDNA truncation homology was the TG combination with 3478MEEN at 107.5 ± 2.6%. The greatest decrease in non-cisplatin ssDNA truncation homology was GG with 3478MEEN showed 93.4 ± 1.5%.

d) **Groove binder**

Differences in cisplatin ssDNA truncation homology around C-x and T-x base combinations were shown with RuP1 and RuP2 (Figure 6.14 d). The greatest increase in non-cisplatin ssDNA truncation homology was TG with RuP1 at 110.8 ± 0.2% while RuP2 showed 110.2 ± 1.0%. The greatest decrease in non-cisplatin ssDNA truncation homology was GG with RuP2 at 84.1 ± 5.5% while RuP1 showed 86.3 ± 2.7%.
Figure 6.14: Vertical bar graphs showing the two base composition of damage sites relative to the cisplatin base binding preference.

Metallo-drugs are separated into DNA binding mode for (a) metallo-covalent binders, (b) DNA strand breakers, (c) metallo-intercalators and (d) metallo-groove binders. Values are expressed as a percentage with error bars representing standard deviations.
6.4 Discussion

The chapter focuses on two approaches to characterise the affinity of several metallo-drugs with different DNA binding modes to disrupt DNA replication by DNA polymerase. The first approach consisted of a methodology that measured the metallo-drug affinity to inhibit DNA replication at the sub-gene level with body labelled ssDNA fluorescence detection. The second approach consisted of a methodology that identified the metallo-drug DNA binding sequence specificity where DNA damage was detected at the single base-pair level with end labelled ssDNA fluorescence detection. Furthermore, by comparing DNA damage profiles of metallo-drugs with different ligand structures it may be possible to better predict the behaviour of novel metallo-drugs in the clinic with comparison to the lead platinum(II) compound cisplatin.

6.4.1 Characterising metallo-drug induced inhibition of DNA replication by Linear Amplification with Rhodamine-labelled dUTP detection

The first approach compared metallo-drugs with different DNA binding modes for their affinity to inhibit DNA replication at the sub-gene level. The methodology combined the Linear Amplification (LA) reaction assay with the application of 5-carboxyrhodamine 6G nucleotide body labelling for fluorescent-based quantification of replicated ssDNA products. This approach proved to be a quick, high throughput PCR-based protocol with safe fluorescent detection which allowed for quantitative comparisons of several metallo-drugs for their capability to inhibit DNA polymerase extension and to serve as an initial optimisation tool for future LA sequence specificity work described in the following section (Section 6.4.2).
6.4.1.1 Methodology development and optimisation

In these experiments, fluorescent quantification of ssDNA replication products was facilitated by the incorporation of dUTP nucleotides labelled with 5-carboxyrhodamine 6G (R6G) fluorophore. The protocol used for R6G coupling reaction with amino-allyl dUTP (AAdUTP) to produce 5-carboxyrhodamine-6G-dUTP (R6GdUTP) has been previously described (Henegariu et al., 2000) with the coupling reaction schematic depicted in Figure 2.1. Post coupling reaction fluorescence densitometry measurements of the reaction mix containing R6G and AAdUTP on agarose slab gel showed that the R6GdUTP yield was 50.1 ± 0.97 % (Appendix B, Figure C1) which is in agreement with previously reported coupling efficiencies of custom made fluorescent dUTPs which achieved yields of between 50 to 60% (Henegariu et al., 2000).

Optimisation of R6GdUTP DNA body labelling was initially carried out by PCR and titration of R6GdUTP with inverse titration of dTTP to assure a constant effective nucleotide concentration (method adapted from Section 2.2.8). Initial optimisation was performed by PCR reaction and not an LA reaction because at the time a PCR protocol with pUC/M13 primers and pUC19 template was being employed. Inspections of agarose slab gel showed that up to 300 ρmol (up to 15 µM per 20 µL PCR reaction) of R6GdUTP nucleotide mix per reaction was the highest concentration to generate a morphologically stable and reproducibly detectable DNA band (Appendix C, Figure C2).

The concentration of R6GdUTP post coupling reaction mix represents the total R6G-coupled and uncoupled AAdUTP nucleotide content in the reaction. Therefore, with consideration of the densitometry measurements for R6GdUTP coupling efficiency where the yield was determined to be approximately 50% – the optimal R6GdUTP incorporation into the PCR product was effectively doubled to 150 ρmol per 20 µL reaction. This amounts are similar to previous work that characterised 80 ρmol of R6G labelled nucleotides required for ssDNA
detection in 20 µL PCR-based DNA sequencing reactions (Seo et al., 2005). Furthermore, PCR-based reactions with 300 pmol (effective ~150 pmol) of R6GdUTP per 20 µL reaction were determined to be optimal for this work as this condition incorporated the most fluorophore whilst retaining DNA band integrity on native slab gel electrophoresis.

The optimised coupling and body labelling of a PCR generated DNA product with R6GdUTP was applied to LA reactions for the current metallo-drug studies described in this chapter. The benefits of employing LA reaction for metallo-drug DNA binding studies is that it characterised intra-strand crosslinks and that DNA replication in this assay is linear, not exponential, as occurs in PCR reactions. Therefore, it can be argued that the quantitative relationships established between DNA/drug amounts and the magnitude of ssDNA product formation by a LA reaction is a more robust form of characterisation (Murray et al., 1992, Moumita and Murray, 2011). The use of pUC19 and the pUC/M13 sequencing primer has previously been well characterised in LA reaction work with platinum(II) metallo-drug DNA binding studies (Murray et al., 1997, Moumita and Murray, 2011).

Native slab gel electrophoresis of the undamaged template amplified by LA reaction produced a discrete band with an apparent molecular weight of 419 bp ± 20 (Figure C2, Figure 6.2 a) which is approximately half of the expected 997 bp which would have an equivalent dsDNA apparent molecular weight of approximately 485 bp. Differences in the expected and actual LA ssDNA full extension product molecular weights can be explained as modifications in electrophoretic mobility due to a number of factors such as the ssDNA conformation (Lerman et al., 1984), ssDNA nucleotide sequence (Konstantinos et al., 2008) and differences in ssDNA ionic charge due to R6G content (Milanova et al., 2011).

Some residual R6G and R6GdUTP molecules can be seen as both high and low molecular weight smears on slab gels due to carry over from the R6G and AAdUTP coupling reaction (Figure C2, Figure 6.2 a, lane 1 & 1'). However, most excess dye was removed by two
rounds of ethanol precipitation as was recommended by the manufacturer (data not shown). Furthermore, native agarose slab gels were used to separate R6GdUTP labelled ssDNA LA products instead of urea based denaturing agarose slab gels (Section 2.2.18) as the loaded samples reacted possibly with the urea to give rise to unstable DNA bands (data not shown). Furthermore, sodium hydroxide based alkaline denaturing electrophoresis was not employed as it was problematic to execute the protocol and quantify the DNA as previously discussed in Section 5.3.1.

6.4.1.2 Comparisons of metallo-drug induced inhibition of DNA replication at the sub-gene level by R6G body-labelling fluorescence quantification

Cisplatin has been well characterised for its ability to inhibit DNA replication by the formation of DNA adducts that block DNA polymerase from reading a DNA template (Jamieson and Lippard, 1999). This was demonstrated in current work as observations and densitometry measurements of agarose slab gels clearly revealed pUC19 treated with cisplatin and subjected to LA reactions using Taq DNA polymerase that induced a decrease in ssDNA synthesis in a dose-dependent manner (Figure 6.2 a). Furthermore, it was calculated that the drug to nucleotide ratio ($r_b$) required to reduce ssDNA replication to 50% and 5% in this assay was 0.0061 and 0.0320, respectively (Table 6.1).

This trend is similar to previous work that analysed Hamster genomic DNA treated with cisplatin where a dose-dependent decrease of a 750 bp dsDNA product analysed by PCR was found to be nearly completely inhibited with an $r_b$ of 0.004 (Jennerwein and Eastman, 1991). However, if we were to consider that PCR generation of dsDNA products synthesises at an exponential rate with two DNA strands whereas LA generation of ssDNA products occurs at a linear rate with one DNA strand, then the $r_b$ that reduced ssDNA replication to 5% in our assay would effectively be $\sim 0.002$ which is closely aligned to what was previously reported (Jennerwein and Eastman, 1991). Furthermore, the discrepancy between our data and that
reported can be attributed to the small differences in DNA replication product length and the
type of PCR-based DNA amplification assay employed with each of these studies.

Another covalent binder metallo-drug that was tested for its ability to inhibit DNA replication
was the cisplatin analogue oxaliplatin. Observations and densitometry measurements of
agarose slab gels clearly showed that pUC19 treated with oxaliplatin and subjected to LA
reactions with Taq DNA polymerase induced a decrease in ssDNA replication in a dose-
dependent manner (Figure 6.2 b). Interestingly, the calculated $r_b$ required for oxaliplatin
to reduce ssDNA replication to 50% was lower than cisplatin at 0.0006, but, then to reduce
ssDNA replication to 5% the $r_b$ for oxaliplatin was higher than cisplatin at 0.0521 (Table 6.1,
Figure 6.6).

The contradicting $r_b$ trends for the 50% and 5% ssDNA replication reduction measurements
between cisplatin and oxaliplatin may be a reflection of the different amounts of DNA
damage with each metallo-drug (Kim et al., 2010). Furthermore, drug/DNA binding studies
previously concluded that oxaliplatin was less effective than cisplatin at inhibiting DNA
replication by PCR stop assay with treated A2780 cell line gDNA (Woynarowski et al., 1998)
which is in line with the current findings for oxaliplatin which induced 5% ssDNA replication
inhibition in this work (Figure 6.6).

Additionally, the LA reaction was used to characterise the ability of the cisplatin analogues
DCBP, DCRP and DACH, for their ability to inhibit DNA replication. These data exhibited a
decrease of ssDNA replication in a dose-dependent manner as observed with cisplatin (Figure
6.2 a) and oxaliplatin (Figure 6.2 b) treatments.

Interestingly, the calculated $r_b$ to reduce ssDNA replication of cisplatinto 50% was similar
with oxaliplatin and DACH at 0.0006 and 0.0002, respectively (Table 6.1). Also, the $r_b$
to reduce the ssDNA replication to 50% was similar with DCBP and DCRP at 0.0019 and
0.0016, respectively (Table 6.1). An explanation for the ssDNA replication inhibition patterns
could relate to the structure homology between oxaliplatin and DACH as both compounds have a cyclohexane non-leaving group where DCBP and DCRP have a larger 2,2ʹ-bipyridine and 2,2ʹ-pyridine non-leaving group, respectively, which may be better suited to sterically hinder Taq DNA polymerase activity.

The increased reactivity for DCBP and DCRP to block Taq DNA polymerase relative to cisplatin and oxaliplatin is likely due to the additional DNA binding by the cationic pyridine ligands which have previously been shown to exhibit hydrogen bonding on the DNA molecule and electrostatic interaction with the DNA phosphate backbone (Roy et al., 2009). The calculated \( r_b \) to reduce ssDNA replication to 5% with DCBP and DCRP were essentially the same at 0.0032 which, again, most likely reflects the pyridine structure homology between the two metallo-drugs. However, the calculated \( r_b \) to reduce ssDNA replication to 5% with DACH at 0.0006 is 80-fold less than cisplatin. Whilst this may be an over statement of the differences due to the small values giving rise to a large fold change, it could be construed that the pattern of DNA replication inhibition between cisplatin and DACH correlates with previous work that described DACH as exhibiting a “high” degree of DNA polymerisation inhibition due to the formation of 1,2-GG interstrand crosslinks (Kasparkova et al., 2010).

Metallo-drugs with different DNA binding modes to cisplatin were also analysed for DNA replication inhibition properties. Densitometry measurements of agarose slab gels revealed pUC19 treated with the platinum(II) metallo-intercalators 56MESS and 3478MEEN (Figure 6.4) which resulted in a decrease in ssDNA replication in a dose-dependent manner. However, 56MESS and 3478MEEN required large \( r_b \) of 0.3177 and 0.2440, respectively, to reduce ssDNA synthesis by Taq DNA polymerase to 5% (Table 6.1, Figure 6.6). The high 56MESS and 3478MEEN \( r_b \) required to induce ssDNA synthesis inhibition is likely attributed to the non-covalent intercalation of these with DNA (Brodie et al., 2004). It has previously been reported that a DNA intercalator (ruthenium(II)-based metallo-drugs) required relatively
high concentrations to inhibit RNA transcription in a dose-dependent manner (Chen et al., 2011).

Conversely, the ruthenium(II)-based groove binders with long pyrrole tails showed little DNA replication inhibition activity. Observations and densitometry measurements of agarose slab gels revealed pUC19 treated with RuP1 or RuP2, and subjected to LA reactions with Taq DNA polymerase, did not result in reductions in ssDNA replication greater than 50% (Figure 6.5). Therefore, an rb calculation for 50% and 5% ssDNA replication reduction to compare performance with other metallo-drugs was not possible. The low DNA replication inhibition profile induced by both ruthenium(II) compounds is likely related to the non-covalent DNA groove binding mode previously characterised with RuP1 (Orkey et al., 2012).

Phleomycin was also subjected to LA reactions with Taq DNA polymerase where it was shown to induce a decrease in ssDNA replication in a dose-dependent manner (Figure 6.3). Furthermore, it was calculated that the rb required to reduce ssDNA replication to 50% and 5% in this assay was 0.0050 and 0.0096, respectively (Table 6.1, Figure 6.6). The reduction in ssDNA replication by phleomycin can be directly attributed to the induced phosphate backbone strand breaks which have been well characterised (Sleigh, 1976, Stern et al., 1974) and would prevent Taq DNA polymerase from replicating beyond a cleavage site as previously described in vitro with E. coli (Farrell and Reiter, 1973).

Conversely, the strand breaker Cu56MESS did not induce a reduction in ssDNA replication below 50% (Figure 6.4 c). The lack of Cu56MESS activity was also characterised in DNA structure studies potentially due to the need for a reducing agent, such as H2O2, to induce Cu56MESS reactivity with a DNA substrate (Krause-Heuer et al., 2012).

In summary, the DNA replication profiles of several metallo-drugs were characterised using pUC19 DNA template and Taq DNA polymerase. Rates of DNA replication inhibition by cisplatin and other metallo-drugs were compared and validated with previously published
data, demonstrating that LA reactions coupled with body-labelled R6G fluorescence quantification is a relatively quick, easy and safe protocol to perform comparative studies to assess the ability of these to inhibit DNA synthesis by Taq DNA polymerase. It is thought that these observations reflect the ability to inhibit eukaryote DNA polymerases and therefore relate to the ability of these metallo-drugs to inhibit DNA metabolism in cells.
6.4.2 Characterising the sequence specificity of metallo-drugs binding to DNA by Linear Amplification and separation by AB3730 fluorescent capillary electrophoresis

The second approach consisted of a methodology that enabled the detection of the DNA binding sequence specificity for a range of metallo-drugs with different DNA binding modes. Again, this methodology employed the Linear Amplification (LA) reaction, however, in this approach the product is coupled with a 5’FAM labelled primer to facilitate fluorescence detection of end-labelled ssDNA products. Another major difference of this approach is the application of an automated Capillary Electrophoresis DNA sequencer coupled with a Laser-Induced Fluorescent detector (CE-LIF) to discriminate product size to base-pair resolution.

6.4.2.1 Methodology development and optimisation

Initial establishment of conditions for the LA reactions considered the DNA template and primer concentration to facilitate a high intensity and reproducible full extension peak on electropherogram traces generated by CE-LIF. It was determined that LA reactions containing 480 ng of linear pUC19 (PvuII) template and 2 pmol of 5’FAM labelled pUC/M13 Primers A and B were sufficient to generate a full extension ssDNA electropherogram peak profile of similar intensity to the LIZ500 size ladder (Appendix D, Figure D1). In hindsight, the concentration of pUC19 template and 5’FAM primer was 10-fold and 2-fold higher, respectively, than concurrent work that used a similar DNA template and standard sequencing primers often used for LA sequence specificity studies (Moumita and Murray, 2011, Nguyen and Murray, 2012).

Sequencing reactions run in parallel to LA reactions of treated sample DNA were facilitated by the incorporation of dideoxynucleotides (ddNTPs) to produce a base-pair resolution ssDNA molecular ladder by CE-LIF. This allowed for peak assignment at base-pair resolution
of electropherogram traces generated by treated DNA template that prevented ssDNA extension by \textit{Taq} DNA polymerase. Sequencing reactions were optimised by the titration of each ddNTP in separate reactions and these were qualitatively assessed for distribution and intensity of peaks to account for most of the DNA sequence.

Qualitative observations of sequencing reaction peak of ddCTP titrated over a concentration gradient showed that 0.5 mM was sufficient to produce numerous peaks with strong intensity (Appendix D, Figure D4). Further LA sequencing optimisation revealed that 1 mM ddATP, 0.5 mM ddGTP and 1.75 mM ddTTP also produced adequate peak profiles for these nucleotides with numerous high intensity peaks. These ddNTP concentrations were similar to previous sequencing work with pUC19 (Nguyen and Murray, 2012). Furthermore, sequencing reactions were able to be successfully reconciled with the published pUC19 sequence on NCBI using both Primer A (Figure 6.9 b) and Primer B (Figure 6.9 a).

Cisplatin, as the lead platinum(II) compound, was utilised for the optimisation of metallo-drug treatment to determine the concentration that produces an electropherogram peak profile that results in neither an under or over-damaged DNA template. Inspection of electropherogram traces of DNA template treated with cisplatin over a concentration gradient revealed that 5 µM produced a peak profile with a reduction in full extension ssDNA product at the expense of an increase in truncated ssDNA products (Appendix D, Figure D3). Furthermore, this metallo-drug concentration was of similar magnitude to previous LA CE-LIF sequence specificity work with cisplatin (Moumita and Murray, 2011).

Consideration was also given to the use of other fluorophores for the detection of ssDNA LA products by CE-LIF. The work previously described in Section 6.4.1 detected ssDNA LA products by body-labelling with R6G fluorophore. Comparisons of CE-LIF electropherogram traces between duplicate LA reactions containing either 150 pmol R6GdUTP body-labelling or 2 pmol 5’FAM primer end-labelling of ssDNA full extension products revealed that the
presence of R6G disrupted the electrophoretic mobility of the full extension peak giving rise to numerous peaks on the electrophoretic trace (Appendix D, Figure D2). The likely explanation for this result relates to the interaction of formamide with the amine reactive R6G fluorophore, which would produce a variety of ssDNA LA products with different ionic charges, giving rise to multiple peaks on an electropherogram trace.

LA reaction sequencing and metallo-drug sequence specificity work was facilitated by the use of two analogue 5′FAM labelled pUC/M13 reverse sequencing primers referred to as “Primer A (22 mer)” and “Primer B (17 mer)”. The rational for this approach was that was an opportunity to generate multiple data sets while concurrently addressing possible sequence bias due to relative length of the LA product from the start of replication. The full extension product size derived from Primer A was 5 bp longer than that derived from Primer B (Figure 6.9). The rationale for using two primers in LA reaction sequencing and metallo-drug sequence specificity work was to increase the quality of replicate data and to aid in the exclusion of artefact peaks from the data set which has previously been identified as an issue in other cisplatin LA CE-LIF generated data sets (Moumita and Murray, 2011).

The detection of DNA damage peaks from LA reactions containing Primer A (22 mer) or Primer B (17 mer) were identified based on a selection criteria where the peak height was 1.5 fold larger than the DMF control electropherogram peak trace (Figure 6.10, Figure E1). Peaks were aligned to the nearest dideoxy sequencing peak according to the common internal LIZ500 standard for Primer A (Table E1) and Primer B (Table E2). Analysis of the DNA damage peaks was performed by comparing the number of ssDNA truncation sites induced by each test metallo-drug, followed by a one-base and two-base composition analysis of the ssDNA truncation sites expressed as a percentage.
6.4.2.2 Metallo-drug induced ssDNA truncation frequency analysis

Initial analysis of the metallo-drug induced ssDNA truncation LA data set was designed to characterise the peak frequency of each treatment. This differs from the first approach (Section 6.4.1) where metallo-drug induced total ssDNA LA product loss was measured by LA reaction. The analysis performed in this section of work considers the relationship between DNA template base composition which averaged 31% cytosine, 24% adenine, 23% guanine and 22% thymine bases and between Primers A and B – and the number of metallo-drug induced ssDNA truncation sites on the linear pUC19 template. The relatively homogenous DNA template base composition appears to have aided in improving the data set by reducing ssDNA truncation site bias towards more abundant bases on the DNA template. This has previously been considered in other LA-based sequence specificity studies with cisplatin and other platinum(II) analogues (Burstyn et al., 2000).

The second generation platinum(II) compound oxaliplatin induced higher frequencies of ssDNA truncations than cisplatin (Figure 6.11a, Figure 6.12a). This contradicts previous findings where cisplatin showed higher lesion frequency than oxaliplatin when analysed by stop-PCR with amplification of c-myc-ORI and β-globin genes (Woynarowski et al., 1998). Furthermore, data presented here also indicated that carboplatin induced a higher frequency of ssDNA truncations than cisplatin (Figure 6.11a, Figure 6.12a), which contradicts previous findings where LA reactions with pUC19 template characterised carboplatin as being a less effective DNA damaging agent than cisplatin (Murray et al., 1997) and could be an artefact of high background noise.

Other platinum(II) covalent binders DCRP and DCBP which contain two pyrrole non-leaving groups exhibited similar ssDNA truncation frequencies to cisplatin (Figure 6.11a, Figure 6.12a). This trend is similar to an analogous platinum(II) metallo-drug cis-[PtCl₂(C₆H₁₁NH₂)₂] which contains two cyclohexane non-leaving groups rather than pyridine.
leaving groups (Murray et al., 1997). Measurements of ssDNA truncation frequency induced by DACH showed it to be less effective than cisplatin (Figure 6.11 a, Figure 6.12 a) which complements previous DNA damage characterisation with plasmid and HeLa cell gDNA templates (Murray et al., 1998).

Other metallo-drugs with different DNA binding modes were also characterised for ssDNA truncation frequencies. Interestingly, the copper(II) strand breaker Cu56MESS induced ssDNA truncations at a similar frequently to cisplatin (Figure 6.11 a, Figure 6.12 a), contradicting previous assertions that Cu56MESS required a reducing agent, such as H2O2, to induce a DNA strand break binding mode (Krause-Heuer et al., 2012). Platinum(II) metallo-intercalators 56MESS and 3478MEEN both showed increased ssDNA truncation frequencies relative to cisplatin (Figure 6.11 a, Figure 6.12 a) which may be a reflection of the DNA binding mode (Brodie et al., 2004) or the degree of induced DNA structure modification, as previously discussed.

The ruthenium(II) metallo groove binders RuP1 and RuP2 both exhibited high levels of ssDNA truncation frequencies relative to cisplatin (Figure 6.11 a, Figure 6.12 a). This result can be explained by the multiple DNA binding modes previously described with RuP1 (Orkey et al., 2012) and sequence specificity data previously published with analogous polypyridyl ruthenium(II) complexes which inhibited DNA polymerisation at different sites to cisplatin which was also contributed to variations in DNA binding modes (Novakova et al., 1995). Phleomycin also showed ssDNA truncation reactivity comparable to other platinum(II) metallo-drugs (Figure 6.11 a, Figure 6.12 a) which is in line with published trends that identified high levels of phleomycin induced degradation of pLJ3 DNA template (Kross et al., 1982).
6.4.2.3 The one-base and two-base sequence specificity of metallo-drug with different DNA binding modes

Initially, a simplified analysis of only the central base at the site where the DNA polymerase paused gives an overview of the reactivity without consideration of the neighbouring residue or nature of the adduct (mono- or bi-functional). The inference as to whether the peak stems from a mono- or bi-functional adduct is made by considering the adjacent bases, which is then considered. Analysis for both mono- or bi-functional adducts have previously been considered for detailed characterisation of metallo-drug DNA binding specificity (Murray et al., 1997, Burstyn et al., 2000).

The platinum(II) covalent binder metallo-drugs cisplatin, carboplatin, oxaliplatin and DACH revealed that each exhibited a high binding preference for purine bases with particular emphasis on guanine bases (Figure 6.11 b, Figure 6.12 b-e), which is concordant with previous studies that characterised the sequence specificity of platinum(II) metallo-drugs by LA reaction (Boudný et al., 1992, Woynarowski et al., 1998, Murray et al., 1997, Murray et al., 1998). The high affinity for platinum(II) covalent binder metallo-drugs to bind to purine bases was also seen with DCRP and DCBP treatments (Figure 6.11 b, Figure 6.12 b-e).

Observations of the two-base binding preference for platinum(II) covalent binder metallo-drugs revealed strong clustering around (higher to lower) GG > GC, AG > AA, GA, GT binding sites (Figure 6.13 a) which showed similar homology to the DNA binding profile exhibited with cisplatin (Figure 6.14 a). These trends agree with previous work with cisplatin, carboplatin, oxaliplatin and DACH for DNA binding preference to GG and AG sites (Kasparkova et al., 2010, Burstyn et al., 2000, Boudný et al., 1992, Murray et al., 1997, Woynarowski et al., 1998).

The single base analysis of phleomycin showed no substantial discrimination for DNA binding preference (Figure 6.11 b) which was reflected as large changes in binding homology.
compared to cisplatin, with the exception of adenine (Figure 6.12 b-e). However, the two base analysis revealed phleomycin exhibited binding preference to GA, GC, GG and AA sites (Figure 6.13 b) which did exhibit some homology with cisplatin DNA binding preference (Figure 6.14 b). This result is similar to previous work with phleomycin which demonstrated DNA binding specificity with GT, GC and AT sites (Kross et al., 1982) and other work that concluded that adenine-thymine base pairs contribute to phleomycin cleavage (Pietsch and Garrett, 1968).

The single base analysis of DNA treated with metallo-intercalator 56MESS, strand breaker Cu56MESS or ruthenium(II) groove binders RuP1 and RuP2 showed no strong discrimination for DNA binding preference between the four bases (Figure 6.11 b) which was reflected as large changes in binding homology with cisplatin (Figure 6.12 b-e). However, the platinum(II) 56MESS analogue 3478MEEN did show a slight increase in guanine base binding preference (Figure 6.11 b) which may reflect results from previous DNA structure characterisation experiments where covalent binder-like DNA binding mode traits were identified as discussed in Sections 3.3, 4.3 and 5.3.

The two base analysis with metallo-intercalator 56MESS and strand breaker Cu56MESS or ruthenium(II) groove binders RuP1 and RuP2 showed no strong discrimination for DNA binding preference for any base-base combination (Figure 6.13 c&d) which was also reflected in the cisplatin homology analysis (Figure 6.14 c&d). However, 3478MEEN showed an increased binding preference to GG sites which, again, may reflect its covalent binder DNA binding mode preference.

Our results did not support previous DNase I footprinting work with 56MESS where sequence specificity was characterised to preferentially bind to GC, ATG, TC, GGC, G, and CA sites (Krause-Heuer et al., 2009a). Furthermore, the results also contradict the intended design of the ruthenium(II) groove binder RuP1 (Orkey et al., 2012) where the pyrrole-
imidazole polyamide tail was supposed to exhibit DNA binding specificity with AT and TA
sites (Wade et al., 1992). A possible explanation for this discrepancy could be due to the
DNase I footprinting assay operating at physiologically relevant temperature of 37 °C while
the LA reaction utilises Taq DNA polymerase at 95 °C.

In summary, the Linear Amplification (LA) reaction coupled with a 5′FAM labelled primer
and separation by CE-LIF was successfully applied to facilitate fluorescence detection of end-
labelled ssDNA products. The DNA binding sequence specificity of a range of metallo-drugs
with different DNA binding modes was successfully quantified to base-pair resolution despite
the high background noise. Furthermore, the metallo-drug DNA binding sequence specificity
of the lead platinum(II) compounds was able to be reconciled with the published literature
which increased the validity of conclusions drawn about novel metallo-drugs included in this
study.
Chapter 7: Characterising the influence of glutathione for titrating metallo-drugs away from their DNA targets

7.1 Introduction

In addition to the formation of drug/DNA adducts, cisplatin has previously been shown to exhibit an affinity to bind to other intracellular constituents (Wang et al., 1996) such as the cytoskeleton (Kopf-Maier and Muhlhausen, 1992), plasmid membrane phospholipids (Speelmans et al., 1996, Speelmans et al., 1997) and intracellular proteins though the formation of covalent bonds with thiol groups (Aull et al., 1980, Pizzo et al., 1988, Zeng et al., 1997, Aull et al., 1979, Kamal, 1996). Experiments have shown that only a small amount of intracellular cisplatin reaches the gDNA to form a covalent adduct (Cepeda et al., 2007). The titration of cisplatin away from forming gDNA adducts and towards other cellular constituents essentially reduces the metallo-drugs bioavailability and contributes to cancer being resistant to treatment (Wong and Giandomenico, 1999, Jamieson and Lippard, 1999, Litterst and Schweitzer, 1988).

At the molecular level, it has previously been demonstrated that platinum(II) metallo-drugs exhibit a higher affinity to form covalent bonds with sulfur donors on proteins and other biomolecules rather than nitrogen donors on DNA (Bose et al., 1995, Reedijk, 1999, Kasherman et al., 2009). This is further exacerbated as much of the cellular content contains thiol ligands (Reedijk, 1999, Rabik and Dolan, 2007).

Glutathione (GSH) – a gamma tripeptide consisting of the amino acids glutamine, cysteine and glycine – is the most abundant non-protein cellular constituent which contains a thiol
ligand on the cysteine residue (Dickinson and Forman, 2002). Cisplatin resistant cell populations often have higher levels of intracellular GSH compared to non-resistant cells (Godwin et al., 1992). As a consequence, drug binding to GSH has been considered during metallo-drug/DNA binding studies and this has lead to the development of the cisplatin analogue picoplatin with a reduced sulfur donor binding affinity relative to DNA adduct formation (Raynaud et al., 1997, Holford et al., 1998).

Several methodologies have been applied to determine the affinity of candidate metallo-drugs to preferentially bind to GSH over DNA. Kinetic assays have previously been used to investigate the affinity of lead platinum(II) metallo-drugs to form covalent thiol bonds with GSH (Hagrman et al., 2004). Another approach analysed the cellular constituents of cell populations treated with cisplatin to determine the distribution of platinum(II) metallo-drug bound to sulfur or nitrogen donor ligands (Kasherman et al., 2009). Also, competitive reaction assays have been performed with HPLC to elucidate whether cisplatin was titrated away from binding to the four nucleotides in the presence of GSH (Volckova et al., 2002).

Work presented in this chapter focuses on two aims (Section 1.6.3). The first aim was to identify test metallo-drugs’ affinity to form thiol bonds with GSH. The second aim was to determine the binding preference of test metallo-drugs for GSH thiol groups or DNA nitrogen donors.

7.2 Investigating the ability of cisplatin analogues and other novel metallo-drugs to form thiol bonds with glutathione

7.2.1 Results

Test metallo-drugs were dissolved in 100% DMF to a final concentration of 1 mM as described in Section 2.1.8. In addition, cisplatin, carboplatin and oxaliplatin were freshly dissolved in DNase-free water to a final concentration of 1 mM to serve as additional controls in the event that the DMF was having an effect. Reduced glutathione (GSH) was dissolved in
DNase-free water to a stock concentration of 280 mM as described in Section 2.1.8. Each reaction mix consisted of 180 mM GSH and 360 µM of each test compound to give a drug to GSH ratio of 1:500 in a final volume of 140 µL incubation buffer. Reactions with phleomycin were made with and without 25 mM DTT. Reactions were replicated in triplicate with water, DMF, DTT, and DMF plus DTT controls.

The rate at which metallo-drugs form thiol bonds with GSH was measured by UV absorbance at 280 nm over a 10 hour period. Additional measurements were made up to 48 hours to ensure GSH thiol-bonding activity was exhausted. The initial absorbance rate was determined during the first 30 to 90 minutes of incubation.

### 7.2.1.1 GSH thiol bond formations after 10 hours of incubation

**a) Lead covalent binders**

Comparisons between water and DMF solvent were carried out to determine if the presence of solvent effected GSH thiol bonding with the lead covalent binder metallo-drugs. The rationale for this comparison will be discussed in Section 7.4.1.

Initially cisplatin, oxaliplatin and carboplatin were made soluble in water and were found to form thiol bonds with GSH after 10 hours (600 minutes) of incubation relative to water control (Figure 7.1a). Cisplatin showed the largest increase absorbance, and therefore in thiol bond formation with UV Au$_{280\text{nm}}$ 0.551 ± 0.006, followed by carboplatin at 0.311 ± 0.003 and oxaliplatin at 0.097 ± 0.000. As expected, water controls exhibited a small increase in UV absorbance which indicates minimal thiol bond formation with UV Au$_{280\text{nm}}$ of 0.061 ± 0.004. These compounds were also dissolved in DMF which is in line with preparations of other tested metallo-drugs (Figure 7.1b). Cisplatin showed the greatest increase in thiol bond formation with UV Au$_{280\text{nm}}$ 0.456 ± 0.002, followed by oxaliplatin at 0.245 ± 0.004 and carboplatin at 0.209 ± 0.002. As expected, the presence of the DMF solvent showed a small
increase in absorbance of $0.199 \pm 0.001$ which is indicative of background GSH thiol bonding with the solvent.

\textit{b) Covalent binders}

The cisplatin analogues DACH, DCRP and DCBP were dissolved and tested for GSH thiol bond activity (Figure 7.2 a). Relative to the DMF control, DACH showed the greatest increase in thiol bond formation with UV $\text{Au}_{280\text{nm}}$ $0.456 \pm 0.002$, followed by DCRP at $0.393 \pm 0.001$. DCBP showed no increase in thiol bond formation with GSH at UV $\text{Au}_{280\text{nm}}$ $0.029 \pm 0.002$ which is less than the DMF control of $0.163 \pm 0.002$ and may possibly be an indication of metallo-drug induced GSH oxidation.

\textit{c) Strand breakers}

DNA strand breaker phleomycin was dissolved in DMSO and tested in the presence and absence of DTT for reactivity with GSH (Figure 7.2 b). Phleomycin alone with GSH showed thiol bond formations at UV $\text{Au}_{280\text{nm}}$ $0.632 \pm 0.006$ which decreased in the addition of DTT to $0.598 \pm 0.001$. The presence of DTT in the DMF control reaction showed no real change in thiol bonding activity with measurements at UV $\text{Au}_{280\text{nm}}$ $0.163 \pm 0.002$ and $0.166 \pm 0.002$. Cu56MESS showed a high thiol bond reactivity profile with UV $\text{Au}_{280\text{nm}}$ at $0.597 \pm 0.011$ (Figure 7.2 b).

\textit{d) Metallo-intercalators}

Metallo-intercalators 56MESS and 3478MEEN did not show significant changes in thiol bond formation with relative to DMF control. 56MESS showed the greatest increase in thiol bond formation with UV $\text{Au}_{280\text{nm}}$ $0.198 \pm 0.005$ while 3478MEEN showed the smallest increase in thiol bond formations at UV $\text{Au}_{280\text{nm}}$ $0.177 \pm 0.002$ (Figure 7.2 c).
e) *Groove binders*

Groove bonders RuP1 and RuP2 did not show significant changes in thiol bond formation with GSH relative to DMF control (Figure 7.2 d). RuP2 showed the greatest increase in thiol bond formation with GSH at UV $\text{Au}_{280\text{nm}}$ $0.198 \pm 0.005$ while RuP1 showed a smaller increase in thiol bonding with GSH at $0.170 \pm 0.014$. 
Figure 7.1: Scatter plot overlayed with Hill non-linear curve of best fit showing kinetic profiles of GSH reacting with cisplatin, carboplatin and oxaliplatin dissolved in water and DMF. UV absorption at 280 nm (y-axis) of GSH reactions for 600 minutes (10 hour) incubation (x-axis). Image shows kinetics of GSH reacting with cisplatin, carboplatin and oxaliplatin dissolved in water. Image (b) shows the kinetics of GSH reacting with cisplatin, carboplatin and oxaliplatin dissolved in DMF.
Figure 7.2: UV absorption time course scatter plot overlayed with Hill non-linear curve of best fit showing kinetic profiles of GSH with various metallo-drugs dissolved in DMF. UV absorption at 280 nm (y-axis) of GSH reactions for 600 minutes (10 hour) incubation (x-axis). Image shows kinetics of GSH reacting with covalent binders DCBP, DCRP and DACH dissolved in DMF. Image (b) shows the kinetics of GSH reacting with the strand breakers phleomycin (with and without DTT) and Cu56MESS dissolved in DMF. Image (c) shows the kinetics of GSH with metallo-intercalator compounds 56MESS and 3478MEEN dissolved in DMF. Image (d) shows the kinetics of GSH with groove binder compounds RuP1 and RuP2 dissolved in DMF.
7.2.1.2 Initial absorbance rates

In this section a kinetic analysis for metallo-drugs to form bonds with GSH was achieved by determining the initial rate of change in UV \( \text{Au}_{280\text{nm}} \) between 30 and 90 minutes of incubation. Initial absorbance rates for all metallo-drugs and controls are summarised in Figure 7.1. These rates are displayed in a vertical bar graph in Figure 7.3 for comparison.

\( \text{a) Control reactions} \)

Control reactions with water showed a low background initial absorption rate of only \( 1.1 \pm 0.05 \times 10^{-3} \text{Au}_{280\text{nm}}/\text{minute} \) (Figure 7.3, bar 1). DMF solvent controls showed an increase in background initial absorbance rate of \( 6.0 \pm 0.33 \times 10^{-3} \text{Au}_{280\text{nm}}/\text{minute} \) (Figure 7.3, bar 2). DMF solvent control reactions supplemented with DTT showed a slight reduction in GSH thiol bond kinetics compared to the DMF control alone with initial absorption rates at \( 5.6 \pm 0.11 \times 10^{-3} \text{Au}_{280\text{nm}}/\text{minute} \) (Figure 7.3, bar 3).

\( \text{b) Lead covalent binders} \)

Lead covalent binders cisplatin, oxaliplatin and carboplatin dissolved in water showed increases in their initial absorbance rate relative to water control (Figure 7.3, bars 4-6). Cisplatin showed the greatest kinetic binding increase at \( 23.0 \pm 0.02 \times 10^{-3} \text{Au}_{280\text{nm}}/\text{minute} \), followed by oxaliplatin at \( 18.0 \pm 0.28 \times 10^{-3} \text{Au}_{280\text{nm}}/\text{minute} \). Carboplatin showed the least kinetic increase for GSH thiol bonding at \( 3.1 \pm 1.69 \times 10^{-3} \text{Au}_{260\text{nm}}/\text{minute} \).

In addition, cisplatin, oxaliplatin and carboplatin dissolved in DMF exhibited similar trends as each showed an increase in the initial absorbance rate relative to DMF control (Figure 7.3, bars 7-9). Cisplatin showed the greatest increase in initial absorption rate at \( 15.2 \pm 0.05 \times 10^{-3} \text{Au}_{280\text{nm}}/\text{minute} \), followed by oxaliplatin at \( 7.2 \pm 0.08 \times 10^{-3} \text{Au}_{280\text{nm}}/\text{minute} \). Carboplatin showed minimal increases in initial absorption rates relative to DMF control with a rate of \( 6.3 \pm 0.46 \times 10^{-3} \text{Au}_{280\text{nm}}/\text{minute} \).
c) **Covalent binders**

Covalent binders DACH, DCRP and DCBP dissolved in DMF showed a mixture of initial absorbance rate kinetics for thiol bonding with GSH between 30 and 90 minutes of incubation relative to DMF control (Figure 7.3, bars 10-12). DACH showed the greatest increase in initial absorbance rate at $13.4 \pm 0.37 \times 10^{-3} \text{Au}_{280\text{nm}}/\text{minute}$, followed by DCRP at $9.8 \pm 5.84 \times 10^{-3} \text{Au}_{280\text{nm}}/\text{minute}$. DCBP showed no kinetic binding affinity for GSH thiol bonding which may indicate GSH oxidation or some experimental error.

d) **Strand breakers**

The strand breaker phleomycin dissolved in DMF and supplemented with DTT showed an increase in initial absorbance rate kinetics for thiol bonding with GSH between 30 and 90 minutes of incubation relative to DMF control (Figure 7.3, bars 15 & 16). Phleomycin in DMF showed an initial absorbance rate of $15.9 \pm 5.65 \times 10^{-3} \text{Au}_{280\text{nm}}/\text{minute}$. Phleomycin in DMF and supplemented with DTT showed a decrease in initial absorbance rate at $14.8 \pm 2.24 \times 10^{-3} \text{Au}_{280\text{nm}}/\text{minute}$. Cu56MESS show a value of $15.0 \pm 2.16 \times 10^{-3} \text{Au}_{280\text{nm}}/\text{minute}$ which is higher than the DMF control (Figure 7.3, bars 17).

e) **Metallo-intercalators**

Metallo-intercalators 56MESS and 3478MEEN dissolved in DMF showed mixed initial absorbance rate kinetics for thiol bonding with GSH between 30 and 90 minutes of incubation relative to DMF control (Figure 7.3, bars 13 &14). Both 56MESS and 3478MEEN showed initial absorbance rates less than the DMF control with values of $5.1 \pm 0.28 \times 10^{-3} \text{Au}_{280\text{nm}}/\text{minute}$ and $4.6 \pm 0.11 \times 10^{-3} \text{Au}_{280\text{nm}}/\text{minute}$, respectively.

f) **Groove binders**

Groove binders RuP1 and RuP2 soluble in DMF showed lower initial absorbance rate kinetics for thiol bonding with GSH between 30 and 90 minutes of incubation relative to DMF control
(Figure 7.3, bars 18 & 19). RuP1 showed an initial absorbance rate of \(3.8 \pm 1.34 \times 10^{-3}\) \(\text{Au}_{280\text{nm}}/\text{minute}\). RuP2 showed an initial absorbance rate of \(3.8 \pm 1.26 \times 10^{-3}\) \(\text{Au}_{280\text{nm}}/\text{minute}\).
Table 7.1: Summary of initial absorption rates for GSH treated with various compounds and solvents. Initial absorption rates (the change in UV absorbance units at 280 nm per minute) with error range (rate error) were calculated between 30 and 90 minutes of GSH incubated with all tested metallo-drugs and phleomycin. Hill non-linear line of best fit curve coefficient of determination ($r^2$) shows correlation of line with data points.

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<th>Rate Error (± SD) ×10$^{-3}$</th>
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Figure 7.3: Initial absorbance rate of GSH treated with various compounds and solvents. Initial absorbance rate (UV absorbance units at 280 nm per minute) was defined between 30 and 90 minutes of incubation (y-axis) and compared between various DNA binding compounds dissolved in water or DMF.
7.3 Competition assay for metallo-drug binding between GSH and DNA

7.3.1 Results

The denaturing assay discussed in Chapter 5 separated ssDNA from DNA that contained metallo-drug intra-strand crosslinks. This enabled the identification of incubation reaction conditions where the metallo-drug completely converted ssDNA into dsDNA-like isoform by inter-strand crosslinks when subjected to denaturing slab gel electrophoresis. Once the minimum concentration of metallo-drug was determined for the drug to completely crosslink the DNA, the assay was modified to include GSH. After the overnight reaction period with metallo-drug and GSH, treated 5′FAM-labelled DNA were separated by denaturing slab gel electrophoresis and bands were subjected to densitometry analysis to determine the reduction in cross-linked DNA formation across the GSH treatment concentration gradient.

The reaction mix for the competition assay consisted of 0.3 µg 5′FAM labelled DNA, and the minimum concentrations of the metallo-drug that induce complete cross-linking of the DNA (determined from data in presented in Chapter 5) which was 5 µM cisplatin, 50 µM oxaliplatin, 1.5 µM DCBP, 15 µM DCRP, 1.5 µM DACH and 150 µM 3478MEEN. Each of these reactions was titrated against an increasing concentration of GSH from 0.005 to 1.5 µM. Further details of the reaction protocol are located in Section 2.2.13.

a) Controls

Each slab gel contained a 100 bp ladder pre-labelled with SYBR which reproducibly yielded molecular markers corresponding to 3000, 2000, 1500, 1000, 900, 800, 700, 600 and 500 bp (Figure 7.4a-f, lane 6). The un-reacted 5′FAM labelled DNA template in the absence of GSH reproducibly showed the ssDNA and dsDNA conformation under denaturing and native conditions, respectively (Figure 7.4). The 5′FAM labelled DNA template treated with over
20-fold of the highest concentration of GSH used in the experiment produced a smear running below 500 bp (Figure 7.4 a-f, lane 3).

It is unclear how such a high concentration of GSH interfered with the assay. However, this was not viewed as an issue at the far lower concentrations of GSH used in the actual assay conditions. This high GSH control was included and these phenomena may be investigated beyond the scope of this thesis.

As previously stated, the 5'FAM labelled DNA template was treated with each metallo-drug at a fixed concentration depending on the \( r_b \) required to induce a cross-linked dsDNA-like isoform under denaturing conditions (Figure 7.4 a-f, lane 1). The metallo-drug \( r_b \) was held constant with 5'FAM-labelled DNA template and concurrently treated with GSH over a concentration gradient of 0.005, 0.015, 0.05, 0.15, 0.5 and 1.5 mM (Figure 7.4 a-f, lanes 8-13). Finally, controls for metallo-drug concentrations higher (Figure 7.4 a-f, lane 2) and lower (Figure 7.4 a-f, lane 1) than the optimal \( r_b \) for complete conversion of ssDNA to dsDNA-like isoform showed two bands indicative of incomplete ssDNA conversion as seen with DCBP treatment (Figure 7.4 c, lane 1).

b) Slab gel densitometry analysis

Densitometry analysis of metallo-drug induced dsDNA-like isoform band retention revealed that prior to complete loss of DNA band detection – DACH (Figure 7.4 e) and DCBP (Figure 7.4 c) required the highest GSH treatment at 1.5 mM to reduce DNA band intensity to 11.23 ± 6.42 % and 4.83 ± 0.96 %, respectively, where 3478MEEN (Figure 7.4 f) required the lowest GSH treatment at 0.05 mM to reduce DNA band intensity to 29.81 ± 5.28 %. Cisplatin (Figure 7.4 a), oxaliplatin (Figure 7.4 b) and DCRP (Figure 7.4 d) showed complete DNA band loss after 0.15 mM GSH treatment which reduced band intensity to 67.37 ± 33.46 %, 91.13 ± 6.41 % and 30.76 ± 6.21 %, respectively.
Densitometry was performed on the metallo-drug induced dsDNA band (Figure 7.4 a-f) to determine the GSH concentration values at which the band intensity of the cross-linked dsDNA was reduced from its initial amount to 50% and 5% are shown in Table 7.2. These values were converted to a GSH to drug to nucleotide ratio, GSH : rb (Figure 7.5).

c) GSH concentrations that induce 50% and 5% dsDNA-like isoform band retention

When taking into account the initial drug to nucleotide ratios that induce the dsDNA-like isoform – DACH showed the highest GSH : rb ratio for 50% band retention at $2.31 \times 10^{-7}$. 3478MEEN showed the lowest GSH : rb ratio for 50% band retention at $1.29 \times 10^{-10}$. For the other compounds, the order (highest to lowest) was DCBP > cisplatin > oxaliplatin > DCRP showed GSH : rb ratios of $9.69 \times 10^{-8}$, $4.01 \times 10^{-8}$, $4.95 \times 10^{-9}$ and $4.02 \times 10^{-9}$, respectively, for 50% retention of the dsDNA band.

DACH showed the highest GSH : rb ratio for 5% band retention at $1.84 \times 10^{-6}$. 3478MEEN showed the lowest GSH : rb ratio for 5% band retention at $6.41 \times 10^{-10}$. In order (highest to lowest) was DCBP > cisplatin > DCRP > oxaliplatin showed GSH : rb ratios of $9.85 \times 10^{-7}$, $8.86 \times 10^{-8}$, $2.65 \times 10^{-8}$ and $9.30 \times 10^{-9}$, respectively for 5 % retention of the dsDNA.
Figure 7.4: Slab gel and semi-log densitometry plots of 5'FAM labelled DNA template treated in competition with various metallo-pharmaceuticals at concentrations that induce covalently bound dsDNA structures and GSH over a concentration gradient. Competition binding assays for 5'FAM labelled DNA uptake were established between a range of metallo-drugs at r₅’s that induced covalent dsDNA conformations and reduced glutathione (GSH) over a concentration gradient of 0.005, 0.015, 0.05, 0.15, 0.5 and 1.5 mM (lanes 8-13). Subsequent slab gel electrophoresis of treated DNA was performed along with a 50 mM GSH control (lane 3), dsDNA conformation control (lane 4), ssDNA isoform control (lane 5) and SYBR labelled 100 bp ladder (lane 6). Image (a) shows cisplatin treatments at 5 µM (lanes 8-13) along with cisplatin (only) controls at 1.5 µM (lane 1) and 15 µM (lane 2). Image (b) shows oxaliplatin treatments at 50 µM (lanes 8-13) along with oxaliplatin (only) controls at 15 µM (lane 1) and 150 µM (lane 2). Image (c) shows DCBP treatments at 1.5 µM (lanes 8-13) along with DCBP (only) controls at 0.5 µM (lane 1) and 5 µM (lane 2). Image (d) shows DCRP treatments at 15 µM (lanes 8-13) along with DCRP (only) controls at 5 µM (lane 1) and 50 µM (lane 2). Image (e) shows DACH treatments at 1.5 µM (lanes 8-13) along with DACH (only) controls at 0.5 µM (lane 1) and 5 µM (lane 2). Image (f) shows 3478MEEN treatments at 150 µM (lanes 8-13) along with 3478MEEN (only) controls at 50 µM (lane 1) and 500 µM (lane 2).
Table 7.2: Summary table showing the concentration of GSH and GSH : $r_b$ ratio that reduces DNA conformation retention to 50% and 5%.

The 5′FAM labelled DNA template was treated with various compounds (column 1) with concentrations (column 2, µM) that produce $r_b$ ratios (column 4) that induce complete conversion of ssDNA to dsDNA conformations. Densitometry analysis of compounds / GSH / DNA competition assays enabled determination of GSH concentrations (column 5, mM) that induced 50% and 5% retention of dsDNA conformation which were then converted to a ratio of GSH : $r_b$ for both 50% and 5% dsDNA retention.

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>µM*</th>
<th>moles</th>
<th>$r_b$</th>
<th>mM</th>
<th>moles</th>
<th>GSH</th>
<th>GSH : $r_b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cisplatin</td>
<td>5</td>
<td>$2 \times 10^{-10}$</td>
<td>0.206</td>
<td>0.207</td>
<td>0.457</td>
<td>$8.28 \times 10^{-9}$</td>
<td>$1.83 \times 10^{-8}$</td>
</tr>
<tr>
<td>Oxaliplatin</td>
<td>50</td>
<td>$2 \times 10^{-9}$</td>
<td>2.064</td>
<td>0.256</td>
<td>0.480</td>
<td>$1.02 \times 10^{-8}$</td>
<td>$1.92 \times 10^{-8}$</td>
</tr>
<tr>
<td>DCBP</td>
<td>1.5</td>
<td>$6 \times 10^{-11}$</td>
<td>0.062</td>
<td>0.150</td>
<td>1.524</td>
<td>$6.00 \times 10^{-9}$</td>
<td>$6.10 \times 10^{-8}$</td>
</tr>
<tr>
<td>DCRP</td>
<td>15</td>
<td>$6 \times 10^{-10}$</td>
<td>0.619</td>
<td>0.062</td>
<td>0.410</td>
<td>$2.49 \times 10^{-9}$</td>
<td>$1.64 \times 10^{-8}$</td>
</tr>
<tr>
<td>DACH</td>
<td>1.5</td>
<td>$6 \times 10^{-11}$</td>
<td>0.062</td>
<td>0.357</td>
<td>2.853</td>
<td>$1.43 \times 10^{-8}$</td>
<td>$1.14 \times 10^{-7}$</td>
</tr>
<tr>
<td>3478MEEN</td>
<td>150</td>
<td>$6 \times 10^{-9}$</td>
<td>6.191</td>
<td>0.020</td>
<td>0.099</td>
<td>$8.01 \times 10^{-10}$</td>
<td>$3.97 \times 10^{-9}$</td>
</tr>
</tbody>
</table>

* These values were previously determined from data presented in Chapter 5.
Figure 7.5: GSH : r_b ratios where DNA isoform retention was reduced to 50% and 5%.

Slab gel densitometry analysis of DNA in competition between GSH and compound uptake yielded GSH : r_b ratios where compound induced dsDNA isoform retention was reduced to (a) 50% and (b) 5%.
7.4 Discussion

This chapter focused on characterising the affinity of several metallo-drugs to preferentially form thiol bonds with glutathione (GSH). To achieve this, two approaches were taken. The first approach consisted of a methodology that measured the affinity for metallo-drugs to form thiol bonds to GSH in the absence of DNA. The second approach employed a competitive reaction to measure the affinity of the metallo-drug to crosslink dsDNA against its ability to form thiol bonds with GSH.

7.4.1 Investigating the ability of cisplatin analogues to form thiol bonds with glutathione

The first approach compared metallo-drugs and the strand breaker phleomycin for their affinity to form thiol bonds with GSH. This methodology consisted of a kinetic assay in which thiol bond formation was measured by UV absorbance over a 48 hour time course to ensure GSH thiol-bonding activity was exhausted. Initial UV absorbance rates were calculated and a comparative analysis was carried out to determine if a relationship existed between the thiol binding activity of the tested metallo-drugs and their structure.

7.4.1.1 Methodology development

The detection for the presence of GSH thiol complexes with tested metallo-drugs and phleomycin was done by measuring the UV absorbance at 280 nm. The basis for this methodology derived from previous work where UV spectra and kinetic analysis of cisplatin-GSH thiol bond complex was determined to have a molar extinction coefficient of 8.05 mM$^{-1}$.cm$^{-1}$ when measured at 280 nm (Ishikawa and Ali-Osman, 1993). However, other cisplatin-GSH kinetic studies have been measured for the presence of thiol bond formations with a similar UV absorbance wavelength at 260 nm (Hagman et al., 2004, Dabrowiak et al., 2002).
In this work, the kinetic incubation reactions contained a ratio of 500:1 in favour of GSH (180 mM) over the tested drugs (360 µM). The rationale to adopt the 500:1 ratio was designed to represent relevant cell phenotype responsive concentrations of intracellular cisplatin and GSH (Eastman, 1996, Eastman, 1991, Souid et al., 1999, Souid et al., 1998). The 500:1 ratio was previously employed in other GSH/metallo-drug kinetic studies (Kasherman et al., 2009, Dabrowiak et al., 2002). However, other GSH/metallo-drug kinetic work concluded that a GSH/cisplatin ratio of 50:1 was also representative of cell phenotype response to treatment (Hagrman et al., 2004).

The conditional relevance for either 50:1 or 500:1 reaction conditions is essentially negated as the excess [GSH] in both cases renders the reaction pseudo first order where the rate of thiol bonding is dependent on metallo-drug concentration (Dabrowiak et al., 2002). The possibility of oxidised GSH (GSSG) disulfide bond formation in influencing UV absorbance measurements was also negated as the rate of reaction is relatively slow in conditions of pH 7.4, 37 °C and does not significantly change after 40 hours of incubation as previously verified by HPLC (Hagrman et al., 2004) and in present water control kinetic data (Figure 7.1 a). The zero time point UV absorbance measurements for tested metallo-drugs and phleomycin in the presence of incubation buffer were subtracted from the kinetic data to negate their UV absorption influence prior to the addition to GSH which is consistent with previously published methods (Dabrowiak et al., 2002).

The GSH/metallo-drug kinetic data was fitted with a Hill non-linear regression to facilitate comparative data analysis which is consistent with other GSH thiol bond kinetic data analysis (Ricci et al., 1995). The kinetic data analysis methodology adopted to compare metallo-drug/GSH thiol bonding considered a linear fit to the data between the first 30 ≤ t ≤ 120 minutes of incubation. This method was adopted from previous work that compared the GSH thiol kinetic rates with platinum(II) metallo-drugs (Hagrman et al., 2004). However, other
work considered a more discrete initial slope interval between $0 \leq t \leq 30$ minutes for their GSH/platinum(II) thiol kinetic data (Dabrowiak et al., 2002).

### 7.4.1.2 Comparative analysis for metallo-drug affinity to form thiol bonds with GSH

This study began by comparing the lead platinum(II) metallo-drugs cisplatin, carboplatin and oxaliplatin dissolved in water for their capability to form thiol bonds with GSH (Figure 7.1a). Initial UV absorption rates of kinetic data revealed that cisplatin had the highest affinity to form thiol bonds with GSH at $23.0 \pm 0.02 \times 10^{-3}$ Au$_{280\text{nm}}$/minute, followed by oxaliplatin and carboplatin at $18.0 \pm 0.28 \times 10^{-3}$ Au$_{280\text{nm}}$/minute and $3.1 \pm 1.69 \times 10^{-3}$ Au$_{280\text{nm}}$/minute, respectively (Figure 7.3, Figure 7.1). These trends are in line with previously reported data that compared cisplatin, oxaliplatin and carboplatin kinetic rates of GSH thiol bond formation (Hagrman et al., 2004). Furthermore, consistency of these data with those published in the literature adds confidence to the results obtained for other tested metallo-drugs presented in this section.

Consideration was given to the effect of the solvent and stored on GSH thiol bond formation as most tested metallo-drugs were dissolved in DMF (Figure 7.1b). Comparisons of water and DMF control reactions revealed that background GSH thiol bond kinetics increased in the presence of DMF to $6.0 \pm 0.33 \times 10^{-3}$ Au$_{280\text{nm}}$/minute (Figure 7.3, Figure 7.1). This increase in background UV absorbance can be explained as a DMF-mediated increase in GSH oxidation to GSSG which has previously been reported (Lyon and Atkins, 2001).

Data presented here show that the presence of DMF in reaction with the lead platinum(II) metallo-drugs and GSH dampens the formation of thiol bonds (Figure 7.1b). This is reflected in the initial UV absorption kinetic rates for GSH thiol bond formation where cisplatin showed the highest activity at $15.2 \pm 0.05$ Au$_{280\text{nm}}$/minute, followed by oxaliplatin and carboplatin at $7.2 \pm 0.08$ Au$_{280\text{nm}}$/minute and $6.3 \pm 0.46$ Au$_{280\text{nm}}$/minute, respectively. However, the order of GSH thiol bond activity with the three platinum(II) metallo-drugs –
(highest) cisplatin > oxaliplatin > carboplatin (lowest) – is consistent with previously published data (Hagrman et al., 2004) and the current GSH kinetic data of the metallo-drugs dissolved in water (Figure 7.1, Figure 7.3, Figure 7.1).

The comparative GSH thiol bond kinetic relationship between the three platinum(II) compounds either freshly dissolved in water or taken from stored samples kept in DMF at -80 °C was retained even though the magnitude of GSH thiol bond formation with the tested metallo-drugs decreased in the presence of DMF solvent. In contrast, carboplatin did not show a dampening in GSH thiol bond formation in the presence of DMF but still retained the GSH thiol bond kinetic relationship between the other two platinum(II) compounds. The retention of the GSH thiol bond kinetic relationship between the three platinum(II) compounds validates further GSH thiol bond kinetic studies with other analogue metallo-drugs dissolved in DMF. It was important to establish that the DMF data was consistent with literature data generated using water stocked as all laboratory stocks of novel drugs are limited in supply and stored in DMF as a matter of practicality.

The initial kinetic rates of GSH thiol bond formation with the platinum(II) metallo-drug DACH dissolved in DMF were lower than cisplatin at 13.4 ± 0.37 Au$_{280\text{nm}}$/minute (Figure 7.2, Figure 7.3, Figure 7.1). This trend was also observed in a previous study that compared $t_{1/2}$ cisplatin and DACH thiol bond formation with GSH (Kasparkova et al., 2010). An explanation for this trend could relate to the differences in the molecular structure between DACH and cisplatin, where DACH possesses a cyclohexane non-leaving group that could sterically hinder efficient thiol bond formation with GSH.

Interestingly, the cisplatin analogues DCBP and DCRP (dissolved in DMF) showed no significant increase in GSH thiol bond activity in comparison to the DMF control (Figure 7.2, Figure 7.3, Figure 7.1). This trend was similar to work with trans-platinum(II) metallo-drugs with pyridine ligands where GSH reactivity was lower than cisplatin (Farrell et al., 1992). The mechanism for this trend could be similar to the steric hindrance suggested with
DACH. Nevertheless, previous work characterised platinum(II) metallo-drugs with pyridine ligands that exhibited additional DNA binding modes other than via the stereo-centre metal group (Roy et al., 2009) which do not appear to have contributed to any increase in GSH thiol bond activity.

The platinum(II) metallo-intercalators 56MESS and 3478MEEN (Figure 7.2 c) along with both ruthenium(II) groove binders RuP1 and RuP2 (Figure 7.2 d) showed no significant increase in GSH thiol bond reactivity (Figure 7.3, Figure 7.1). An explanation for this trend could relate to the non-covalent DNA binding mode previously determined in studies with 56MESS, 3478MEEN and RuP1 (Brodie et al., 2004, Orkey et al., 2012, Krause-Heuer et al., 2009a). Furthermore, the bulky ligand structures exhibited by 56MESS, 3478MEEN, RuP1 and RuP2 could likely contribute to steric hindrance for GSH to form thiol bonds.

In contrast, the 56MESS copper(II) analogue Cu56MESS showed significant thiol bond reactivity to GSH with a similar magnitude to cisplatin (Figure 7.2 c, Figure 7.3, Figure 7.1). The affinity for GSH to react with copper(II) complexes and reduce them to copper(I)-GSH conjugates has previously been described in NMR (Corazza et al., 2004) and in vitro cell studies (Ciriolo et al., 1990). It is likely that a similar reduction reaction took place with Cu56MESS and GSH to form a copper(I)-GSH complex.

Phleomycin was incubated with GSH in the presence of the reducing agent DTT to facilitate comparable reaction conditions for DNA strand breaking activity previously described in the literature (Sleigh, 1976, Stern et al., 1974, Kross et al., 1982) and in the DNA structure studies in Chapters 3 to 5. The GSH incubation reactions with phleomycin showed an increase in thiol bond kinetics with comparable magnitude to cisplatin which was negligibly influenced by the presence of DTT (Figure 7.2 b, Figure 7.3, Figure 7.1). The increased GSH thiol bond kinetics with phleomycin was unsurprising as it has previously been shown that phleomycin requires a reducing compound with a thiol ligand to facilitate DNA strand breakage (Sleigh, 1976).
In summary, GSH thiol bond kinetic reaction studies were performed with a range of metallo-drugs with different DNA binding modes and the DNA strand breaker phleomycin. Kinetic rates for GSH thiol bond formation with lead platinum(II) metallo-drugs cisplatin, oxaliplatin and carboplatin were able to be correlated with previously published trends. Furthermore, the kinetic rates for GSH thiol bond formation with candidate metallo-drugs and the DNA strand breaker phleomycin were able to be compared to GSH thiol bond rates exhibited by cisplatin and reconciled with relevant published literature.

7.4.2 Competition assay for metallo-drug binding between GSH and DNA

The second approach employed a competitive reaction methodology where selected metallo-drugs were titrated away from forming interstrand cross links (ICLs) with dsDNA in favour for forming thiol bonds with GSH. The methodology used previously established drug concentrations, where a dsDNA template formed predominantly interstrand cross links (ICLs). However, in this instance GSH was titrated into the treatment reaction. This facilitated the basis of a competitive reaction where the affinity of the metallo-drugs to be titrated away from forming ICLs with a DNA substrate in the presence of GSH was measured.

7.4.2.1 Methodology development

Data generated from Chapter 5 enabled the identification of metallo-drug/DNA reaction parameters necessary to facilitate complete conversion of a ssDNA template into a dsDNA-like isoform under H-bond melting conditions though the formation of ICLs. Candidate metallo-drugs and their \( r_0 \) parameters that facilitated the complete inhibition of DNA strand separation under H-bond melting conditions are derived from the data produced in Chapter 5 and include cisplatin, oxaliplatin, DCBP, DCRP, DACH and 3478MEEN.

Control conditions in this study considered the effect of GSH in reaction with the 5’FAM dsDNA template in the absence of metallo-drug when subjected to urea-based denaturing agarose slab gel electrophoresis. Inspection of the denaturing agarose slab gels revealed that
the 5′FAM DNA template treated with GSH did not produce a ssDNA band as expected but rather a low apparent molecular weight smear similar to that seen with excess primer after a PCR reaction (Figure 7.4 a, lane 3). This trend contradicts previous work which showed an EcoRI linearised pBR322 plasmid DNA template treated with GSH then subjected to denaturing alkaline agarose slab gel electrophoresis revealed a single ssDNA band (Park et al., 2011). An explanation for this result may be attributed to a difference between the urea-based (this study) and alkaline-based denaturing agarose slab gel protocols which prevented the formation of the ssDNA band in the presence of GSH. Furthermore, initial observations of the urea denaturing slab gels clearly showed that the retention of the dsDNA-like isoform by ICLs subjected to a concentration gradient of GSH was dependent on the metallo-drug treatment (Figure 7.4).

The presence of GSH in the reaction between DNA and the metallo-drug appears to have reduced the cross linking of the DNA as measured by the retention of the dsDNA-like isoform band on urea denaturing agarose slab gel in three ways. (1) The presence of GSH titrated the metallo-drug away from forming ICLs with the DNA substrate as the assay was designed to do. (2) The presence of GSH competed with the metallo-drug for the DNA substrate to induce the low apparent molecular weight 5′FAM-DNA smear observed in the GSH control condition. (3) The presence of GSH affected the retention of the dsDNA-like isoform by cross-linking with a platinum(II) compound partially reacted with DNA to form a GSH-DNA complex, which has previously been reported (Eastman, 1987). This may have contributed to the low apparent molecular weight smear.

This activity associated with GSH in the current experimental system is interesting as the strength of the metallo-drug/DNA association appears to be directly challenged by the affinity of GSH to disrupt DNA conformation as observed on urea denaturing agarose slab gel. Therefore, analysis of the data presented in this Section needs to consider GSH activity with the tested metallo-drug and DNA substrate. The data was analysed by comparing the
calculated GSH : \( \tau_b \) ratio which normalised the metallo-drug and DNA concentration and produced a value that reflected the concentration of GSH required to reduce the metallo-drug induced dsDNA-like isoform to 50% and 5%.

**7.4.2.2 Comparative analysis for metallo-drug affinity to retain the inhibition of DNA strand separation by ICL in the presence of GSH**

DACH showed the highest GSH : \( \tau_b \) ratio out of all tested compounds which indicated that a high concentration of GSH was required to titrate the metallo-drug away from forming ICLs with the DNA template (Figure 7.4 e, Table 7.2, Figure 7.5). This was followed (in decreasing order) by DCBP (Figure 7.4 c), cisplatin (Figure 7.4 a), and then oxaliplatin (Figure 7.4 b) and DCRP (Figure 7.4 d) which were of similar magnitude (Table 7.2, Figure 7.5). The order of GSH-thiol bond affinity between cisplatin, oxaliplatin, DACH and DCRP was similar to the GSH : \( \tau_b \) exhibited in previous work (Section 7.4.1) (performed in the absence of DNA) suggesting that the presence of DNA did not affect the rate of metallo-drug titration towards GSH. Rather, the presence of GSH dictated the rate of metallo-drug titration away from the DNA substrate which can be rationalised as GSH was the predominant constituent in the reaction mix.

The metallo-intercalator 3478MEEN showed the lowest GSH : \( \tau_b \) ratio out of all tested compounds which suggested that a small concentration of GSH was required to titrated the metallo-drug away from forming ICLs with the DNA template (Figure 7.4 f, Table 7.2, Figure 7.5). This trend may reflect the non-covalent \( \pi-\pi \) intercalation previously characterised with 3478MEEN (Krause-Heuer et al., 2009a) where the presence of GSH displaced the metallo-drug association with the DNA substrate as a consequence of the relatively weak association. It is unlikely that 3478MEEN possessed a stronger preference to titrate towards GSH binding over DNA as that trend is not consistent with previous GSH-thiol
bond kinetic data which concluded that steric hindrance prevented that kind of activity from occurring (Section 7.4.1).

In summary, work presented here offers an alternative methodology to characterise the metallo-drug affinity to titrate towards DNA or GSH. Trends were consistent with previous GSH-thiol kinetic data and published literature. Further improvements to address the apparent urea-based denaturing agarose slab gel protocol effect on retaining the ssDNA conformation in the presence of GSH should be further investigated.
Chapter 8: General Discussion

This chapter summarises and compares key discussion points from the work described in previous chapters. The main considerations are presented. Firstly, issues that have arisen during the methodology development and interpretation of biological data are discussed. Secondly, issues related to the validation of biological data by comparing cisplatin trends to those previously published in the literature are discussed. Finally, interesting trends identified with tested metallo-drugs are highlighted and discussed.

Work presented in this thesis employed several methodologies to elucidate the biological effects of selected metallo-drugs for binding to DNA substrates. These methodologies aimed to predict the likely chemo-pharmaceutical properties of the tested metallo-drugs by comparison to the lead platinum(II) compound cisplatin. The first theme considered the classes of metallo-drugs based on their DNA binding mode and the implications of binding to the DNA structure. The second theme measured the affinity at selected metallo-drugs to inhibit DNA replication by DNA polymerase and determine the sequence specificity of metallo-drug/DNA binding sites. Finally, the third theme considered the affinity of metallo-drugs to be titrated away from binding to DNA nitrogen donor ligands in preference to binding to GSH sulfur donor ligands.

8.1 Characterisation of metallo-drug induced DNA helical conformation alterations and hindrance of H-bond melting for dsDNA strand separation

Chapters 3 to 5 employed three methodologies to characterise and compare several classes of metallo-drugs for their affinity to modify DNA structure. The first methodology analysed selected metallo-drugs for their ability to modify the structure of native plasmid DNA conformations and associate the modifications to the compound’s DNA binding mode (Chapter 3). The second experiment utilised a novel application of free solution capillary
electrophoresis to quantifiably compare the effectiveness of selected metallo-drugs to induce structural modifications to a linear dsDNA substrate (Chapter 4). Finally, the third methodology analysed selected metallo-drugs for their capability to form ICLs and prevent dsDNA strand separation under denaturing conditions (Chapter 5).

8.1.1 Metallo-drug studies with plasmid DNA

Initial work with native plasmid DNA intended to measure the angle of metallo-drug induced negatively supercoiled plasmid conformation unwinding which has been standard practice for measuring DNA structure distortions in a variety of studies with platinum(II) compounds (Suchánková et al., 2009, Keck and Lippard, 1992, Kostrhunova et al., 2010, Krause-Heuer et al., 2009a). However, isolation of negatively supercoiled plasmid DNA by caesium chloride density gradient centrifugation as previously described (Keck and Lippard, 1992) was not able to be performed due to limited resources. Therefore, an approach was taken which set out to differentiate metallo-drugs with different DNA binding modes by considering the structure modification of all three plasmid DNA conformations.

This approach is similar to previous metallo-drug plasmid DNA structure studies which differentiated compounds by their DNA binding mode. For example, ruthenium(II) and copper(II) compounds were shown to exhibited nuclease cleaving activity (Hernández-Gil et al., 2012, Patel et al., 2011) using similar techniques to those presented in this thesis. Another example described the plasmid DNA structure modification of aluminium(III) π-π base stacking metallo-intercalators which accumulate and increase the overall molecular weight of the DNA substrate (Rajendran et al., 2008).

The data presented in this thesis suggest that metallo-drugs with different DNA binding modes were able to be differentiated by measuring the mobility patterns of three plasmid DNA conformations on agarose slab gel as discussed. The DNA covalent binders cisplatin and DACH have been well characterised for their ability to unwind negatively supercoiled
plasmid DNA (Kasparkova et al., 2010). This trend and magnitude of negatively supercoiled plasmid DNA unwinding between cisplatin and DACH was also replicated in the current data set (Figure 3.2). Furthermore, closer inspection of cisplatin and DACH agarose slab gels along with other analogue covalent DNA binder metallo-drugs oxaliplatin (Figure 3.2 b), DCBP (Figure 3.2 c) and DCRP (Figure 3.2 d) clearly showed a unique pattern of plasmid DNA migration where the three bands converge upon treatment with compounds that share this common DNA binding mode.

This pattern of plasmid DNA mobility on agarose slab gel induced by the covalent binder metallo-drugs contrasts with other platinum(II) compounds which exhibited non-covalent modes of DNA binding. The metallo-intercalator 56MESS has previously been characterised to adopt a π-π base stacking intercalation DNA binding mode (Krause-Heuer et al., 2009b). In the current work, 56MESS clearly induced a unique plasmid mobility pattern on agarose slab gel where all three plasmid conformations were retained and increased in apparent molecular weight in a dose-dependent manner (Figure 3.4 a). The contrast of plasmid DNA mobility patterns between DNA covalent binders and non-covalent DNA intercalator metallo-drugs such as cisplatin and 56MESS, respectively, showed that differentiation of novel metallo-drugs by DNA binding mode can be achieved by considering the migration pattern of the three plasmid DNA conformations on agarose slab gel across a concentration gradient.

8.1.2 Detection of metallo-drug induced structure modifications to a linear dsDNA substrate by free solution capillary electrophoresis

The novel application of free solution capillary electrophoresis in the critical conditions was applied to measure metallo-drug induced structure modifications to a linear dsDNA substrate. Initial optimisation work considered the sodium borate buffer concentration and DNA electropherogram peak profiles as discussed in Sections 4.3.1 and 4.3.2. Further optimisation
work also considered the concentration of cisplatin necessary to detect changes in linear
dsDNA structure by free solution CE-CC as described in Section 4.3.3.

Data presented here showed that free solution CE-CC was able to be employed to quantifiably
detect and compare metallo-drugs with different DNA binding modes which induced structure
modifications to a linear dsDNA substrate independent of the DNA molecular weight. For
example, the strand breaker Cu56MESS exhibited negligible reactivity with the DNA
substrate (Figure 4.6 c) which is consistent with the plasmid DNA mobility work (Figure 3.4
c) and published literature which described the need for H2O2 to facilitate DNA strand
breaking (Krause-Heuer et al., 2012).

Analysis of electropherogram peak data revealed that cisplatin increased the linear dsDNA
substrate mobility in contrast to other tested metallo-drugs with covalent DNA binding
modes, such as DACH, which decreased the DNA substrate mobility (Figure 4.6 a). This
pattern of DNA mobility suggests that cisplatin condensed the DNA substrate and prevented
counter-ion penetration into the double helix structure according to previously published
DNA CE-CC theory (Stellwagen et al., 1997, Stellwagen et al., 2003). This trend disagrees
with previously published data which concluded that cisplatin and its analogue DACH both
unwind and relax dsDNA substrates upon covalent binding (Kasparkova et al., 2010). A
definitive explanation for the discrepancy between cisplatin and the analogue platinum(II)
metallo-drug trends cannot be offered at this point and is the subject of further investigation.

8.1.3 Determination of a metallo-drug’s DNA binding mode by a two-point
comparative analysis that measured for changes to a linear dsDNA substrate
structure and apparent molecular weight

Work presented in this thesis utilised several methodologies to measure metallo-drug induced
structure modifications to a linear dsDNA substrate. The experiments discussed in Chapter 3
analysed native plasmid DNA (one was a linear dsDNA structure as discussed in Section
3.3.1) for metallo-drug induced changes in apparent molecular weight as a measure of DNA structure modification. The free solution CE-CC experiments discussed in Chapter 4 described a data set which quantitatively measured the metallo-drug induced structure modifications with a linear dsDNA substrate. A comparative analysis of these two data sets was carried out to differentiate the test metallo-drugs by their DNA binding mode. The data sets consisted of the linear pUC19 straight line regression fit data which measured for changes in DNA molecular weight (Table 3.1) and the free solution CE-CC peak mobility ratio data which measured for changes in DNA structure (Table B2). Both data sets were then plotted on a scatter graph to qualitatively correlate trends for metallo-drug induced structure modifications to a linear dsDNA substrate (Figure 8.1).

A relationship existed between metallo-drug induced modifications to the linear dsDNA substrate and DNA binding mode (Figure 8.1). For example, metallo-drugs which share a covalent DNA binding mode such as oxaliplatin, DCBP, DCRP and DACH (Chaney et al., 2005, Kasparkova et al., 2010, Zou et al., 1993, Holford et al., 1998) clearly cluster on the bottom left quadrant on the scatter plot. On the other hand, the metallo-intercalator 56MESS (Brodie et al., 2004) is clearly differentiated from the covalent binder metallo-drugs and clusters down on the bottom right hand quadrant of the scatter plot. The clustering patterns to the bottom half of the scatter plot indicate that both covalent binders and metallo-intercalators compounds relax DNA structure.

However, the data clustering to the right half of the graph indicate that the accumulation of 56MESS on the DNA substrate appears to have contributed to an overall increase in DNA molecular weight according to the scatter plot distribution (Figure 8.1). This conclusion agrees with the plasmid DNA mobility data previously discussed where accumulation of the metallo-intercalator 56MESS contributed to an overall increase in the molecular weight of three plasmid DNA conformations.
Interestingly, the metallo-intercalator 3478MEEN data clusters between the covalent binders and metallo-intercalators on the scatter plot (Figure 8.1). This suggests that 3478MEEN adopts both covalent binder and intercalator DNA binding modes. This trend of dual DNA binding modes adopted by 3478MEEN was also identified with the plasmid DNA mobility data discussed.

Cisplatin’s position on the scatter plot is distinctly separated from all other tested compounds (Figure 8.1). This is due to the CE-CC data generated by cisplatin treatment that produced an unexpected increase in DNA mobility as previously discussed in Sections 4.3.4 and 8.1.2. However, cisplatin’s data points on the 2D plot are on the left hand side of the graph as are the other tested covalent binder metallo-drugs which provides an indication of DNA binding mode homology.

Analysis of Cu56MESS showed negligible DNA reactivity (Figure 8.1) which was previously identified in the plasmid DNA mobility (Figure 3.4 c) and CE-CC DNA mobility (Figure 4.6 c) work and can be attributed to the reported need for H₂O₂ to facilitate copper-mediated DNA strand breaking (Krause-Heuer et al., 2012). Interestingly, Cu56MESS clustered close to the DNA strand breaker phleomycin (Figure 8.1) which suggests that both compounds exhibit a similar DNA binding mode. However, phleomycin induced a small increase in DNA molecular weight which may be indicative of the compound’s ability to relax the double helix structure upon DNA cleavage or reflect (all or some part of) phleomycin covalently bound to the DNA molecule thereby contributing to an increase in molecular weight (Stern et al., 1974, Sleigh, 1976, Kross et al., 1982).

Observations of the scatter plot data with the two ruthenium(II) metallo-drugs to the upper half of the graph indicated that both compounds contributed to a condensation of the linear dsDNA substrate (Figure 8.1). However, the addition of another pyrrole ligand on to the RuP2 groove binding tail shifted the data set far to the right which indicates a significant
increase in the linear dsDNA substrate’s molecular weight (Figure 8.1). An explanation for
the RuP2 mediated increase in DNA molecular weight compared to RuP1 could relate to the
additional pyrrole ligand on RuP2 which appears to have substantially increased the metallo-
drug’s ability to bind and accumulate on to the DNA substrate (Orkey et al., 2012, Wade et
Figure 8.1: Scatter plot showing changes in linear dsDNA apparent molecular weight and structure after treatment with metallo-drugs. Linear pUC19 dsDNA template was treated with metallo-drugs, subjected to native slab gel electrophoresis and data was fitted with a linear regression where the straight line gradient was used to compare metallo-drug effectiveness at modifying DNA apparent molecular weight (x-axis). PCR generated dsDNA template DNA was treated with the same metallo-drugs, however, the treated DNA was subjected to free solution CE-CC where the mobility of DNA peaks A (■), B (●), C (▲) and D (▼) were monitored and converted to a ratio of migration change relative to water control to compare metallo-drug effectiveness at modifying DNA helical structure (y-axis). DNA mobility ratio controls are shown for the average of peaks A, B, C and D treated with DMF solvent (---) where values above or below the y-intercept either increase or decrease DNA mobility, respectively. An additional DNA mobility ratio control for the average of peaks A, B, C and D treated with DMF solvent and DTT (---) was included, however, this is only relevant for DNA treated with phleomycin as this was the only compound where DTT was present to facilitate strand breaking.
8.1.4 Interstrand cross link assay

It is a well established practice for metallo-drug studies to analyse novel compounds for their ability to form ICLs on a dsDNA substrate, as the biological consequences of DNA cross-linking are well characterised for its disruption to DNA metabolism (Todd and Lippard, 2009). Lead platinum(II) metallo-drugs such as cisplatin have been well characterised for their ability for form ICLs on a dsDNA substrate by employing traditional alkaline denaturing agarose slab gels and $^{32}$P end labelling methodologies (Mlcouskova et al., 2012, Halámiková et al., 2008, Kasparkova et al., 2010). Furthermore, the application of this type of methodology allows for the differentiation between metallo-drugs that exhibit a covalent DNA binding mode or strand breaking nuclease activity from compounds with non-covalent DNA binding modes.

ICL assay work presented in this thesis began by applying updated methodologies as discussed in Section 5.3.1. The first of these methodologies replaced the established alkaline denaturing agarose slab gels (Mlcouskova et al., 2012) with a urea-based denaturing agarose slab gel (Hegedus et al., 2009). The second methodology update replaced the $^{32}$P 5'-labelled dsDNA substrate (Woynarowski et al., 1998) with a FAM fluorophore to reduce health risks and enhance the efficiency of experimental practice. The application of the updated methodologies to quantify metallo-drug induced ICLs on a linear dsDNA substrate with platinum(II) compounds cisplatin and DACH (Figure 5.6) correlated with previously published data using traditional methodologies with platinum(II) compounds (Kasparkova et al., 2010).

The ICL assay and application of the updated methodologies clearly differentiated tested compounds with covalent DNA binding mode and strand breaking nuclease activity from metallo-drugs that do not form covalent bonds with a linear dsDNA substrate (Section 5.3.2). For example, as expected all tested covalent binders – oxaliplatin, DCBP, DCRP, DACH and
cisplatin (Figure 5.2) – exhibited ICL activity. Furthermore, the DNA strand breaker phleomycin induced a degraded smear though multiple cleaving of the linear dsDNA substrate phosphate backbone which agrees with previously published trends (Farrell and Reiter, 1973).

Other tested metallo-drugs such as the ruthenium(II) groove binders (Figure 5.5), metallo-intercalator 56MESS (Figure 5.4 a) and strand breaker Cu56MESS (Figure 5.4 b) all showed a lack of ICL activity on the linear dsDNA substrate as expected due to their previously reported non-covalent DNA binding modes (Orkey et al., 2012, Brodie et al., 2004, Krause-Heuer et al., 2012). In contrast, the platinum(II) metallo-intercalator 3478MEEN did show ICL reactivity with the dsDNA substrate at high dose (Figure 5.4 b) which contradicts its previously described non-covalent DNA binding mode (Brodie et al., 2004). Furthermore, the ICL reactivity trend exhibited by 3478MEEN provides further evidence that this metallo-drug adopts a dual DNA binding mode which is consistent with previous DNA structure data discussed in this thesis (Chapters 3 and 4) and the comparative analysis discussed in Section 8.1.3.

8.2 Analysis of test metallo-drugs for their DNA binding sequence specificity and ability to inhibit DNA replication by DNA polymerase

Chapter 6 employed the LA reaction and applied two methodologies to characterise the affinity of several metallo-drugs with different DNA binding modes to inhibit DNA replication and determine the sequence binding specificity by DNA polymerase. The first methodology measured the metallo-drug affinity to inhibit DNA replication at the sub-gene level via the incorporation of a fluorescent nucleotide to label the newly synthesised ssDNA for subsequent fluorescent detection (Section 6.4.1). The second methodology identified the metallo-drug DNA binding sequence specificity where DNA extension inhibition was
measured at the single base-pair level though the use of a fluorescently labelled primer to facilitate ssDNA fluorescence detection (Section 6.4.2).

### 8.2.1 Measuring the metallo-drug induced inhibition of DNA replication by a DNA polymerase

This work employed a fluorescent body labelled methodology to quantify ssDNA LA products by incorporating a R6GdUTP nucleotide (Henegariu et al., 2000) with optimisation work discussed in Section 6.4.1.1. The initial intention for the R6GdUTP fluorescent detection method was to apply it to an *in vitro* transcription assay to characterise metallo-drug ability to inhibit RNA synthesis (Jung and Lippard, 2006) with possible single-base resolution detection by CE-LIF as discussed with the LA work in Section 6.4.2. However, the separation of body labelled ssDNA with Rhodamine 6G fluorophores by CE-LIF proved to be problematic as the electropherogram traces did not produce replicable full extension peaks with minimal artefacts, such as those with a 5’FAM end labelled protocol (Figure D2), which is further discussed in Section 6.4.1.1.

The current work applied the R6GdUTP body labelling fluorescent detection method in LA DNA replication studies with the intention to serve as a quick protocol to quantitatively compare test metallo-drug’s ability to inhibit DNA replication which is similar to previously described PCR-based approaches (Jennerwein and Eastman, 1991). Data discussed in Section 6.4.1.2 showed that the application of the R6GdUTP and LA methodologies was able to quantitatively detect metallo-drug induced inhibition of DNA replication. For example, the lead platinum(II) metallo-drug cisplatin was shown to induce an inhibition of R6G labelled ssDNA LA products (Figure 6.2 a) through the formation of covalent adducts with the DNA template which has previously been reported in the literature (Murray et al., 1992, Jennerwein and Eastman, 1991).
Furthermore, correlations between metallo-drug DNA binding mode and the magnitude of DNA replication inhibition were also able to be established. For example, the covalent binder DACH showed a higher degree of DNA replication inhibition than cisplatin (Figure 6.2 e & Figure 6.6) which is in agreement to previously reported trends (Kasparkova et al., 2010). On the other hand, other tested platinum(II) metallo-drugs such as 56MESS and 3478MEEN were also shown to be capable of inhibiting DNA replication (Figure 6.4) which is also consistent with previously reported trends (Krause-Heuer et al., 2009a). However, the degree of DNA replication inhibition was clearly greater with covalent binders such as cisplatin and DACH than the non-covalent metallo-intercalators 56MESS and 3478MEEN (Figure 6.6).

### 8.2.2 Characterising the metallo-drug sequence specificity for DNA binding

This section of work set out to characterise the DNA binding sequence specificity of selected metallo-drugs according to their DNA binding mode (Section 6.4.2). The method employed primers with a 5ʹFAM modification to enable the fluorescent detection of ssDNA LA products by CE-LIF. The use of LA reaction methodologies coupled with fluorescent detection and size separation by CE-LIF has been described previously in work that set out to determine cisplatin’s preferential DNA binding sites on conserved sequences (Moumita and Murray, 2011).

Initial optimisation of these protocols considered the LA reaction primer and DNA template quantities, ddNTP concentrations for DNA sequencing reactions and cisplatin concentration for adequate DNA damage distribution as discussed in Section 6.4.2.1. One issue of concern related to the inspection of metallo-drug DNA damage and DMF control overlayed electropherogram traces generated by the 22 mer 5ʹFAM primer which appeared to exhibit a higher background noise than LA reactions with the 17 mer 5ʹFAM primer (Figure 6.10). This made it harder to distinguish real DNA damage sites with the 22 mer primer. However,
downstream analysis of replicate metallo-drug induced DNA damage LA data revealed distinguishable trends for DNA base binding preferences which agreed with the literature.

Analysis of covalent binder drugs such as cisplatin, DACH, oxaliplatin and carboplatin all showed high preference for binding to purine bases (Figure 6.11 b). Furthermore, the two-base analysis revealed that these platinum(II) covalent binder metallo-drugs exhibited specific sequence binding preference for GG, AG and GC sites (Figure 6.13 a). These DNA sequence specificity binding trends are in line with previously published data that showed platinum(II) covalent binder metallo-drugs exhibit high preference for purine bases (Kasparkova et al., 2010, Burstyn et al., 2000, Boudný et al., 1992, Murray et al., 1997, Woynarowski et al., 1998).

Analysis of 56MESS damage did not significantly reveal any preferential DNA binding sites (Figure 6.13 c) which contradicts previous DNaseI footprinting work that identified 56MESS at similar concentrations preference for guanine sequences (Krause-Heuer et al., 2009a). Furthermore, work with the ruthenium(II) groove binder RuP1 did not reveal any significant binding sites (Figure 6.13 d) which contradicts the intended design of the pyrrole-imidazole polyamide tail which should exhibit a binding preference for AT and TA sites at similar concentrations (Orkey et al., 2012, Wade et al., 1992). The results obtained by 56MESS and RuP1 may be due to under damage as previous LA work showed these compounds were less effective at inhibiting DNA replication than analogue platinum(II) metallo-drugs like cisplatin as discussed in Section 6.4.1.

Interestingly, the 56MESS analogue platinum(II) metallo-intercalator 3478MEEN did show significant preference for binding to GG sequence sites (Figure 6.13 c). This may be a reflection on the previous DNA structure characterisation work, which identified that 3478MEEN could exhibit dual intercalation and covalent DNA binding modes (Chapters 3 to 5 and 8.1.3). Future characterisation of 3478MEEN mode of DNA binding will need to be carried out to verify the current findings.
8.3 Influence of glutathione sulfur donors for titrating metallo-drugs away from nitrogen donor DNA targets

Chapter 7 utilised two experiments to determine the binding preference of several test metallo-drugs for DNA nitrogen donor ligands or GSH sulfur donor ligands. The first approach consisted of a methodology that measured the affinity for metallo-drugs to form thiol bonds to GSH (Section 7.4.1). The second approach employed a competitive reaction methodology where the affinity of selected metallo-drugs to be titrated away from forming ICLs with a dsDNA template in favour for forming thiol bonds with GSH was measured (Section 7.4.2).

8.3.1 Metallo-drug thiol kinetic assay with GSH

This work was based on a methodology previously published where the kinetics of thiol bond formations between GSH and platinum(II) metallo-drugs cisplatin, carboplatin and oxaliplatin were measured (Hagrman et al., 2004). Data presented in the current work for GSH thiol bond reactivity between cisplatin, oxaliplatin and carboplatin (Figure 7.3) matched previously defined trends (Hagrman et al., 2004). However, thiol bond formation between GSH and the three platinum(II) metallo-drugs appeared to be reduced when dissolved in DMF (Figure 7.3).

Other tested covalent DNA binder platinum(II) metallo-drugs like DACH showed a slightly decreased GSH reactivity compared to cisplatin (Figure 5.3) which is consistent with previously published data (Kasparkova et al., 2010). On the other hand, platinum(II) metallo-intercalators 56MESS and 3478MEEN (Figure 5.2 c) along with both ruthenium(II) groove binders RuP1 and RuP2 (Figure 5.2 d) showed no significant increase in GSH thiol bond reactivity (Figure 5.3, Table 5.1). This is likely due to their large bulky ligands which could sterically hinder GSH binding and their previously reported non-covalent mode of DNA binding (Orkey et al., 2012, Brodie et al., 2004).
Interestingly, the strand breaker Cu56MESS appeared to exhibit a GSH kinetic reaction profile similar to the DNA strand breaker phleomycin (Figure 5.3). An explanation for this trend could be related to the DNA strand breaker mode previously reported for Cu56MESS (Krause-Heuer et al., 2012) and phleomycin (Sleigh, 1976, Pietsch and Garrett, 1968, Farrell and Reiter, 1973, Kross et al., 1982). Furthermore, the need for a reducing agent did not appear to be required to facilitate this trend of GSH kinetic bond activity as shown with the phleomycin positive or negative DTT controls.

8.3.2 Competitive assay to measure metallo-drug titration between GSH and DNA

This assay consisted of a competitive reaction to measure test metallo-drug’s affinity to bond with GSH sulfur donor ligands or DNA nitrogen donor ligands. The methodology employed the ICL assay data (Section 5.1) where concentrations of test metallo-drugs were determined that completely prevented strand separation of a linear dsDNA substrate under denaturing conditions. These conditions were then challenged by titrating GSH to facilitate a competitive reaction with DNA which was quantified by densitometry measurements of the ICL dsDNA-like conformation.

The experimental design restricted the type of metallo-drugs that were able to be tested to compounds that were able to induce ICL on a dsDNA substrate. Out of all tested metallo-drugs, 3478MEEN appeared to exhibit the highest preference to form thiol bonds with GSH compared to binding to DNA (Figure 7.5) which may be a reflection of the previously characterised non-covalent \( \pi-\pi \) intercalation with DNA or some unexpected affinity for GSH (Krause-Heuer et al., 2009a). On the other hand, the covalent binder metallo-drug DACH appeared to exhibit a higher preference to bind to a DNA substrate in the presence of GSH (Figure 7.5). To our knowledge, this trend has not been previously observed using a large DNA substrate.
8.3.3 Two-point comparative analysis to assess metallo-drug binding preference for DNA nitrogen donor ligands or GSH sulfur donor ligands

A comparative analysis of two data sets was carried out and plotted on a 2D scatter graph (Figure 8.2) to better elucidate test metallo-drugs’ preferential binding to DNA nitrogen donor ligands or GSH sulfur donor ligands. The first dimension data set is derived from the GSH kinetic data described in Section 7.2 (Figure 7.1). The data set for the second dimension is derived from the competitive reaction assay described in Section 7.3 where metallo-drug preferential binding for GSH or DNA was examined (Table 7.2).

As expected, the data distribution for cisplatin to the upper left quadrant of the scatter plot suggests a higher binding preference for GSH over DNA (Figure 8.2). This is consistent to previously reported trends which characterised cisplatin for exhibiting stronger preference to form bonds with sulfur donors over nitrogen donors (Bose et al., 1995, Reedijk, 1999, Kasherman et al., 2009). Data distribution for DACH to the upper right quadrant on the scatter plot suggested the metallo-drug exhibited preferential binding for both DNA and GSH (Figure 8.2). On the other, the data distribution for DCBP to the lower left quadrant suggests this metallo-drug exhibits low binding preference for both DNA and GSH (Figure 8.2).
Figure 8.2: 2D scatter plot mapping GSH : r_b ratios where dsDNA isoform retention was reduced to 50% and 5% and initial absorbance rates for metallo-drugs forming thiol bonds with GSH. Slab gel densitometry analysis of DNA in competition between GSH and metallo-drug uptake yielded GSH:r_b ratios where compound induced dsDNA isoform retention was reduced to 50% (●) and 5% (●). This is plotted against the initial UV absorbance rates of metallo-drug thiol bonding with GSH. Data points in the lower left quadrant indicate metallo-drugs have no affinity for either GSH or DNA. Data points in the upper right quadrant indicate metallo-drugs that have high affinity for both DNA and GSH. Data points in the upper left and lower right quadrants indicate that tested metallo-drugs have high affinity to bind to GSH and DNA, respectively.
Chapter 9: Conclusions & Future directions

The work described in this thesis set out to develop and apply several methodologies to determine the potential of test metallo-drugs for further investigations as anti-cancer compounds. Analysis centred on the rational drug design concept where the biological activity of test metallo-drugs was compared to the lead platinum(II) compound cisplatin. The methodologies employed in this study focused on three core themes that aimed to characterise the test metallo-drugs:

- DNA binding mode and degree of double helix structure modification;
- DNA replication inhibition by a DNA polymerase and the sequence specificity of metallo-drug/DNA binding sites;
- affinity to be titrated away from binding to the therapeutically relevant DNA nitrogen donor ligands in preference to binding to GSH sulfur donor ligands.

9.1 Conclusions

9.1.1 Methodology development

The first theme applied three methodologies to elucidate the efficacy of several test metallo-drugs to bind and modify DNA structure. The first experiment produced evidence that metallo-drugs with covalent and non-covalent DNA binding modes were able to be differentiated by analysing the mobility patterns of three native plasmid DNA conformations on agarose slab gel. The second experiment provided evidence that a novel application of free solution CE-CC could quantify metallo-drug induced structure modifications on a linear dsDNA substrate. Furthermore, differentiation of test metallo-drugs into more specific DNA binding modes was able to be achieved by 2D scatter plot analysis which considered test metallo-drug induced changes in linear plasmid DNA molecular weight and structure modification of a linear dsDNA substrate by CE-CC. The third experiment within this theme
investigated the potential for test metallo-drugs to form interstrand crosslinks on a 5′FAM linear dsDNA substrate with a novel application of a urea-based denaturing slab gel electrophoresis. Validation of these new protocols was confirmed by comparing trends between cisplatin and other analogue compounds to those published in the literature.

The second theme applied the LA reaction in two methods to elucidate the potential of test metallo-drugs to inhibit DNA replication by a DNA polymerase and determine the sequence specificity of metallo-drug/DNA binding sites. The first experiment employed the novel application of Rhodamine 6G labelled nucleotides to quantify DNA replication. Data generated from this experiment identified a relationship that showed higher degrees of ssDNA synthesis inhibition with metallo-drugs that exhibit higher strength DNA binding modes. The second experiment employed a 5′FAM labelled primer in a LA reaction with separation of ssDNA molecules by CE-LIF. Analysis of the cisplatin induced DNA replication truncation sites corresponded to known purine base binding preference trends previously identified in the literature which aided in validating data generated from other test metallo-drugs in the current study.

The third theme set out to determine the affinity of test metallo-drugs to be titrated away from binding to their therapeutically significant DNA nitrogen donor ligands in preference for binding to GSH sulfur donor ligands. The first experiment consisted of a GSH kinetic study to measure metallo-drug thiol bond reactivity where trends with the lead platinum(II) compounds cisplatin, oxaliplatin and carboplatin agreed with published literature. Furthermore, the presence of DMF showed a significant dampening effect on metallo-drug reactivity with GSH which may have implications of DNA binding rates. Finally, a novel in vitro competitive assay and analysis protocol was developed which quantifies test metallo-drug binding preference between DNA and GSH.
9.1.2 Interesting metallo-drug trends

Analysis of DNA structure studies with the platinum(II) metallo-intercalator 3478MEEN suggested that this compound exhibited dual covalent binder and $\pi-\pi$ base stacking DNA binding modes. Evidence for this trend was shown in the comparative DNA structure analysis 2D scatter plot where 3478MEEN clustered between the covalent binder and intercalator compounds. Analysis of the ICL assay revealed that 3478MEEN was capable of preventing dsDNA strand separation under denaturing conditions possibly in a similar manner to covalent binders like cisplatin. The final piece of evidence that 3478MEEN adopts dual DNA binding modes was in the LA sequence specificity work where 3478MEEN exhibited a high binding preference for GG sites which is analogous to platinum(II) covalent binder metallo-drug trends.

The copper(II) metallo-drug Cu56MESS showed negligible DNA reactivity which is consistent with published trends where a reducing agent like $\text{H}_2\text{O}_2$ was required. Interestingly, the 2D scatter plot analysis of linear dsDNA substrate modification of molecular weight and structure showed that Cu56MESS and another non-metallo-drug DNA strand breaker, phleomycin, exhibit similar DNA modification profiles. Furthermore, Cu56MESS in the absence of a reducing agent exhibited a similar GSH reactivity profile with phleomycin which suggests the need for a reducing agent may not be necessary for reactions with other biological substrates.

The 2D scatter plot analysis of metallo-drug DNA structure modification showed that the ruthenium(II) groove binder RuP2 with its additional pyrrole ligand compared to RuP1 could substantially increase the molecular weight of a linear dsDNA substrate. Furthermore, the GSH kinetic data revealed that metallo-drugs with non-covalent DNA binding modes tended to exhibit lower GSH thiol bond formations than compounds that display covalent DNA binding modes.
9.2 Future directions

9.2.1 Further methodology development and applications

Further work is required to explain why the three native plasmid DNA conformations exhibit different migration profiles that correlate to metallo-drugs with covalent and non-covalent DNA binding modes. The use of free solution CE-CC proved to be a highly sensitive tool to quantify metallo-drug induced changes to DNA structure, however, the cisplatin induced increase in DNA mobility in contrast to other tested platinum(II) covalent binder metallo-drugs requires further work to be explained. Furthermore, future CE-CC studies could analyse changes in mobility of linear dsDNA substrates with different lengths or nucleotide composition. In a similar fashion, the ICL assay could be applied in a comparative analysis of dsDNA substrates with different lengths or nucleotide composition to correlate test metallo-drugs’ base binding specificities.

Further investigations could be pursued which use R6GdUTP fluorescent nucleotides in vitro transcription assays to characterise test metallo-drugs’ ability to inhibit RNA expression. In reduced GSH reactivity with lead platinum(II) metallo-drugs needs to be further investigated as it may affect the results of kinetic assays of other test metallo-drugs with GSH or DNA. Finally, further work needs to be carried out to explain why ssDNA products were not observed in the GSH/DNA competitive reaction assay where it was assumed that titration of metallo-drug to GSH would leave the DNA substrate in its native state under denaturing conditions.

Further work with metallo-drugs and GSH could include picoplatin as a control drug for comparison to the novel drugs as it exhibits a preference for DNA binding over GSH. Such a control would allow better comparisons with the current experimental data and improve the predictability of novel metallo-drug designs.
9.2.2 Further exploration of metallo-drug trends

The apparent covalent and intercalator dual DNA binding modes exhibit by 3478MEEN is interesting and could be further investigated as the analogue 56MESS did not exhibit these trends, suggesting that additional methyl ligands may be playing a role. The notable increase in RuP2 mediated DNA molecular weight in comparison to its analogue RuP1 appears to be due to the additional pyrrole ligand on RuP2 but requires further characterisation. The LA sequence specificity studies with metallo-drugs 56MESS, 3478MEEN and RuP1 did not correlate with previously published DNaseI footprinting studies or the ruthenium(II) compounds intended DNA binding design for TA or AT regions which require further investigation. Furthermore, the LA procedure needs to be repeated in the opposing pUC19 strand to further verify the DNA damage sites and sequence specificity data.
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Appendix A: pUC19 quantification supplementary data

Supplementary data provided for the experiments described in Section 3.2 for quantitative replicate measurements of native pUC19 on agarose slab gels. Quantification was done for the apparent molecular weight of the three pUC19 conformations against the 1 kb size standard ladder (Table A1). Densitometry measurements of DNA band intensity were done to quantify the population of the three native pUC19 conformations which were converted into a percentage (Table A2).

Table A1: Replicate apparent molecular weight measurements of DNA bands representing the three conformations of native pUC19 along with straight line regression coefficient of the 1 kb ladder size standard. Data was averaged and standard deviation calculated.

<table>
<thead>
<tr>
<th>Nicked</th>
<th>Linear</th>
<th>Supercoiled</th>
<th>1 kb Ladder</th>
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<td>2439</td>
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<td>2957</td>
<td>2551</td>
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<tr>
<td>Std Dev (±)</td>
<td>181</td>
<td>131</td>
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Table A2: Replicate densitometry measurements of DNA bands representing the three conformations of native pUC19. Data was converted into a percentage of the total population of the three plasmid conformations. Percentages were then averaged and standard deviation calculated.

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<tr>
<th>Densitometry (Au, minus background)</th>
<th>Percentage of Total Population (%)</th>
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<td>251</td>
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<tr>
<td>Average (%)</td>
<td>27.61</td>
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<td>Standard Deviation (±)</td>
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Appendix B: CE-CC mobility ratio supplementary data

Supplementary data provided for the CE-CC work described in Section 4.2. Tables show the mobility of peaks A, B, C and D along with their calculated mobility ratios which were normalised with the water control. The first table shows the control conditions and cisplatin concentration gradient which is described in Section 4.2.2 (Table B1). The second table shows the calculated values for all other tested compounds which is described in Section 4.2.3 (Table B2).

Table B1: Summary of peak mobility ratios of treated cisplatin DNA. Four DNA peaks (A, B, C and D) were monitored for changes in mobility due to treatment with water, 5% DMF and cisplatin at 0.015, 0.05 and 0.15 µM. The average mobility (mobility) and standard deviation (± StdDev) of these peaks are presented. Peak mobility was converted to a ratio of DNA treatment to water control which allowed for a more direct comparison between treatments. The average ratio (ratio) and standard deviation (± StdDev) of these peaks are presented.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
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<tbody>
<tr>
<td></td>
<td>Mobility</td>
<td>± StdDev</td>
<td>Mobility</td>
<td>± StdDev</td>
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<td>3.16E-11</td>
<td>3.68E-08</td>
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<td>DMF Control</td>
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<td>3.69E-08</td>
<td>7.66E-11</td>
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<td>Cisplatin 0.015 µM</td>
<td>3.84E-08</td>
<td>1.58E-10</td>
<td>3.90E-08</td>
<td>1.94E-10</td>
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<tr>
<td>Cisplatin 0.05 µM</td>
<td>1.058</td>
<td>0.003</td>
<td>1.060</td>
<td>0.004</td>
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<td>Cisplatin 0.15 µM</td>
<td>3.88E-08</td>
<td>1.62E-10</td>
<td>3.94E-08</td>
<td>0.00E+00</td>
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<td>1.069</td>
<td>0.004</td>
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<td>1.072</td>
<td>0.005</td>
<td>1.068</td>
<td>0.004</td>
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Table B2: Summary of peak mobility ratios of treated DNA with test metallo-drugs.

Four DNA peaks (A, B, C and D) were monitored for changes in mobility due to treatment. Treatment consisted of 0.015 μM of each compound tested with appropriate controls (water, 5% DMF, 25 mM DTT and 5% DMF and 25 mM DTT). Peak mobility was converted to a ratio of DNA treatment to water control (presented in brackets).

<table>
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<tr>
<th>Treatment</th>
<th>Controls</th>
<th>Covalent Binders</th>
<th>Strand Breaker</th>
<th>Metallo-intercalators</th>
<th>Groove Binders</th>
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<td>(Peak Mobility Ratio – DNA Treatment : Water Control)</td>
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<td></td>
<td>Mobility (Ratio) ± StdDev</td>
<td>Mobility (Ratio) ± StdDev</td>
<td>Mobility (Ratio) ± StdDev</td>
<td>Mobility (Ratio) ± StdDev</td>
<td>Mobility (Ratio) ± StdDev</td>
</tr>
<tr>
<td>Water Control</td>
<td>3.62E-08 (1.000) 6.81E-10 (0.000)</td>
<td>3.66E-08 (1.000) 8.63E-10 (0.000)</td>
<td>3.77E-08 (1.000) 6.58E-10 (0.000)</td>
<td>3.85E-08 (1.000) 6.40E-10 (0.000)</td>
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<tr>
<td>DMF Control</td>
<td>3.70E-08 (1.021) 6.40E-10 (0.002)</td>
<td>3.75E-08 (1.023) 7.80E-10 (0.003)</td>
<td>3.86E-08 (1.024) 5.99E-10 (0.002)</td>
<td>3.95E-08 (1.022) 5.48E-10 (0.003)</td>
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<td>DTT Control</td>
<td>3.69E-08 (1.020) 9.28E-10 (0.006)</td>
<td>3.73E-08 (1.019) 1.11E-09 (0.006)</td>
<td>3.84E-08 (1.018) 9.44E-10 (0.007)</td>
<td>3.91E-08 (1.018) 8.63E-10 (0.006)</td>
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<td>DMF+DTT Control</td>
<td>3.75E-08 (1.036) 1.04E-09 (0.009)</td>
<td>3.79E-08 (1.034) 1.16E-09 (0.007)</td>
<td>3.91E-08 (1.036) 9.33E-10 (0.007)</td>
<td>3.97E-08 (1.032) 7.83E-10 (0.003)</td>
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<td>Cisplatin</td>
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<td>Oxaliplatin</td>
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<td>DACH</td>
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<td>3.66E-08 (1.001) 2.95E-10 (0.032)</td>
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<td>Cu56MESS</td>
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<td>56MESS</td>
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<td>3478MEEN</td>
<td>3.62E-08 (1.000) 5.99E-10 (0.002)</td>
<td>3.66E-08 (0.999) 7.32E-10 (0.004)</td>
<td>3.76E-08 (0.998) 4.33E-10 (0.006)</td>
<td>3.84E-08 (0.999) 5.21E-10 (0.003)</td>
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<tr>
<td>RuP1</td>
<td>3.71E-08 (1.025) 8.45E-10 (0.004)</td>
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<td>3.93E-08 (1.041) 1.38E-09 (0.018)</td>
<td>4.02E-08 (1.046) 1.48E-09 (0.021)</td>
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<td>RuP2</td>
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<td>3.76E-08 (1.028) 7.32E-10 (0.004)</td>
<td>3.87E-08 (1.025) 4.33E-10 (0.006)</td>
<td>3.98E-08 (1.035) 7.03E-10 (0.001)</td>
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Appendix C: Rhodamine-6G-dUTP DNA body labelling optimisation supplementary data

Supplementary data provided for experiments described in Section 6.4.1. Confirmation of R6GdUTP labelled was done by agarose slab gel (Figure C1) and densitometry quantification was performed to determine coupling reaction yield (Table C1). Optimisation of R6GdUTP concentration for PCR body fluorescence labelled was done by titration of coupling reaction product and qualitative observation by agarose slab gel electrophoresis for stable DNA band morphology (Figure C2).

![Agarose slab gel of caboxy-Rhodamine 6G (R6G) and AminoAllyl-dUTP labelled with R6G (R6GdUTP). Lane 1 shows free R6G and lanes 2 to 4 show triplicate loading of R6GdUTP reaction mix containing AAdUTP labelled R6G and free R6G.](image)

Figure C1: Agarose slab gel of caboxy-Rhodamine 6G (R6G) and AminoAllyl-dUTP labelled with R6G (R6GdUTP). Lane 1 shows free R6G and lanes 2 to 4 show triplicate loading of R6GdUTP reaction mix containing AAdUTP labelled R6G and free R6G.
Table C1: Densitometry data of free R6G and R6GdUTP. Densitometry measurements (Au) of R6G and R6GdUTP were determined and subtracted from background (BG) measurements, averaged and converted to a percentage to determine the coupling reaction yield.

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<td>Au</td>
<td>Au-BG</td>
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<td>Average (%)</td>
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Figure C2: Fluorescent image of native agarose slab gel of PCR product body labelled with concentrations of R6GdUTP. PCR reactions were supplemented with various concentrations of R6GdUTP to determine optimal concentration for stable DNA band migration on slab gel electrophoresis. Lane 1 shows a 1kb ladder pre-stained with SYBR Green 1. Lane 2 is of a non-flurophore labelled PCR product. Lanes 3 to 7 show descending concentrations of R6GdUTP. Lane 8 shows flurophore control containing R6G only.
Appendix D: 5’FAM DNA end labelling LA optimisation

Supplementary data

Supplementary data provided for various LA reaction optimisations for experiments described in Section 6.4.2. Optimisation first considered DNA template and primer concentrations for electropherogram full extension ssDNA peak detection (Figure D1). Comparisons were made for electropherogram peak detection of a full extension LA product between end-labelled FAM and body-labelled R6G fluorophore (Figure D2). Optimisation of cisplatin treatment of the linear pUC19 (PvuII) was performed to determine the concentration that would give the best resolvable peak distribution for sequence specificity studies (Figure D3). An example sequencing reaction optimisation with ddCTP to enable base-pair resolution sequencing was also shown (Figure D4).
Figure D1: Electropherogram traces showing optimisation data for DNA template and primer concentration to product LA full extension ssDNA product. Optimisation for LA reactions considered linear pUC19 (PvuII) DNA template and Primer B quantities. Combinations for LA optimisation were (a) 480 ng DNA template and 2 pmol primer, (b) 480 ng DNA template and 1 pmol primer, (c) 240 ng DNA template and 2 pmol primer, and (d) 240 ng DNA template and 1 pmol primer. The full extension peak is highlighted (blue peak) with peak height (H, Au) and size (S, bp) according to LIZ500 size marker (red line, orange peaks).
Figure D2: Electropherogram traces comparing replicate LA reaction ssDNA full extension products with fluorophore detection by end-labelled FAM and body-labelled R6G. Optimal LA reaction conditions were applied to determine if (a) R6GdUTP body-labelled ssDNA products could be resolvable by fluorescent AB3730 capillary electrophoresis in comparison to (b) FAM end-labelled ssDNA products. The red arrow (→) indicates the full ssDNA extension peak.
Figure D3: Electropherogram traces comparing LA reactions with DNA template treated with various concentrations of cisplatin. Optimisation of cisplatin and DNA incubation conditions were done to determine conditions that produce electropherogram trace with resolvable peak distribution for sequence specificity studies. Linear pUC19 (PvuII) was treated with cisplatin with concentrations of (a) 0.15 µM, (b) 0.5 µM, (c) 1.5 µM, (d) 5 µM and (e) 15 µM. The red arrow (➔) indicates the full ssDNA extension peak.
Figure D4: Electropherogram traces showing example sequencing reaction optimisation with various concentrations of ddCTP. Optimal LA reaction conditions were applied to determine the ddCTP concentration that would enable base-pair resolution sequencing peak data. The ddCTP concentration gradient supplemented into each LA reaction consisted of (a) 0.05 mM, (b) 0.25 mM, (c) 0.5 mM and (d) 1 mM. The red arrow (➡️) indicates the full ssDNA extension peak.
Appendix E: 5′FAM DNA end labelling LA optimisation

supplementary data

Supplementary data provided for metallo-drug sequence specificity LA reaction data for experiments described in Section 6.4.2. Electropherogram data showing metallo-drug induced ssDNA truncation peaks overlayed with DMF control trace is provided for both 5′FAM-pUC/M13(Rev) Primer A (22 mer) and Primer B (17 mer) (Figure E1). Tables showing the alignment of the DNA damage peaks with the dideoxy sequencing peaks for LA reactions are shown for both Primer A (22 mer) (Table E2) and Primer B (17 mer) (Table E1).
<table>
<thead>
<tr>
<th>DCRP</th>
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<tbody>
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</table>

Appendix E
Figure E1: Overlayed electropherograms showing metallo-drug induced ssDNA truncation peaks and DMF control peaks with linear pUC19 (PvuII) using 5′FAM-pUC/M13(Rev) Primer A (22 mer) and Primer B (17 mer). Electropherogram traces of metallo-drug induced ssDNA truncation peaks (▬) and DMF solvent control background peaks (▬) along with the LIZ500 size standard alignment (●) using the 5′FAM labelled 17 & 22 mer primers. Tested compounds include cisplatin, carboplatin, oxaliplatin, DACH, DCRP, DCBP, phleomycin, 56MESS, Cu56MESS, 3478MEEN, RuP1 and RuP2. Labelled x-axis values (□) of ssDNA truncation peak positions are relative to LIZ500 size standard ladder.
Table E1: Alignment of metallo-drug induced DNA damage peaks with dideoxy sequencing peaks for LA reactions using Primer B (17 mer). The table shows the 5’ to 3’ base sequence (column 1) along with the pUC19 NCBI database (Accession No: M77789) base sequence position (column 2) and dideoxy sequencing apparent molecular weight according to the LIZ500 size standard (column 3). Metallo-drug DNA damage peaks with their LIZ500 aligned apparent molecular weights are listed and positioned to the nearest sequencing peak value (columns 4-15). The alignment of the DNA damage peaks then allows for the assignment of a base to the metallo-drug induced DNA damage site for downstream sequence specificity analysis. In these LA reactions, Primer B (17 mer) was utilised.

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<th>Phleomycin</th>
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Table E2: Alignment of metallo-drug induced DNA damage peaks with dideoxy sequencing peaks for LA reactions using Primer A (22 mer). The table shows the 5’ to 3’ base sequence (column 1) along with the pUC19 NCBI database (Accession No: M77789) base sequence position (column 2) and dideoxy sequencing apparent molecular weight according to the LIZ500 size standard (column 3). Metallo-drug DNA damage peaks with their LIZ500 aligned apparent molecular weights are listed and positioned to the nearest sequencing peak value (columns 4-15). The alignment of the DNA damage peaks then allows for the assignment of a base to the metallo-drug induced DNA damage site for downstream sequence specificity analysis. In these LA reactions, Primer A (22 mer) was utilised.

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