CHAPTER 1

Introduction
1.1 Existing platinum-based anticancer therapies

Existing anticancer therapies have limited selectivity for tumour tissue compared to healthy tissue. This causes adverse side-effects for the patient and limits the dose able to be administered. Sequence and structural differences between healthy and cancerous DNA are anticipated to emerge in the near future as a consequence of the human genome mapping project.1 By taking advantage of the unique characteristics of DNA found in tumours, anticancer drugs could be selectively targeted to tumours, potentially minimising adverse side effects.

1.2 Platinum anticancer drugs

Platinum complexes currently make up one of the three most widely used groups of anticancer drugs in the world.2-3 The anticancer activity of cisplatin (cis-[PtCl₂(NH₃)₂], see Figure 1.1, (1)) was discovered serendipitously in the 1960s.4 Since 1978 it has been used in the clinic against a variety of cancers, including testicular, ovarian, head and neck, bladder, cervical, lymphoma and melanoma. Treatment with cisplatin often causes severe side effects such as nausea, vomiting, nephrotoxicity, neurotoxicity, myelotoxicity, and emetogenesis.2 These side effects arise mainly as a result of the limited selectivity of cisplatin for tumour cells as compared to healthy cells,5 and may also be due to reactions with thiol-containing species in blood plasma, such as cysteine and human serum albumin.6-11 In spite of its widespread clinical use, many tumours are unresponsive to cisplatin treatment due to intrinsic (eg. colon cancer, non-small-cell lung cancer) or acquired resistance (eg. ovarian cancer, small-cell lung cancer).12 The cellular mechanisms of cisplatin resistance have been identified and recently reviewed.13-16 The main factors that modulate resistance include decreased drug accumulation, increased levels of intracellular thiols that can deactivate cisplatin, and an increased capability of cells to repair or tolerate DNA damage caused by cisplatin. Other processes have also been implicated.
The search for platinum anticancer drugs with improved activity and selectivity, minimal side effects and circumvention of cisplatin resistance has produced thousands of complexes; of which few significantly improve upon the performance of cisplatin. This is reflected in the fact that less than 1% of such complexes have entered clinical trials.\textsuperscript{17} Carboplatin, cis-[Pt(NH\textsubscript{3})\textsubscript{2}(CBDCA)] where CBDCA = 1,1-cyclobutanedicarboxylic acid (see Figure 1.1, (2)), has achieved widespread clinical approval.\textsuperscript{2, 18} It has the advantage of being less toxic than cisplatin, although it is only effective against the same range of tumours.\textsuperscript{2} Oxaliplatin ([Pt(oxalato)(1R,2R-chxn])], chxn = cyclohexane-1,2-diamine, see Figure 1.1, (3)) was approved in France, the UK and other European countries in 1996 and in the US in 2002 for clinical use against advanced colorectal cancer\textsuperscript{2, 19, 20}; and is the only platinum complex to have displayed activity against colorectal cancer thus far.\textsuperscript{20} In spite of these successes, the toxicity, limited spectrum of activity, and resistance of such drugs remain significant obstacles to overcome.

1.3  Platinum(IV) complexes

The majority of platinum drugs studied have been in the platinum(II) oxidation state. More recently there has been increasing interest in the use of platinum(IV) complexes.\textsuperscript{21, 22} Platinum(IV) drugs are thought to hold substantial promise due to their relatively low reactivity, which should enable the drug to arrive at the tumour
site intact, reducing the incidence of side reactions, and hence minimising the toxic side effects associated with chemotherapy. The axial functional groups may endow the complex with a lipophilic nature, that when combined with the low reactivity of platinum(IV) species should enhance the cellular uptake of such drugs.\textsuperscript{22} Platinum(IV) complexes are believed to act as prodrugs, requiring reduction to their active platinum(II) analogues prior to exerting anticancer activity.\textsuperscript{2,23} As such, the reduction potential of platinum(IV) complexes is expected to influence their biological activity.\textsuperscript{22,24}

\textit{In vitro} studies involving platinum(IV) complexes have produced some promising results. Kelland et al\textsuperscript{25} studied the \textit{in vitro} cytotoxicity of numerous ammine/amine platinum(IV) trans-dicarboxylate species (analogues of satraplatin, a complex currently undergoing Phase 3 clinical trials) against human ovarian carcinoma cell lines with cisplatin resistance of varying degrees. A number of these complexes displayed cytotoxicities up to 840 times that of cisplatin, with significant selectivity towards the most resistant cell lines. The highest cytotoxicity was displayed by the most lipophilic species. This has been attributed to the ease of entry of the complexes into cells where they undergo reduction to the active platinum(II) species.\textsuperscript{25}

A similar study was performed by Jolley and coworkers.\textsuperscript{26} The platinum(IV) species investigated (analogues of satraplatin) consisted of ethane-1,2-diamine derived ligands containing alcohol, carboxylic acid, and acetate substituents. The \textit{in vitro} cytotoxicity of these complexes was considerably poorer than that of the complexes studied by Kelland.\textsuperscript{25,26} This was thought to be due to their relatively low lipophilicity and/or the negative charge of the corresponding platinum(II) complex, which may cause repulsion with the DNA phosphodiester backbone.\textsuperscript{26}

Rationally designed platinum(IV) agents have been developed and tested in recent years. Such agents often possess substituents designed to improve the selectivity of the drug. For example, Kratochwil et al have developed analogues of satraplatin, containing iodo ligands. Irradiation of such complexes with visible light was found to
induce non-reversible binding to DNA,\textsuperscript{27} stereospecific reactions with 5'-GMP\textsuperscript{28}, and cytotoxicity. One such complex displayed 35 \% greater growth inhibitory activity \textit{in vitro} in human bladder cancer cells when irradiated with light during the first 1.5 hr of drug exposure compared to the equivalent sample that remained in the dark.\textsuperscript{27} Platinum(IV)-diazide compounds have been shown to display similar properties, although they possess better stability under physiological conditions than the iodo complexes.\textsuperscript{29} In addition, the platination sites of these complexes were found to be similar to those observed for cisplatin.\textsuperscript{30} Thus there exists the potential of photoactivating platinum(IV) prodrugs specifically in and around tumour sites.

A novel platinum(IV) complex possessing ethacrynic acid in its axial positions has been developed. Ethacrynic acid (EA) is a clinically used diuretic known to inhibit glutathione-S-transferase (GST) isozymes that are overexpressed in cisplatin resistant cells lines. It was hoped that the platinum(IV) complex would be targeted to GST enzymes in human cancer cells and be reduced \textit{in vivo}, releasing both the cytotoxic platinum(II) moiety, as well as the EA moiety and thus potentially showing effectiveness against cisplatin resistant cell lines. This complex has been shown to have promising cytotoxicity and the ability to inhibit GST activity to a greater extent than EA itself.\textsuperscript{31}

It is somewhat disappointing that positive results with platinum(IV) complexes have not yet translated into agents with significant improvements upon the clinical activity of cisplatin. Only a small number of platinum(IV) species have entered clinical trial in recent years, yet their antitumour activity remains lower than that of cisplatin and they have had to be withdrawn for a variety of reasons.\textsuperscript{22} The lower than expected clinical efficacy of platinum(IV) drugs may be due to their low reactivity with DNA, the efflux of platinum(IV) species from cells,\textsuperscript{32} or their rapid reduction \textit{in vivo}.\textsuperscript{22, 26}

In an attempt to develop platinum(IV) species with a reduced rate of reduction \textit{in vivo}, Hambley \textit{et al} investigated a series of novel platinum(IV) complexes with a variety of reduction potentials, and moderate to high \textit{in vitro} activities. Several difficult to reduce complexes had activities similar to the parent platinum(II) complex,
indicating the possibility of developing platinum(IV) complexes that possess both high activity and reduced side effects in humans.\textsuperscript{33}

Hambley and coworkers have undertaken extensive studies on a series of platinum(IV) complexes possessing a common platinum(II) moiety. This series consists of platinum(IV) analogues of cisplatin or [PtCl\textsubscript{2}(en)] with either chloro, acetato or hydroxo axial ligands, see Figure 1.2.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{structures.png}
\caption{Structures of the platinum(IV) cisplatin analogues used by Hambley and coworkers.}
\end{figure}

Investigation of such model complexes should aid in an improved understanding of the mechanism of action of platinum(IV) agents and assist in the rational design of future drugs. The reduction potentials of the complexes were shown to be dependent on the axial ligands.\textsuperscript{34} The model platinum(IV) complexes cover a relatively large range of reduction potentials, following the order chloro>acetato>hydroxo. A correlation between their reduction potentials and several of their biological properties has been observed. Table 1.1 shows some of these properties. In addition to those shown in the table, a reduction potential correlation has also been shown for the rate of binding of the complexes to 5'-GMP,\textsuperscript{35} and albumin, as well as protein binding in RPMI.\textsuperscript{11}
Table 1.1: Biological properties of a series of platinum(IV) complexes. Reduction potentials (versus Ag/AgCl),\textsuperscript{32} cytotoxicity against A2780 ovarian cancer cell lines (µM),\textsuperscript{24, 32} platinum uptake into A2780 cells (24 hr, 30 µM),\textsuperscript{32} and proportion of intracellular platinum(IV) in A2780 cells following 2 hr incubation with the complexes.\textsuperscript{36} These properties show a close correlation to the reduction potential of the complexes.

<table>
<thead>
<tr>
<th>Axial group X (PtX(NH$_3$)$_2$)</th>
<th>$E_p$ (mV)</th>
<th>IC$_{50}$ (µM)</th>
<th>Cell uptake (nmol Pt/mg protein)</th>
<th>% Pt(IV) (2 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cl$_2$</td>
<td>-</td>
<td>2.5</td>
<td>2.45</td>
<td>1</td>
</tr>
<tr>
<td>Cl$_4$</td>
<td>-260</td>
<td>3.3</td>
<td>3.82</td>
<td>5</td>
</tr>
<tr>
<td>Cl$_2$(OAc)$_2$</td>
<td>-635</td>
<td>17.9</td>
<td>0.78</td>
<td>33</td>
</tr>
<tr>
<td>Cl$_2$(OH)$_2$</td>
<td>-880</td>
<td>22.0</td>
<td>0.74</td>
<td>54</td>
</tr>
</tbody>
</table>

It appears the cytotoxicity of the complexes shown in Table 1.1 leaves much to be desired. However, it should be noted that cytotoxicity assays taken alone may be an inadequate basis upon which to assess the clinical efficacy of platinum(IV) agents. The cytotoxicity results shown in Table 1.1 suggest that difficult to reduce platinum(IV) complexes will be less active than complexes that are easily reduced, though this does not reflect clinical experience with platinum(IV) agents. For example, iproplatin, which has a similar reduction potential to the dihydroxo complex shown in Figure 1.2 (\textsuperscript{6}), showed good activity in clinical trials. This suggests that a significant proportion of difficult to reduce platinum(IV) complexes of this type may arrive intact at the site of action, perhaps corresponding to fewer side reactions and hence lower side effects associated with treatment.\textsuperscript{24, 32} In fact, as indicated in Table 1.1, it has been shown that such complexes are able to enter cells as platinum(IV) and thus avoid deactivation in the extracellular medium. This correlated closely with reduction potential, with a higher percentage of platinum(IV) in cells that had been treated with complexes having relatively low reduction potentials.\textsuperscript{36, 37} Hence, reduction potential is an important property to consider in the design of future platinum(IV) therapeutics.

Further inspection of Table 1.1 indicates that the cellular uptake of the platinum(IV) complexes is relatively low compared to cisplatin. Hall et al suggest that this may be because the neutral platinum(IV) complexes are able to easily diffuse in and out of
cells, whereas the platinum(II) complexes essentially become ‘trapped’ inside the cells after undergoing aquation and thus becoming positively charged species.\textsuperscript{32} Rational design of future platinum(IV) drugs may perhaps involve inclusion of a functionality that would hold the agent within the cell or the tumour.

1.4 DNA-Targeting

Two grooves or channels are characteristic of the secondary DNA structure. These grooves differ significantly in size and hence are known as the major and minor grooves.\textsuperscript{38} The major groove has a larger number of potential hydrogen bonding sites than the minor groove. Hence the major groove is richer in information and is the site at which most sequence-selective drugs bind. Small drug molecules (<1000 Daltons) tend to occupy the minor groove and may also be sequence selective in nature.\textsuperscript{39}

Most DNA binding drugs currently in use have very little sequence selectivity. A number of strategies exist for increasing the selectivity of drug/DNA interactions. For instance, drugs may be designed to have particular characteristics that increase affinity for DNA. These may include the ability to take part in electrostatic interactions, hydrogen bonds, hydrophobic interactions, intercalative interactions, covalent bonds, and inert or labile coordination bonds.\textsuperscript{40-42}

The three main categories of drugs that interact with DNA include the alkylators, which form covalent bonds with DNA bases; DNA groove binders, which bind in either the major or minor groove by electrostatic interactions, hydrogen bonds and van der Waals interactions; and intercalators.\textsuperscript{39} Intercalators will be discussed further below.

1.5 Intercalators

Intercalators are polycyclic planar heterocycles, usually aromatic and often positively charged.\textsuperscript{43} Electrostatic and van der Waals interactions allow the intercalators to
insert between the base pairs of double-helical DNA, causing a lengthening and unwinding of DNA\textsuperscript{44, 45} which may prevent its replication and transcription,\textsuperscript{46} cause cleavage of the DNA,\textsuperscript{47} or inhibit the synthesis of DNA and RNA.\textsuperscript{48} Intercalators alone have little sequence selectivity, yet they bind rapidly to double-stranded DNA.\textsuperscript{49} The properties of intercalators can be enhanced by coupling to a moiety possessing sequence recognition elements.

1.6 Anthraquinone intercalators

The properties of anthraquinone intercalators have been extensively studied. Several compounds containing the anthraquinone skeleton have been found to possess good antitumour activity, such as doxorubicin and daunorubicin.\textsuperscript{50-55} (Figure 1.3, (7a) and (7b), respectively).

![Figure 1.3: The structures of doxorubicin (7a) and daunorubicin (7b).](image)

Daunorubicin is active against acute lymphoblastic leukemia and acute myeloblastic leukemia whilst doxorubicin is active against a variety of tumours such as soft-tissue
and osteogenic sarcomas, paediatric malignancies, leukemia, bladder, breast, lung and thyroid tumours. Clinical use of these drugs is restricted by the severe nature of their side effects. Both drugs cause dose-limiting myelosuppression, stomatitis, nausea, vomiting, alopecia, bone marrow suppression, and cumulative, irreversible cardiac toxicity which may cause fatal congestive heart failure.

In the search for less toxic derivatives, many analogues containing the anthraquinone skeleton have been synthesised. Two compounds in particular have emerged as suitable clinical replacements: mitoxantrone and amenantantrone (Figure 1.4, (8a) and (8b), respectively).

Mitoxantrone is effective against breast cancer, lymphoma and acute leukemia. It can cause myelosuppression, nausea, vomiting, stomatitis, mucositis, alopecia and cardiotoxicity with cumulative doses. However, the incidence of acute toxicity is considerably less than that caused by doxorubicin. It is believed that mitoxantrone inhibits DNA and RNA synthesis. The interaction of mitoxantrone with DNA is both intercalative and non-intercalative, inducing non-protein DNA strand scission. It has not been established whether the side chains reside in the major or minor groove when mitoxantrone is bound to DNA. However when the ring system is intercalated there is evidence to suggest that the side chains are found in...
The side chains seem to endow mitoxantrone with selectivity for interaction with GC-rich tracts of DNA, and have been identified as a key factor in the nuclear localisation, DNA binding and inhibition of DNA synthesis. There is also evidence to suggest that the cytotoxicity of mitoxantrone is partly the result of its interaction with topoisomerase II, the enzyme responsible for the supercoiling of DNA. Mitoxantrone forms a stable complex with the enzyme, inhibiting its activity and causing DNA breakage and cell death.

Hundreds of analogues of mitoxantrone have been synthesised in the search for a more active and less toxic drug. Compounds containing the anthraquinone skeleton with modified side chains have produced several positive results. DNA cleavage upon irradiation has been shown to be catalysed by cationic anthraquinones containing ammonium-substituted side chains (Figure 1.5). There are two distinct pathways by which the strand cleavage can be initiated. The excited quinones can either cause electron transfer from a nearby DNA base, or they can abstract a hydrogen atom from the deoxyribose component of the nucleic acid backbone. Regardless of the cleavage pathway, the anthraquinone is regenerated to its ground state, fully oxidised form and appears to be able to cycle indefinitely through this catalytic process. Hence, repeated use of anthraquinones as robust photonucleases for extended periods of time as small molecules or tethered to DNA recognition elements is a possibility. It was also found that anthraquinones containing the ammonium chain substituted in the 1-position of the anthraquinone ring did not cause DNA cleavage, even after prolonged periods of irradiation. However, those substituted in the 2-position of the anthraquinone skeleton, are efficient strand cleavers. The DNA binding of the 1-substituted anthraquinones is significantly weaker than their 2-substituted analogues.
(9a): \( R_1 = \text{CONH(CH}_2\text{)}_4\text{NH}_3^+, \ R_2 = R_3 = H \)
(9b): \( R_1 = \text{SO}_2\text{NH(CH}_2\text{)}_4\text{NH}_3^+, \ R_2 = R_3 = H \)
(9c): \( R_2 = \text{CONH(CH}_2\text{)}_4\text{NH}_3^+, \ R_1 = R_3 = H \)
(9d): \( R_2 = \text{SO}_2\text{NH(CH}_2\text{)}_4\text{NH}_3^+, \ R_1 = R_3 = H \)
(9e): \( R_2 = R_3 = \text{SO}_2\text{NH(CH}_2\text{)}_4\text{NH}_3^+, \ R_1 = H \)

**Figure 1.5:** The structures of the cationic anthraquinone derivatives.\(^{65}\)

Studies on related anthraquinone analogues have produced similar results. These anthraquinone analogues, shown in Figure 1.6, were found to produce GG-selective DNA cleavage. The dominant pathway by which this occurs is electron transfer from a DNA base to the excited intercalated anthraquinone. Some excited non-intercalated anthraquinones are also able to cause DNA cleavage. This occurs spontaneously, in a non-selective fashion when the excited anthraquinone abstracts a hydrogen atom directly from the deoxyribose backbone of the DNA.\(^{66}\)

(10a): \( R = \text{CH}_2\text{CH}_2\text{N(CH}_2\text{CH}_3\text{)}_2\text{HCl} \)
(10b): \( R = \text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}_2\text{HCl} \)
(11): \( R = \text{CH}_2\text{CH}_2\text{N(CH}_2\text{CH}_3\text{)}_2\text{HCl} \)

**Figure 1.6:** The anthraquinone derivatives studied by Breslin et al\(^{66}\) for their potential as light-activated agents that initiate DNA cleavage.
The quinone skeleton of anthraquinone compounds is believed to have intrinsic hypoxic selectivity, that is, in regions of low oxygen tension found in many solid tumours the compound is reduced to its active form. The bioreductive activation cycle for quinones is shown in Figure 1.7.

\[
\text{quinone} \xrightarrow{2e^-} \text{semiquinone radical anion} \xrightarrow{0_2^-} \text{hydroquinone} \xrightarrow{20_2} \text{quinone}
\]

**Figure 1.7**: The quinone bioreductive cycle.

The cellular tumour environment is known to have high bioreductive enzyme activity relative to normal tissues. Such enzymes can cause one or two-electron reduction of the quinone skeleton to the semiquinone free radical or the hydroquinone, respectively. The one-electron reduction to the semiquinone radical can be reversed in the presence of molecular oxygen to regenerate the quinone, and also form the superoxide anion. The superoxide radicals can subsequently produce DNA lesions, peroxidative damage to membrane lipids, alteration of subcellular organelles, etc, either directly or following conversion to hydroxy radicals. The
semiquinone radicals can react with lipids, macromolecules and thiols to cause cell damage. In the absence of oxygen (that is, in a hypoxic environment) the semiquinone will be the predominant species. The toxicity of the semiquinone in hypoxic tissue should far exceed the damage caused by superoxide formation in well-oxygenated tissue. The dose-limiting cardiotoxicity of doxorubicin is believed to partly be due to such damaging redox cycling.

Other anthraquinone derivatives possess hypoxic selectivity predominantly by virtue of their side chains. Compound (12) is one such example that was found to be active against hypoxic cells. Upon reduction in hypoxic conditions, compound (13), a potent topoisomerase II inhibitor and tight DNA binding agent, is formed.

![Figure 1.8](image)

**Figure 1.8:** Under hypoxic conditions, compound (12) is converted to compound (13), a potent topoisomerase II inhibitor.

The antiviral antibiotics netropsin and distamycin are known to selectively bind to AT-rich sequences of DNA. This property has been utilised in attempts to produce sequence selective DNA binding agents. Helissey et al covalently linked these drugs to an anthraquinone moiety believed to be capable of DNA intercalation and photocleavage. The presence of the anthraquinone did not alter the sequence specific recognition properties of the drug, yet the DNA cleavage was found to be nonspecific in most cases. Boitte et al conducted a similar study involving the attachment of two netropsin moieties to each of the side chains of mitoxantrone. This conjugate retained the AT sequence specificity characteristics of netropsin, rather
than the GC sequence selectivity of mitoxantrone. This has been attributed to the
exocyclic 2-amino group of guanine, which hinders the access of the netropsin moiety
into the minor groove and in turn interferes with the intercalation of the
anthraquinone ring. This conjugate molecule is less cytotoxic than mitoxantrone, and
yet more growth-inhibitory than netropsin. 59

1.7 Platinum-intercalator complexes

DNA is the main cellular target of the chemotherapeutic agent, cisplatin. Cisplatin is
believed to exert its toxic effects by binding to DNA via inert coordination bonds to the
N7 atoms of guanine or adenine. 73 Hence the complex resides in the major groove of
the DNA, interfering with normal cellular functions such as replication and
transcription of DNA. 13 One strategy to improve upon the clinical performance of
cisplatin involves attachment of a platinum moiety to a group possessing intrinsic
affinity for DNA, 74, 75 such as an intercalator.

A platinum-intercalator conjugate generally consists of a platinum centre tethered to
an intercalator through a flexible linker chain and a stable bond to the metal centre.
For the conjugate to be biologically active it will ideally remain intact under biological
conditions, or until the binding of either of the components to DNA. A further
requirement is that the two components remain functionally interdependent and the
conjugate is able to both intercalate and platinate DNA. In addition, the platinum
centre must contain at least one labile group that is able to react with a DNA
nucleobase nitrogen atom to form a stable coordinative bond. 43

It is hypothesised that the intercalating moiety of such a platinum-intercalator
conjugate will rapidly transport the platinum moiety to the vicinity of its DNA target,
potentially minimising reactions with biomolecules 76 and perhaps hindering efflux of
the complexes from the cell. Thus the platinum centre may be held within the cell for
sufficient time to allow it to exert its anticancer effects. 32 Side reactions have been
implicated in the deactivation of cisplatin, 7–9, 11, 77 the adverse side effects associated
with cisplatin chemotherapy, and the resistance of tumours towards platinum-based drugs. In addition, these novel platinum complexes may bind to DNA with different kinetics, mechanism or sequence specificity as compared to cisplatin, thus potentially overcoming cisplatin resistance. Several platinum-intercalator complexes have been studied and a number have been shown to possess many of the hypothesised advantages over cisplatin. These studies have also been motivated by the fact that cisplatin is often administered in combination with intercalative drugs such as actinomycin and adriamycin. Consequently, tethering of the two regimes into a single drug may further enhance synergistic effects.

1.7.1 Increased rate of DNA reaction

Platinum phenanthridinium complexes (see Figure 1.9) have been shown to damage DNA more rapidly than cisplatin, with some requiring only 30 min to produce the same DNA damage as 18 hr incubation with cisplatin. Complexes with shorter polymethylene chains generally resulted in more extensive DNA damage. Observations in intact human cancer (HeLa) cells were similar, with more efficient DNA damage produced by complexes with shorter linker chains, and up to a 6-fold enhancement of the rate of DNA damage compared to cisplatin. The complexes also showed good antitumour activity in mice bearing P388 wild-type leukaemia.

![Figure 1.9: Structures of the platinum(II) phenanthridinium complexes.](image-url)
Intercalator-tethered phenazine-1-carboxamide platinum(II) complexes ([PtCl₂L], where L are phenazine ligands with varying lengths of polymethylene linker chain, see Figure 1.10), have been shown to increase the rate and extent of DNA platination as compared to [PtCl₂(en)], even in the presence of thiols which are known to interfere with the interaction of cisplatin with DNA. Complexes with longer polymethylene linker chains (ie. n = 3, 4, 5) were more cytotoxic than cisplatin, [PtCl₂(en)], or the ligands on their own. Molecular models reveal that complexes with polymethylene chains of length longer than n = 2 allow for both platination and intercalation to take place simultaneously and hence cause a large extent of DNA unwinding. The platinum binds covalently to DNA bases while the intercalator lies in the DNA major groove.⁷⁸, ⁸⁴

![Figure 1.10: Structures of the platinum(II) phenazine carboxamide complexes.](image1.png)

1.7.2 Activity in cisplatin-resistant cell lines

Many platinum-acridine complexes have been shown to possess activity in cisplatin-resistant cells. For example, 9-anilinoacridine platinum complexes, based on ethane-1,2-diamine and propane-1,3-diamine tethered intercalators, have been studied (Figure 1.11). Those based on ethane-1,2-diamine had similar in vitro toxicities against wild type P388 leukemia and cisplatin resistant P388 cell lines, and those based on propane-1,3-diamine were approximately 8-fold more active in the resistant line. However, the cytotoxicities were not significantly greater than the corresponding ligands in isolation. In contrast, the standards (cisplatin, ethane-1,2-diamine and
propane-1,3-diamine platinum complexes) were approximately 10 times less active against the resistant cell line compared to the wild type line. Most of the intercalator complexes were found to have no in vivo activity against wild-type P388 leukaemia. It was suggested that the intercalator complexes may bind so as to direct the platinum away from the major groove of DNA. Hence it would not be possible for simultaneous platination and intercalation to be occurring. They conclude that future studies should involve complexes of varying topologies and improved solubilities.

\[ \text{HN} \quad \text{(CH}_2\text{)}^n \quad \text{NH}_2 \ 	ext{Pt} \ 	ext{Cl}_2 \]

\( \text{(16a)}: \ n = 2 \)
\( \text{(16b)}: \ n = 3 \)
\( \text{(16c)}: \ n = 4 \)
\( \text{(16d)}: \ n = 5 \)

\[ \text{HN} \quad \text{(CH}_2\text{)}^n \quad \text{NH}_2 \ 	ext{Pt} \ 	ext{Cl}_2 \]

\( \text{(17a)}: \ n = 1, R = R' = H \)
\( \text{(17b)}: \ n = 2, R = R' = H \)
\( \text{(17c)}: \ n = 3, R = R' = H \)
\( \text{(17d)}: \ n = 4, R = R' = H \)
\( \text{(17e)}: \ n = 5, R = R' = H \)
\( \text{(17f)}: \ n = 3, R = \text{OMe} \)
\[ \text{R'} = \text{CONH(CH}_2\text{)}\text{NMe}_2 \]

**Figure 1.11:** Structures of the 9-anilinoacridine platinum(II) complexes. (16a-d) are based on ethane-1,2-diamine and (17a-f) are based on propane-1,3-diamine.
Acridine carboxamide derivatives of [PtCl$_2$(en)] have also been studied. In general, they were found to be more active and cytotoxic than the free ligands, though they had similar potencies. The acridine-4-carboxamide platinum complexes were more cytotoxic than the acridine-2-carboxamides. The complexes displayed equal *in vitro* activity against wild-type and cisplatin-resistant P388 leukaemia cell lines, in contrast to cisplatin.$^{86}$ The sequence specificity of DNA damage was determined in plasmid DNA and it was found that the most intense damage sites were similar to cisplatin, though the medium and weak damage sites displayed slight differences.$^{87}$ Damage caused by two such complexes (Figure 1.12, (18) and (19)) was monitored in intact human cancer (HeLa) cells and directly compared to that in purified HeLa DNA. It was found that the position and relative intensity of damage was similar in both systems for the acridine-4-carboxamide complex studied, whilst damage caused by the acridine-2-carboxamide complex was undetectable. This correlated with antitumour activity in mouse models with the acridine-2-carboxamide complex displaying little activity compared to [PtCl$_2$(en)] and the acridine-4-carboxamide complex.$^{88}$

*Figure 1.12:* Structure of the acridine carboxamides with (18) showing a 2-carboxamide complex and (19) showing a 4-carboxamide complex.
1.7.3 Alterations in DNA sequence specificity

The DNA sequence specificity of the vast majority of platinum-intercalator complexes is dominated by the platinum centre due to its high selectivity for cross linking to runs of adjacent purine bases in the major groove of double stranded DNA. This results in DNA adduct profiles closely resembling those of cisplatin. More recently, platinum-intercalator complexes that break away from this paradigm have been developed. For example, platinum 9-aminoacridinecarboxamide complexes react rapidly with DNA with different sequence specificity than that of cisplatin. Complexes with shorter chain lengths (n = 2, 3) exhibited DNA adducts significantly different from those of cisplatin. Those with longer chains (n = 4, 5) tended to closely resemble the sequence specificity of cisplatin, preferring runs of consecutive guanine bases. This was the first report to show altered DNA sequence specificity for a cisplatin analogue. The sequence selectivity was retained in intact human cells, although the specificity differences compared to cisplatin were less distinct in cells than in purified plasmid DNA.

Platinum-acridinylthiourea conjugates have recently been developed and found to have different sequence selectivity than cisplatin. The complexes were rationally designed such that the metal is unable to bind in the G-rich region of the major groove, the dominant sites at which cisplatin is known to bind. The minor groove directed intercalating agent is attached to the platinum centre through a thiourea sulfur. The coordination sphere of the platinum consists of one chloro leaving group, whilst the sulfur atom and bidentate amine should act as non-leaving groups. Thus cross-linking with purine bases on DNA should be prevented and only monofunctional covalent DNA adducts should be allowed to form, with sequence and groove specificity dominated by the intercalator. Upon reaction of one such complex (R1 = CH₃, R2 = H, R3 = CH₃, see Figure 1.13, (20)) with calf thymus DNA, a unique array of monofunctional adducts was formed, 80% of which were at guanine-N7, and 20% at adenine sites, most likely at the N3 position in the minor groove. Adenine damage sites are unprecedented in the biocoordination chemistry of platinum anticancer
complexes thus far and suggest that the intercalator, rather than the platinum moiety, dictates the platination sites in a significant proportion of adducts.\textsuperscript{43, 89} Such findings open the door for development of platinum drugs significantly different from those currently in use. The same complex has also demonstrated promising activity, with high potency in HL-60 leukaemia cells. Micromolar cytotoxicities were observed in the ovarian cell line 2008 and its cisplatin resistant variant, C13\textsuperscript{*}. This indicated no advantage over the acridine ligand in isolation, though suggests that the complex is capable of partially circumventing acquired cisplatin resistance in vitro.\textsuperscript{90} A more recent study investigated the in vitro cytotoxicity of two related complexes (with various substitution patterns for R1 – R3, see Figure 1.13, (20)). They were found to have cytotoxicities in the low micromolar range, representing activity similar or superior to cisplatin in cisplatin sensitive and resistant A2780 ovarian cancer cells, lung cancer cells (NCI-H460), and colon cancer cells (RKO).\textsuperscript{91}

\textbf{Figure 1.13:} Structure of the platinum-acridinylthiourea complexes, where R1, R2, R3 = H or CH\textsubscript{3}.

\textit{1.8} Platinum – anthraquinone complexes

Platinum-anthraquinone complexes have been extensively studied. Attachment of a platinum complex to the amine group on the sugar of doxorubicin resulted in a
complex, (21), that possesses activity against cisplatin and adriamycin-resistant murine leukemias and activity comparable to that of adriamycin in solid tumours.\textsuperscript{92, 93}

\begin{center}
\includegraphics[width=0.5\textwidth]{complex.png}
\end{center}

\textbf{Figure 1.14}: The adriamycin-Pt(II) complex (21) studied by Pasini.\textsuperscript{92}

Several complexes consisting of an anthraquinone linked to a platinum(II) moiety via an (aminoalkyl)amino side chain have been examined. Figure 1.15 shows the structures of such complexes. Structure activity guidelines have been established for this class of complex. It was found, in general, that platinum complexes of anthraquinones substituted in position 2 were significantly less potent than the analogous complexes that have been substituted in position 1. In fact, their activity was equal to or lower than that of the isolated ligands.\textsuperscript{80} The length of the alkyl chain linking the anthraquinone to the platinum moiety affected the activity of the complex. In complexes based upon [PtCl\textsubscript{2}(en)], (22), linker chains consisting of 2 or 3 three methylene groups endowed the complex with significant activity. Those with longer linker chains were 100-fold less potent.\textsuperscript{80, 94-96} Three such complexes, containing short linker chains, had \textit{in vitro} and \textit{in vivo} activity similar to that of cisplatin.\textsuperscript{95} These complexes were more active than the [PtCl\textsubscript{2}(en)] platinum species and the free ligand from which they were derived. In addition, they were also more active than a 1:1 mixture of the intercalator ligand with [PtCl\textsubscript{2}(en)]. Hence chemically tethering the two moieties is preferable to administering the two components in isolation.\textsuperscript{95} The activity
of similar complexes based on cis-PtCl$_2$ (23) was found to be quite low and unaffected by linker chain length.$^{94}$

![Chemical structures](image)

**Figure 1.15**: The platinum-anthraquinone complexes studied by Gibson and coworkers.$^{80, 94-96}$ Complex (22) is a bidentate based upon [PtCl$_2$(en)], complex (23) is a monodentate based upon cisplatin, and complex (24) is a platinum-anthraquinone complex with platinum:anthraquinone ratio of 1:2. The complexes shown here have been substituted in the 1-position of the anthraquinone. The 2-position is indicated on complex (22).

Other variations on the structure of these anthraquinone intercalators have been attempted. Altering the structure of the linking atoms between the intercalator and the platinum moiety (an ether linkage rather than an NH group) resulted in little variation of activity.$^{95, 96}$ Tethering multiple anthraquinone intercalators to each platinum atom resulted in lower *in vitro* cytotoxic activity of the 1:2
platinum:intercalator complex, (24), as compared to the corresponding 1:1 complexes.\textsuperscript{95}

Gibson and coworkers have developed a series of platinum-anthraquinone complexes that are structurally different from the platinum-intercalator complexes studied previously (Figure 1.16, (25) and (26)). Rather than linking the anthraquinone in an essentially irreversible fashion to the platinum moiety, which is the case for the structures found in Figure 1.15, it was linked in such a way that it would dissociate from the molecule. The anthraquinone should act as a carrier molecule to deliver the active platinum moiety to its DNA target prior to dissociation into its separate constituents.\textsuperscript{94, 97} The free ligands were found to be moderately active and the corresponding platinum complexes were 10-fold more active. However, when compared to the analogous compounds where the anthraquinone was irreversibly linked to the platinum species, their potencies were approximately 10-fold lower. Interestingly, there was no difference observed between the activity of the platinum species and the platinum-anthraquinone species. These observations may indicate that these compounds are ineffective carrier ligands, though low aqueous solubility may have contributed to their low activities.\textsuperscript{97}
Figure 1.16: The second generation platinum-anthraquinone complexes studied by Gibson.\textsuperscript{94, 97} The spacer group in (26) is either (CH\textsubscript{2})\textit{n} or (CH\textsubscript{2})\textit{3}NHCO(CH\textsubscript{2})\textit{2}.

Dinuclear platinum complexes containing anthraquinone moieties (see Figure 1.17) have recently been used by Reedijk et al\textsuperscript{98-100} to model polynuclear cationic platinum complexes that have emerged as a novel class of anticancer drugs\textsuperscript{101-103}. In A2780 ovarian carcinoma cells, the platinum complexes (28), and the ligands from which they were derived (27), exhibited good cytotoxicity (in the lower micromolar range). Those with shorter chains were more cytotoxic than those possessing longer chains. Upon extraction of DNA from A2780 cells that had been treated with the platinum-anthraquinone complexes (and cisplatin for comparison), it was found that the complexes bound to DNA to a greater extent than cisplatin, with a similar chain length dependence to that observed for their toxicity. The ligands were found to partly circumvent resistance in the A2780cisR (cisplatin resistant) cell line, whilst the platinum complexes were relatively ineffective in this cell line, possibly due to deactivation of the complexes in the cytosol.\textsuperscript{98, 99} In U2-OS human osteosarcoma cells, compounds with shorter chains were approximately 10 times more active than cisplatin, whilst those with longer chains had similar cytotoxicity to cisplatin. The ligands and the complexes were able to overcome cisplatin resistance in the cisplatin
resistant U2-OS cell line.\textsuperscript{100} The intrinsic fluorescence of these complexes allowed their cellular processing to be monitored using time-lapse digital fluorescence microscopy. It was found that in parental A2780 ovarian carcinoma cells, the ligands and the complexes were processed differently. Both species entered the cells and were initially seen in the cytosol. After 24 hr, the ligands were visible in the cytosol surrounding the nucleus and in the nucleoli within the nucleus. In contrast, the platinum complexes were localised in the lysosomes after 24 hr.\textsuperscript{99} In cisplatin resistant A2780 cells, the ligands were processed in an identical fashion as in the parent cell line, whereas the platinum complexes were encapsulated by the lysosomes at the beginning of their incubation and remain as such.\textsuperscript{98} The cellular processing was somewhat different in cisplatin sensitive and resistant U2-OS cells, with both the anthraquinones and the platinum-anthraquinones rapidly entering the cell and accumulating in the nucleus. The anthraquinone ligands subsequently accumulated in the Golgi complex, were taken up by lysosomes and then transported to the cell surface. The platinum complexes were subsequently excreted from the cell via the Golgi.\textsuperscript{100}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{structures}
\caption{Structures of the anthraquinones (27) and platinum-anthraquinone complexes (28) used by Reedijk et al.\textsuperscript{98-100}}
\end{figure}
1.9 Project aims

There are currently many limitations to the use of platinum anticancer drugs (see Section 1.2). These include resistance of tumours to platinum chemotherapy, the limited range of tumours against which the drugs are effective, and the toxicity of the drugs. The toxicity of such agents is largely a consequence of the lack of specificity for either their DNA target and/or tumour cells as opposed to healthy cells. The two general classes of platinum complexes chosen for this study hold significant potential for overcoming such toxicity and specificity issues:

a) **Platinum(IV) complexes:** The relative inertness of platinum(IV) complexes may potentially allow them to arrive at the tumour site intact, thus avoiding deactivation and side reactions and resulting in reduced toxicity. The structures of the platinum(IV) complexes used for the current study are shown below in Figure 1.18.

\[
\begin{align*}
(29) & \quad \text{cis-}[\text{PtCl}_2(\text{NH}_3)_2] \quad \text{(cisplatin)}, \\
(30) & \quad \text{cis-}[\text{PtCl}_4(\text{NH}_3)_2] \quad \text{(Cl4)}, \\
(31) & \quad \text{cis,trans,cis-}[\text{PtCl}_2(\text{OAc})_2(\text{NH}_3)_2] \quad \text{(OAc)}, \text{ and} \\
(32) & \quad \text{cis,trans,cis-}[\text{PtCl}_2(\text{OH})_2(\text{NH}_3)_2] \quad \text{(OH)}.
\end{align*}
\]

**Figure 1.18:** Structures of platinum(IV) cisplatin analogues used in the current study. (29) = cis-[PtCl₂(NH₃)₂] (cisplatin), (30) = cis-[PtCl₄(NH₃)₂] (Cl4), (31) = cis,trans,cis-[PtCl₂(OAc)₂(NH₃)₂] (OAc), and (32) = cis,trans,cis-[PtCl₂(OH)₂(NH₃)₂] (OH).

b) **Platinum-intercalator complexes:** The high affinity of the intercalator for DNA should act to rapidly transport the drug to DNA, minimising reactions along the way and holding the platinum moiety at its DNA target within the nucleus of the cell for sufficient time to allow its
anticancer effects to be exerted. Such a drug may potentially be less toxic and more specific than cisplatin. The intercalators chosen for this study were anthraquinones, the structures of which are shown below in Figure 1.19. These particular complexes have been studied previously by Gibson and coworkers. They found that the position at which the anthraquinone ring is substituted has a significant effect on their activity, with 1-substituted complexes having better activity than the 2-substituted complexes. The 1- and 2-substituted anthraquinones and platinum-anthraquinone complexes studied here were chosen to ascertain whether the substitution pattern caused any significant changes to their properties within cells.

Figure 1.19: Structures of the anthraquinones ((33) = 1C3, (35) = 2C3) and platinum-anthraquinone complexes ((34) = Pt1C3, (36) = Pt2C3) used in the current study.

The aim of the work detailed herein was to study the distribution of the above complexes (Figure 1.18 and 1.19) in a variety of biological systems to gain insight into the specificity of such complexes and hence the factors that may contribute to
their toxicity. The biological systems used, in order of increasing complexity, are as follows:

A. **DNA:** The interactions of the platinum(IV) cisplatin analogues (shown in Figure 1.18) with DNA were investigated. This was designed to model what may happen if a platinum(IV) complex were to arrive at its DNA target intact.

B. **Cancer cells:** The distribution of the platinum-anthraquinone complexes (shown in Figure 1.19) in cancer cells (A2780 ovarian, and DLD-1 colon) was investigated. This allowed us to ascertain the specificity of the complex for its DNA target, the subcellular organelles in which it accumulated, how rapidly it accumulated in cells, and the pathways it followed to be excreted by the cells.

C. **Multicellular tumour spheroids:** The penetration and distribution of platinum(IV) complexes in DLD-1 spheroids (an established model of solid tumours) was investigated. The results of this study should allow speculation concerning the situation in a real tumour environment and may be the basis upon which the effectiveness of a drug can be tested in a preclinical situation.
CHAPTER 2

Interactions of Platinum Complexes with DNA
2.1 Introduction

Platinum(IV) complexes are known to be more inert than their platinum(II) analogues.\textsuperscript{22, 24} Hence it is possible that such complexes arrive at DNA whilst still intact. Upon arrival, the complex may bind to DNA directly as a platinum(IV) moiety or undergo reduction to the more active platinum(II) analogue.\textsuperscript{22, 24} It is now generally accepted that platinum(IV) complexes act as prodrugs that must be reduced to their active platinum(II) analogues in order to bind to DNA and exert anticancer activity,\textsuperscript{104-107} however, studies have shown that reactive platinum(IV) complexes are able to bind directly to DNA or to DNA fragments such as nucleobases and nucleotides (and their analogues), given high temperatures and/or long reaction times.

One study has shown that 9-methylhypoxanthine (mHyp), a nucleobase guanine analogue, formed both platinum(II) and platinum(IV) products when reacted at 80 °C for 1-2 weeks with some representative platinum(IV) complexes.\textsuperscript{108} A later study found that at milder physiological temperatures (37 °C) the reaction between mHyp and a model platinum(IV) complex (\textit{mer}-[PtCl\textsubscript{3}(dien)]Cl, dien = N-(2-aminoethyl)ethane-1,2-diamine) was incomplete after 50 hr, yet contained both platinum(II) and platinum(IV) products. Using proton NMR, these products were shown to be coordinated to mHyp. The presence of 9-methylxanthine, the oxidation product of mHyp, was also detected in the reaction mixture, suggesting that the platinum(IV) complex caused oxidative damage to the nucleobase. It also indicated that the nucleobase caused reduction of the platinum(IV) complex. The equivalent reaction using the platinum(II) analogue occurred approximately 50 times more rapidly.\textsuperscript{109}

Reactions of the nucleotide, 5'-guanosine monophosphate (5'-GMP), with \textit{mer}-[PtCl\textsubscript{3}(dien)]Cl gave similar results. Platinum(II) and platinum(IV) products coordinated to 5'-GMP were detected in substantial quantities only after eight days at 37 °C.\textsuperscript{109} Similarly, [Pt(OAc)\textsubscript{4}(en)] was found to bind directly as platinum(IV) to 5'-
GMP, although formation of the product took several weeks\textsuperscript{110}. Choi \textit{et al} detected the presence of a platinum(IV) intermediate in the reaction of tetraplatin, a readily reduced platinum(IV) complex, with 5\textquotesingle-GMP. However, it rapidly formed a platinum(II)-5\textquotesingle-GMP product.\textsuperscript{35} In later work, Choi \textit{et al} showed that tetraplatin causes the oxidation of guanine to 8-oxo-guanine in guanosine-5\textquotesingle-monophosphate (GMP), 2\textquotesingle-deoxyguanosine-5\textquotesingle-monophosphate (dGMP), d(GG), and a double-stranded oligonucleotide.\textsuperscript{111, 112} This suggests that oxidative damage by platinum(IV) complexes may be an additional mechanism of anticancer action that platinum(II) anticancer drugs are incapable of.

Direct reaction between platinum(IV) and DNA has been shown to be sterically feasible by molecular modelling,\textsuperscript{34} as well as experimentally possible under certain conditions. Iproplatin and oxoplatin were found to coordinate to DNA when an excess of platinum complex was used, although only 10 \% bound after a 12 day reaction period at 28 \textdegree{}C. In contrast, their platinum(II) analogues reacted with DNA almost quantitatively over the same time period. Monofunctional adducts, as well as bifunctional inter- and intra-strand adducts of DNA with oxoplatin were detected.\textsuperscript{113-115}

Recent work within our research group has investigated the DNA binding properties of various platinum(IV) analogues of [PtCl\textsubscript{2}(en)], having axial chloro, hydroxo, or acetato groups. Each of the complexes was found to exhibit some DNA binding, with the extent of binding following the same trend as the reduction potential of the complexes. That is, the most easily reduced complexes were found to react with DNA to the greatest extent. Given the absence of any known reducing species in the reaction mixtures, it was hypothesised that the complexes may be binding as platinum(IV), or that the DNA may be causing the reduction and subsequent binding of the complexes.\textsuperscript{116}

The work outlined in this chapter is an extension of the above mentioned platinum(IV) DNA binding studies. The aim of this work was to ascertain whether representative platinum(IV) complexes are capable of binding to DNA directly as platinum(IV)
species, or whether DNA or a species in the reaction mixture is able to reduce the complexes. The results of this work should also provide an indication as to the behaviour of platinum(IV) intercalator complexes in the presence of DNA.

XANES spectroscopy was used to monitor the oxidation state of the platinum complexes following incubation in the presence of DNA for various periods of time. This technique has been used previously in our research group as a method to determine the proportion of platinum(II) and platinum(IV) present in biological samples. Analysis of XANES spectra of several platinum(II) and platinum(IV) complexes, including those used in this study, revealed that the peak height of platinum(IV) complexes is substantially greater than that of platinum(II) complexes. In fact, the ratio of the peak height (parameter a) to the post-edge minimum height (parameter b) is characteristic of the oxidation state and a linear relationship exists between this ratio and the proportion of platinum(II) or platinum(IV) present in any given mixture (within an error of approximately 5 %).36,117

2.2 Methods

2.2.1 Complexes examined

The following complexes were examined:
cis-[PtCl2(NH3)2] (cis), cis-[PtCl4(NH3)2] (Cl4), cis,trans,cis-[PtCl2(OAc)2(NH3)2] (OAc), and cis,trans,cis-[PtCl2(OH)2(NH3)2] (OH). The complexes were synthesised as described previously.37 The structures of the complexes are shown in Figure 2.1. Each platinum complex was dissolved in TE buffer to a final concentration of 1 mM. Sodium chloride was added to the stock solutions to prevent aquation of the complexes.
CHAPTER 2: Interactions of Platinum Complexes with DNA

Figure 2.1: Structures of the platinum complexes used in this study: (1) = cis-[PtCl$_2$(NH$_3$)$_2$] (cisplatin), (2) = cis-[PtCl$_4$(NH$_3$)$_2$] (Cl4), (3) = cis,trans,cis-[PtCl$_2$(OAc)$_2$(NH$_3$)$_2$] (OAc), and (4) = cis,trans,cis-[PtCl$_2$(OH)$_2$(NH$_3$)$_2$] (OH).

2.2.2 Sample preparation

The following procedures are based upon those outlined by Dolman$^{116}$ for DNA binding studies with platinum(IV) complexes. A small amount of calf thymus DNA was dissolved in TE buffer and UV-vis spectroscopy was used to determine the concentration of the solution (using the absorbance at 260 nm, 50 µg/mL DNA = 1 absorbance unit). TE buffer, followed by DNA (0.32 µg/µL) and then the platinum solution (to a final concentration of 200 µM) were mixed together and then incubated at 37 °C for the required time period (0, 4, 24, 48 hr). Upon completion of the incubation period, the reaction solution was placed into a polycarbonate sample holder with a sample well size of 8 mm x 1.5 mm x 1.5 mm, and secured with Kapton tape windows. The reaction was quenched by plunging in liquid nitrogen.

2.2.3 XANES spectra

The Pt L3 edge XANES spectra were obtained at the Australian National Beamline Facility (ANBF) on bending magnet beamline 20B at the KEK Photon Factory, Tsukuba Synchrotron Radiation Laboratory, Tsukuba, Japan, using a Si(111) channel-cut monochromator. The storage ring delivered a current of 250-400 mA at 2.5 GeV.
The samples were pre-chilled in liquid nitrogen prior to insertion into a Cryodone REF-1577-D22 closed-cycle cryostat. Samples were maintained at 15 K with a Neocera LTC-11 temperature controller unit. Energies were calibrated with a Pt foil, the first inflection point being assigned as 11.5671 keV. Four scans of the XANES spectra were recorded in fluorescence mode, using a Canberra ten-element detector. The scans were averaged using weights based on the signal-to-noise ratios, with each spectrum checked individually prior to averaging. The XANES spectra displayed herein were smoothed using 3-point adjacent averaging, and a/b ratios were calculated from the raw spectra.

2.3 Results and Discussion

2.3.1 Cisplatin in the presence of DNA

Cisplatin was used as the “control” sample for this experiment. Figure 2.2 shows the normalised XANES spectra obtained following 0, 4, 24, and 48 hr incubation of cisplatin in the presence of calf thymus DNA. As expected, the spectra retain a typical platinum(II)-like appearance for the duration of the experiment, indicating that the oxidation state of the platinum complex does not vary. The corresponding a/b ratios (see Table 1.1) are also consistent with the presence of platinum(II) complexes, with each ratio lying approximately within the range determined previously for numerous platinum(II) complexes structurally similar to cisplatin (Pt(II) a/b = 1.52 ± 0.08).

The overlayed spectra (and peak height ratio) reveal a slight increase in the peak height with increasing incubation times. The XANES spectra are known to be sensitive to the coordination sphere of the complex. Hall et al reported that the peak height ratio of platinum(IV) complexes was subtly dependent upon the ligand donor atoms, increasing as the number of “hard” oxygen donor atoms increased. This effect has also been reported by others. Lytle et al observed an increase in the peak height when oxygen was chemisorbed onto the surface of a platinum catalyst.
Hence the observed increase in peak height ratio of cisplatin in the presence of DNA overtime may be due to a change in the coordination sphere of cisplatin, which is most likely the result of aquation of the complex and/or reaction with DNA.

**Figure 2.2:** Normalised XANES spectra of cisplatin incubated in the presence of DNA for 0, 4, 24, 48 hr.

<table>
<thead>
<tr>
<th>Incubation time (hr)</th>
<th>a/b ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.49</td>
</tr>
<tr>
<td>4</td>
<td>1.55</td>
</tr>
<tr>
<td>24</td>
<td>1.61</td>
</tr>
<tr>
<td>48</td>
<td>1.60</td>
</tr>
</tbody>
</table>

It is widely accepted that aquation of cisplatin precedes its binding to DNA. The predominant pathway then involves reaction of the monoaquated species (cis-[PtCl(NH$_3$)$_2$(OH$_2$)]$^+$) with a DNA base, usually guanine, forming a monofunctional DNA adduct. Ring closure to form a bifunctional adduct may occur either directly from the monofunctional adduct or may involve aquation of the second chloro ligand followed by rapid ring closure. Cisplatin aquation has been the subject of
extensive study.\textsuperscript{121,122,127-133} In one such study,\textsuperscript{122} performed at 25 °C in the presence of 9 mM NaClO4, the monoaquated species was the first product to be observed, accounting for 8 % of the platinum within 2 hr of reaction. The diaquated species (cis-[Pt(NH$_3$)$_2$(OH$_2$)$_2$]$^{2+}$) was first detected after 3.5 hr, and subsequent formation of bridged dinuclear Pt species was evident after 10.5 hr. After 40 hr of reaction, cisplatin accounted for 23 % of the total Pt; the monoaquated species, 65 %; the diaquated species, 7 %; and the dimeric species, approximately 5 %. In the same study, cisplatin aquation was found to be slowed down by 30 – 40 % in the presence of oligonucleotides. The monoaquated species only accounted for a minor percentage of the reaction mixture, most likely due to a rapid subsequent reaction to form DNA adducts. The diaquated cisplatin species was not detected, neither was an aquated monofunctional DNA adduct which may precede ring closure to a bifunctional adduct. Another study,\textsuperscript{123} using chicken erythrocyte DNA, performed at 37 °C in the presence of 3 mM NaCl and 1 mM NaH$_2$PO$_4$, found that the half life of formation of monoaquated species was 1.9 hr, after which the species rapidly (within minutes) formed a monofunctional DNA adduct. Subsequent aquation of the remaining chloro ligand then occurred with a half life of 2.1 hr, followed by rapid closure to a bifunctional DNA adduct.

The increase in peak height ratio, observed with cisplatin incubated in the presence of DNA, may be explained by a similar reaction scheme to those outlined in the above mentioned papers. A small quantity of cisplatin may become monoaquated, regardless of the chloride in the reaction mixture (present to hinder aquation of the complex). This singly aquated species may then form a monofunctional adduct with DNA, and subsequently ring close to a bifunctional adduct, possibly via an aquated monofunctional DNA adduct. In the absence of large quantities of chloride, a significant proportion of the cisplatin is likely to have reacted in this manner within approximately 4 hr. The chloride present in the reaction mixture may have hindered this process such that 24 hr incubations are required for a significant proportion of cisplatin to undergo reaction. During the proposed reaction, the coordination sphere of the platinum centre changes from Cl$_2$N$_2$ to ClON$_2$ to ClN$_3$ to ON$_3$ to N$_4$. These changes involve loss of chloro ligands (“softer” donor groups) and replacement with
oxygen and nitrogen donor groups ("harder" donors). Thus an increase in peak height ratio over the course of such a reaction would be expected. The slight increase in peak height ratio observed in this experiment may be consistent with some cisplatin undergoing a reaction of this sort, resulting in a mixture of platinum products (perhaps with intact cisplatin still present), containing some species with relatively "hard" donor groups. After 48 hr incubation, reaction with DNA should be complete.

The presence of a small post-edge feature just above the whiteline peak in the XANES spectra of all platinum(II) and platinum(IV) complexes containing chloro ligands in the coordination sphere has been previously reported. The intensity of this peak was found to be dependent upon the number of chloro ligands in the complex. The more chloro ligands present, the higher the intensity of the peak, whilst the feature was absent in complexes devoid of chloro ligands. Further work is required to assess whether the intensity of this peak can be used to quantitatively monitor the number of chloro ligands present in a platinum(II) or platinum(IV) complex. Upon closer inspection of the XANES spectra, shown in Figure 2.3, such a post-edge feature is visible in the spectra for 0 and 4 hr incubation times, whilst it is absent in the 24 and 48 hr spectra. The peak is more pronounced in the 0 hr spectrum. This provides a further indication that cisplatin undergoes reaction involving loss of chloro ligands from the coordination sphere.
Figure 2.3: XANES spectra of cisplatin incubated in the presence of DNA highlighting the presence of a post-edge feature in the 0 hr (black) and 4 hr (red) spectra.

2.3.2 Platinum(IV) complexes in the presence of DNA

Figures 2.4, 2.5, 2.6 show the normalised XANES spectra obtained following 0, 4, 24, 48 hr incubation of the platinum(IV) complexes with calf thymus DNA (Figure 2.4: cis-[PtCl$_4$(NH$_3$)$_2$]; Figure 2.5: cis,trans,cis-[PtCl$_2$(OAc)$_2$(NH$_3$)$_2$]; Figure 2.6: cis,trans,cis-[PtCl$_2$(OH)$_2$(NH$_3$)$_2$]). The spectra of each of the complexes remains essentially unchanged over the entire duration (48 hr) of the experiment, retaining a typical platinum(IV)-like appearance.\textsuperscript{36}

Table 2.2 shows the a/b ratios for the platinum(IV) complexes at each individual incubation time. It has been determined previously that the a/b ratio for platinum(IV) complexes of this type is 2.51 ± 0.13.\textsuperscript{36} A more detailed study found that the average peak height ratio for the tetrachloro, diacetato, and dihydroxo analogues of cisplatin and transplatin is 2.34 ± 0.10.\textsuperscript{117} The values for the OAc and OH complexes tend to be higher than those for the Cl$_4$ complex. A similar trend was observed by Hall et al.,\textsuperscript{117} where it was concluded that separate standard curves were required for each platinum(IV) complex due to the coordination sphere-dependent variation seen in the peak height ratio. This trend was related to the number of “hard” donor atoms in the
complexes, as discussed above. The OAc and OH complexes have more “hard” oxygen donor ligands than the tetrachloro complexes and hence they tend to have higher peak height ratios. In the study by Hall et al, it was found that the tetrachloro complexes studied (cis-[PtCl₄(NH₃)₂] (Cl₄) and trans-[PtCl₄(NH₃)₂]) had lower peak height ratios than the other platinum(IV) complexes studied. cis-[PtCl₄(NH₃)₂] gave a peak height ratio of 2.26, and trans-[PtCl₄(NH₃)₂], 2.19, with an average of 2.22 (range 0.07). In the present study, the values for Cl₄ in the presence of DNA for all incubation times (average 2.31 ± 0.03) were within 0.08 of the value reported by Hall et al (2.26), though several lie outside the range calculated by including both the cis and trans complexes (average 2.22, range 0.07). In the Hall study, the dihydroxo and diacetato analogues of cisplatin and transplatin had an average ratio of 2.40 ± 0.04. The corresponding value for the current study, calculated by averaging the peak height ratio of OAc and OH for all incubation times, gave a very similar value of 2.43 ± 0.02.

Figures 2.4, 2.5, 2.6 exhibit the post-edge feature discussed above. The intensity of this peak decreases in the order Cl₄, OAc, OH (intensities are 0.10, 0.06 – 0.07, 0.04, respectively, as compared to the post-edge minima of the curves). In the Cl₄ spectra, the intensity of the post-edge peak decreases over the course of the incubation with DNA, as seen in the zoomed in version of the XANES spectra in Figure 2.7. This is indicative of changes in the coordination sphere of the complex during the experiment, namely, loss of some chloro ligands. This may be due to aquation and/or direct reaction of the platinum(IV) species with DNA. These observations are consistent with the findings of Hall et al who followed the aquation of cis-[PtCl₄(¹⁵N)₂] at 25 °C for 42 hr using [¹H,¹⁵N] 2D HSQC NMR. Platinum(IV) mono- and di-aquated species (substituted in the axial positions) were the first to be detected, followed by a platinum(IV) mono-aquated species (substituted in an equatorial position trans to an amine ligand).

In the OAc spectra, the post-edge peaks of the 0, 4 and 24 hr spectra overlay one another quite closely, whereas in the 48 hr spectrum, the peak is more intense. This is a rather surprising result and would seem to imply that the complex gains chloro
ligands during the course of the incubation, though this seems to be an unlikely possibility. In the OH spectra, the post-edge peaks overlay one another closely, indicating very few changes in the coordination sphere of the complex over the course of the DNA incubation. This result is consistent with the reported inertness of the OH complex to both reduction and aquation.\textsuperscript{34} Hall \textit{et al} reported no aquation of the OAc or the OH complex over a period of 96 hr and little or no aquation after several weeks.\textsuperscript{137}

![Normalized XANES spectra of Cl4 incubated in the presence of DNA for 0, 4, 24, 48 hr.](image)

**Figure 2.4:** Normalised XANES spectra of Cl4 incubated in the presence of DNA for 0, 4, 24, 48 hr.
**Figure 2.5:** Normalised XANES spectra of OAc incubated in the presence of DNA for 0, 4, 24, 48 hr.

**Figure 2.6:** Normalised XANES spectra of OH incubated in the presence of DNA for 0, 4, 24, 48 hr.
CHAPTER 2: Interactions of Platinum Complexes with DNA

Table 2.2: The ratio of the peak height (parameter a) to the post-edge minima (parameter b) each of the platinum(IV) complexes incubated in the presence of DNA for 0, 4, 24, 48 hr.

<table>
<thead>
<tr>
<th>Complex</th>
<th>Cl4</th>
<th>OAc</th>
<th>OH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation time (hr)</td>
<td>a/b ratio</td>
<td>a/b ratio</td>
<td>a/b ratio</td>
</tr>
<tr>
<td>0</td>
<td>2.30</td>
<td>2.42</td>
<td>2.45</td>
</tr>
<tr>
<td>4</td>
<td>2.34</td>
<td>2.42</td>
<td>2.46</td>
</tr>
<tr>
<td>24</td>
<td>2.30</td>
<td>2.43</td>
<td>2.41</td>
</tr>
<tr>
<td>48</td>
<td>2.28</td>
<td>2.42</td>
<td>2.44</td>
</tr>
<tr>
<td>average</td>
<td>2.31 ± 0.03</td>
<td>2.42 ± 0.01</td>
<td>2.44 ± 0.02</td>
</tr>
</tbody>
</table>

Figure 2.7: XANES spectra of Cl4 incubated in the presence of DNA highlighting the presence of a post-edge feature. This peak decreases in intensity over the course of the incubation.

These results indicate that the complexes remain as platinum(IV) species throughout the duration of the incubation. Hence, within the time period investigated, DNA/TE buffer is unable to reduce the platinum(IV) complexes, or any platinum(IV) metabolites that may be present, to platinum(II) products to a significant extent.
Choi and coworkers detected a platinum(IV) intermediate in the reaction of tetraplatin with 5'-GMP that later formed a platinum(II)-5'-GMP product. Later work of this group showed tetraplatin to be capable of oxidising guanine in various DNA fragments. Specifically, within 1 day, the oxidised product was detected in the reaction mixture of tetraplatin with dGMP, and 2 days for the equivalent reaction with GMP. Earlier investigations of platinum(IV) complexes reacting with DNA fragments also inferred that platinum(IV) complexes are able to cause the oxidation of DNA fragments. However, this was only observed to occur when much harsher conditions than those adopted by Choi et al (longer reaction times and/or higher temperatures) were used. Under the conditions of the present study, the platinum(IV) complexes did not cause significant oxidation of DNA, as evidenced by the platinum(IV)-like appearance and peak height ratio of the XANES spectra over the duration of the experiment. Given longer reaction times and/or higher temperatures, these complexes may cause oxidation of DNA. The tetrachloro complex is the most likely candidate, given its more positive reduction potential compared to the other complexes. The milder conditions needed for tetraplatin to oxidise DNA are likely to be due to its more positive reduction potential compared to the platinum(IV) species used by Dolman (or any of the similar previous studies reported in the literature).

The corresponding DNA binding studies performed by Dolman (as mentioned above) used a series of platinum(IV) complexes based upon [PtCl₂(en)], a structural analogue of cisplatin. It should be noted that this series of platinum(IV) complexes has the same axial groups and similar reduction potentials to the cisplatin derivatives used in the present study. Dolman reported that for 24 hr incubation time periods, [PtCl₄(en)] exhibited 13 % binding at a concentration of 20 μM, and 4 % binding at 177 μM; cis,trans-[PtCl₂(OAc)₂(en)] exhibited 5 % binding at a concentration of 19 μM, and 3 % at 160 μM; and cis,trans-[PtCl₂(OH)₂(en)] displayed no significant DNA binding at any of the platinum concentrations tested. The results are shown in Figure 2.8. In the current studies, a platinum concentration of 200 μM was used, at which less than 5 % binding would be expected. This
concentration was chosen to ensure sufficient platinum signal to obtain high quality XANES spectra. However, it is unclear whether there is sufficient DNA present at this concentration to cause reduction of the complexes. Future work should include repeats of these studies at lower platinum concentrations where higher platinum binding would be expected. When considered in light of the current studies, Dolman’s DNA binding results suggest that either the complexes bind to DNA as platinum(IV) species, or that trace platinum(II) impurities are present that could account for the small percentage of platinum-DNA binding that was observed. Direct platinum(IV)-DNA binding seems to be inconsistent with previous work that found that such direct binding was only detected when much harsher reaction conditions and/or lengthy reaction times were used (see above). For example, the interactions of cis,trans,cis-[PtCl$_2$(OH)$_2$(NH$_3$)$_2$] (oxoplatin) with DNA have been the subject of some previous research. In one study, Brabec detected some (approximately 10 %) direct binding of oxoplatin to DNA in a 12 day reaction period at 28 °C. Another study found that oxoplatin was not capable of binding to PM2 DNA after 18 hr at 37 °C in a buffer medium containing chloride ions or in a tris-nitrate buffer medium.

Figure 2.8: Percentage binding of platinum complexes following 24 hr incubation.

It is possible that small quantities of a platinum(II) complex may have been present in the reaction mixtures used by Dolman. Previous work has shown that the presence of small quantities of a platinum(II) complex can catalyse the reaction between
platinum(IV) complexes and DNA fragments.\textsuperscript{35, 142} Roat et al observed that in a reaction mixture containing 90 \% cis-[PtCl\(_4\)](NH\(_3\))\(_2\) and 10 \% cisplatin in the presence of 9-methylxanthine, a 1:1 ratio of platinum(II):platinum(IV)-nucleobase products formed within 10 hr at 37 °C.\textsuperscript{142} Product formation appeared to level off after this time period. The presence of platinum(II) impurities in the reaction mixtures of the current study, remaining in the sample as an artefact of the synthetic procedure, may not be detectable using XANES. Hence platinum(II) catalysis may have occurred during the current XANES experiment with the Cl\(_4\) complex, and is also the most likely explanation for the platinum-DNA binding observed by Dolman for this same complex.

2.4 Conclusions

The present work indicates that cisplatin undergoes changes in its ligand donor atoms when incubated in the presence of a TE buffered aqueous solution of DNA. This is likely to be the result of aquation of the complex and subsequent reaction with DNA.

The results also suggest that DNA, or any other species present in the reaction mixtures, are incapable of reducing the platinum(IV) complexes used in this study to platinum(II) species over the time period studied. The complexes either bind directly to DNA as platinum(IV) species, or remain in the vicinity of the DNA with minimal interactions. There is some indication that the tetrachloro complex undergoes changes in its coordination sphere over the course of the incubation, which may be due to direct or catalysed reaction with DNA and/or aquation.

When considered in light of the DNA binding studies performed by Dolman on similar platinum(IV) complexes, and other research into the interactions of platinum(IV) complexes with DNA and DNA fragments, the present study indicates that the platinum-DNA binding observed by Dolman was likely to be due to the presence of
some platinum(II) impurities in the reaction mixtures, causing catalysis of the reaction with DNA.
CHAPTER 3

Cellular Distribution of Platinum Complexes
3.1 Introduction

Whilst DNA is known to be a vital cellular target of cisplatin, much remains unknown regarding the pathway followed by cisplatin to enter cells and reach genomic DNA. Recent work has addressed this question using a number of different techniques and cell lines. However, no definitive answer has yet been found. In the following two sections, selected studies that have approached this problem will be described, focussing on work that has utilised synchrotron radiation induced X-ray emission (SRIXE) and fluorescence imaging techniques.

3.1.1 Synchrotron radiation-induced X-ray emission (SRIXE)

Hall et al. used synchrotron radiation-induced X-ray emission (SRIXE) to examine elemental distribution within A2780 human ovarian cancer cells that had been treated with cisplatin and platinum(IV) complexes (analogues of cisplatin containing axial chloro, acetato or hydroxo groups, see Figure 1.18 for structures). The SRIXE technique allows two-dimensional mapping of elemental distribution with sub-micron accuracy. In addition, the location of the platinum moiety is directly monitored and not merely inferred indirectly by, for example, imaging of chemically modified analogues of the drug. It was found that cisplatin and the platinum(IV) analogues accumulated in the nuclei of the cells after 24hr incubation, and the distribution of the platinum(IV) complexes was similar to that of cisplatin (note that zinc elemental maps were used to identify cell nuclei). This suggested that the majority of the platinum(IV) species had been reduced, an observation confirmed qualitatively by collecting micro-XANES spectra at points of high platinum concentration within the cells. The observation was also confirmed quantitatively by obtaining XANES spectra of bulk cell samples and quantifying the percentage of remaining platinum(IV). SRIXE coupled with XANES represents a powerful technique whereby both the distribution and oxidation state of a drug within a biological system can be determined. The disadvantage of the SRIXE technique is the difficulty of identifying
small subcellular organelles, such as mitochondria, Golgi apparatus, endoplasmic reticulum, lysosomes, etc.

SRIXE has been used in the current study to investigate the distribution of platinum-anthraquinone complexes in cancer cells. This technique allows determination of the distribution and uptake of such complexes relative to cisplatin. As outlined in Chapter 1, the intercalating moiety of platinum-anthraquinone complexes should rapidly transport the complex to its DNA target and hence we expect such complexes to accumulate more rapidly, more specifically, and to a greater extent in the nuclei of the cells than cisplatin.

3.1.2 Fluorescence imaging

The spatial distribution of fluorescence within cells can be detected with high sensitivity and signal specificity (within a few hundred nanometres). Numerous cellular distribution studies with fluorescently labelled platinum complexes have been performed, commonly using laser scanning confocal microscopy. This technique eliminates out-of-focus light and hence high resolution images can be obtained. Conventional wide-field fluorescence microscopy has also been used. The resolution of this technique tends to be lower than for confocal microscopy, although recent advances have diminished this disparity. The choice between techniques is dependent upon factors such as sample thickness and fluorescence depth.\textsuperscript{153}

Reedijk and colleagues have investigated the cellular distribution of a fluorescently labelled platinum complex (a model of cisplatin) using digital fluorescence microscopy (a form of wide-field fluorescence microscopy).\textsuperscript{98, 99, 143} The cisplatin model consisted of a non-fluorescent fluorescein derivative attached to a platinum moiety (CFDA-Pt, where CFDA = carboxyfluorescein diacetate). CFDA-Pt becomes fluorescent following cellular uptake and acetate hydrolysis. The complex was shown to initially distribute throughout the entire cell, then accumulate in the nucleus after 2-3 hr and subsequently in the Golgi apparatus after 6-8 hr (determined by colocalisation experiments with a fluorescent Golgi selective stain) and up to 72 hr
after the initial incubation. After 24 hr, no significant fluorescence was observed in
the nuclei of the cells, indicating that very little platinum remained bound to DNA.
Findings were similar when immunocytochemical studies were performed with a
related non-fluorescent platinum complex (DNP-Pt, where DNP = dinitrophenyl). The
platinum complexes were shown to have similar DNA binding properties to cisplatin,
and their cellular distributions were distinct from those of the free ligands, suggestive
of distribution characteristics dominated by the platinum moiety, rather than the
fluorescent ligand. However, the complexes remain questionable models of
cisplatin because they are likely to act quite differently to cisplatin in cellular
environments.

Safaei et al conducted a similar study with a fluorescein-labelled cisplatin analogue
(F-DDP) using digital deconvoluting microscopy in fixed 2008 human ovarian
carcinoma cells. F-DDP was shown to mimic the behaviour of cisplatin, being subject
to the same mechanisms by which cisplatin resistant 2008 cells are resistant to
cisplatin, and having cellular distributions significantly different from those of the
fluorophore in isolation. Staining with organelle-specific markers revealed that F-DDP
was sequestered into the lysosomes, Golgi, and secretory compartments of the cells.
The role of organelles in the distribution of the complex within cells was determined
by treatment of the cells with agents that selectively block certain trafficking
pathways. The secretory pathway was shown to play an important role, perhaps in
efflux of the complex from cells.

Liang et al studied the trafficking and localisation of a cisplatin model complex (the
fluorescent Alexa Fluor molecule attached to a platinum moiety), in live cells derived
from human KB epidermoid carcinoma cells (KB-3-1). The use of this complex as an
appropriate model of cisplatin was justified by the differences seen in the kinetics
and distribution of the complex in cisplatin resistant cells as compared to parent
cells. They found that the complex was readily internalised by the cells and during the
first 30 min of the incubation, the complex moved from the membranes and into the
Golgi apparatus. After 30 min, the complex moved to the nuclei of the cells, such that
by 1 hr, it was localised in both the nuclei and Golgi. In contrast, the cisplatin
resistant cell lines showed much less fluorescence within the cells, with only punctate cytoplasmic staining and little present in the cell nuclei. Studies indicate that cellular resistance is due to inappropriate trafficking of cell membrane binding proteins for cisplatin.\textsuperscript{151}

It is also of interest to investigate the cellular distribution of intrinsically fluorescent platinum complexes. Dinuclear platinum complexes containing anthraquinone moieties have been used by Reedijk \textit{et al} to model a novel class of anticancer drugs. However, the platinum-anthraquinone complexes were also interesting in their own right, displaying good DNA binding and cytotoxicity properties. The intrinsic fluorescence of these complexes allowed their cellular processing to be monitored using time-lapse digital fluorescence microscopy. In live parental A2780 ovarian carcinoma cells, such complexes entered the cells and after 24 hr were localised in the lysosomes. However, in the cisplatin resistant phenotype, the complexes were encapsulated by the lysosomes at the beginning of their incubation. The lack of fluorescence within the nuclei of the cells was attributed to quenching of the anthraquinone fluorescence when in the presence of DNA.\textsuperscript{98, 99} Processing of the complexes in U2-OS human osteosarcoma cells was somewhat different, with rapid initial accumulation in the nuclei, followed by uptake into the Golgi apparatus, believed to be involved in transport of the platinum complexes out of the cells.\textsuperscript{100}

In the current study, the live cell distribution of intrinsically fluorescent platinum-anthraquinone complexes was investigated using laser scanning confocal microscopy. Such studies allow us to gain insights into the mechanism of action of such complexes by determining the pathways by which they are accumulated and excreted from the cells, the organelles in which they accumulate, how rapidly they accumulate, etc.

\textbf{3.1.3 Aims}

The aim of the work detailed in this chapter was to determine whether the presence of the intercalating moiety in platinum-anthraquinone complexes endowed the
complexes with favourable biological properties. Monolayers of A2780 ovarian cancer cells and DLD-1 colon cancer cells have been used in these studies to investigate properties such as platinum cellular uptake, cytotoxicity, and cellular distribution.

3.2 Methods

3.2.1 Synthesis

The structures of the ligands and complexes used in this study are shown below in Figure 3.1.
Figure 3.1: Structures of the compounds used in the current study. (1) = cisplatin, (2) = JM118, (3) = 1C3, (4) = Pt1C3, (5) = 2C3, (6) = Pt2C3, (7) = doxorubicin.

3.2.1.1 Materials

All solvents and reagents were of laboratory grade and were used as received without further purification, unless otherwise stated. 1-chloroanthraquinone and propane-1,3-diamine were obtained from Aldrich. Tetraethylammonium chloride monohydrate was obtained from Lancaster, ammonium chloride from Ajax Chemicals, and cisplatin
from the Institute of Drug Technology, Victoria, Australia. Doxorubicin hydrochloride
was purchased from Sigma.

3.2.1.2 Instrumentation

Diffuse reflectance infrared Fourier transform spectra (DRIFTS) were measured on a
BIO-RAD FTS-40 or FTS-7 spectrophotometer. Potassium bromide was used as both
the background and the matrix over the range 400 – 4000 cm\(^{-1}\). \(^1\)H and \(^{13}\)C NMR
spectra were recorded at 300 K on a Bruker AMX 300 MHz spectrometer. All spectra
were recorded using commercially available solvents (Aldrich) of >99.6 % isotopic
purity and referenced to TPS (3-(trimethylsilyl)propionic acid) or solvent isotopic
impurities. \(^{195}\)Pt NMR spectra were recorded at 300 K on a Bruker AMX 400 MHz
spectrometer. Chemical shifts were referenced to Na\(_2\)[PtCl\(_6\)]. Elemental analyses (C,
H, N) were performed by the Microanalytical Service of the Australian National
University, Canberra, ACT or Chemical and Micro Analytical Services, Belmont,
Victoria.

1-[(3-Aminopropyl)amino]-anthracene-9,10-dione, 1C3 (Figure 3.1, (3))
The following synthetic procedure is based upon that outlined by Barasch \textit{et al.}\(^{71}\)
Propane-1,3-diamine (15 mL, 180 mmol) was added to a solution of 1-
chloroanthraquinone (10 g, 41 mmol) in dry toluene (350 mL, dried over anhydrous
Na\(_2\)SO\(_4\)) and refluxed for 18 hr. The red suspension was evaporated to dryness under
reduced pressure and resuspended in chloroform (500 mL). Gaseous hydrochloric
acid was bubbled through the suspension for approximately 2 days, or until the
mixture became pale pink in colour. The product was extracted into an aqueous layer
with several washings of water until the organic layer became pale pink in colour.
Using 3 M NaOH, the pH of the aqueous layer was adjusted to approximately 8,
followed by subsequent extraction of the final product into chloroform. The solution
was evaporated to dryness under reduced pressure, giving a red solid (7.67 g, 27.4
mmol, 77 % yield). \(^3\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 9.74 (broad, 1H), 8.24 (m, 2H), 7.72
(m, 2H), 7.57 (dd, 1H), 7.51 (t, 1H), 7.07 (dd, 1H), 3.41 (m, 2H), 2.93 (t, 2H), 1.91
(m, 2H), 1.38 (broad, 2H); $^{13}$C NMR (300 MHz, CDCl$_3$) δ 185.10, 183.89, 151.86, 135.41, 135.11, 134.75, 134.02, 133.13, 133.01, 126.81, 126.75, 117.94, 115.75, 113.06, 40.68, 39.94, 32.92; IR (KBr, cm$^{-1}$): 3356 m, 3273 m, 3182 m, 2934 m, 2862 m, 1662 s, 1628 s, 1594 s, 1572 s, 1511 s, 1470 m, 1465 m, 1409 m, 1303 s, 1272 s, 1236 m, 1176 m, 1160 m, 1071 m, 1019 m, 972 m, 916 m, 830 m, 806 m, 786 m, 735 s, 709 s, 673 m, 662 m, 598 m, 549 m, 481 m, 419 m.

Amminetrichloroplatinate(II), [PtCl$_3$(NH$_3$)$_3$]–

The following synthetic procedure is based upon that outlined by Cai et al.$^{154}$ Cisplatin (4.0 g, 13.3 mmol), tetraethylammonium chloride monohydrate (2.94 g, 16.0 mmol), and ammonium chloride (0.20 g, 3.7 mmol) were dissolved in N,N-dimethylacetamide (150 mL). The reaction mixture was heated at 100°C, purging with a slow stream of N$_2$ for 8-10 hr. The solution changed colour from yellow to orange, and its volume evaporated to approximately 50 mL during the course of the reaction. Hexane/ethyl acetate (1:1, 300 mL) was added to the solution and it was kept overnight at -20°C. The supernatant was decanted off, and the solid extracted using 60 mL of acetonitrile. The remaining solid, assumed to be a mixture of ammonium chloride and cisplatin, was washed with water to recover unreacted cisplatin. Water (5 mL) was added to the acetonitrile solution and the acetonitrile was removed under reduced pressure, resulting in an orange aqueous solution of [PtCl$_3$(NH$_3$)$_3$]– (11.6 mmol, 87 % yield).

cis-1-[[3-Aminopropyl]amino]-anthracene-9,10-dione]aminedichloroplatinum(II), Pt1C3 (Figure 3.1, (4))

The following synthetic procedure is based upon that outlined by Gibson et al.$^{95}$ A solution of 1C3 (1.65 g, 5.9 mmol) in methanol (approximately 2 L) was added to an aqueous solution of [PtCl$_3$(NH$_3$)$_3$]– (6.0 mmol) over a period of approximately one hour. Additional methanol was added, as required, to ensure that minimal precipitation occurred. The solution was stirred at room temperature for approximately 3 weeks, during which time a dark red precipitate formed. The solid was filtered and washed with methanol. The resulting red solid was ground to a fine powder, resuspended in methanol (500 mL), stirred for several days, then filtered and washed with methanol.
The process of resuspension was repeated until elemental analysis revealed that the product did not contain unreacted 1C3. The final product was obtained as a red solid (0.83 g, 1.47 mmol, 25 % yield). $^{195}$Pt NMR (DMF/HCl): $\delta$ -2157 ppm. Analysis calculated for PtC$_{17}$H$_{19}$N$_3$O$_2$Cl$_2$: C, 36.25; H, 3.40; N, 7.46. Found: C, 36.37; H, 3.29; N, 7.32.

2-[(3-Aminopropyl)amino]-anthracene-9,10-dione, 2C3 (Figure 3.1, (5)) AND
cis-2-[(3-Aminopropyl)amino]-anthracene-9,10-dione]aminedichloroplatinum(II), Pt2C3 (Figure 3.1, (6))
These compounds were synthesised by Leanne T. Ellis, as described previously.$^{155}$

cis-[PtCl$_2$(NH$_3$)(cyclohexylamine)], JM118 (Figure 3.1, (7))
JM118 was synthesised by Dr Timothy W. Failes using a procedure based upon that outlined previously by Cai et al.$^{154}$

3.2.2 Cell culture
The parental human ovarian carcinoma (A2780) cell line was used in this study. The cell line was obtained from Dr Lloyd Kelland (CRC Centre for Cancer Therapeutics, Institute of Cancer Research, Sutton, UK). Cells were maintained in exponential growth as monolayers in RPMI-1640 growth medium (TRACE Bioscience) supplemented with 2 mM glutamine (Sigma-Aldrich) and 5 % fetal calf serum (TRACE Bioscience) at 37 °C in 5 % CO$_2$. Trypsin and MTT were obtained from Sigma-Aldrich.

The human colon adenocarcinoma (DLD-1) cell line was also used in this study. The cell line was provided by Dr Roger Phillips (University of Bradford, UK). Cells were grown as monolayers in RPMI-1640 growth medium containing Glutamax I and 25 mM HEPES (Life Technologies – Invitrogen) and supplemented with 10 % v/v heat inactivated foetal bovine serum (Life Technologies – Invitrogen) and penicillin/streptomycin (100 IU/mL and 100 mg/mL respectively, Life Technologies – Invitrogen) at 37 °C in 5 % CO$_2$. The research described herein using DLD-1 cells was
performed in the laboratory of Dr Richard Callaghan, Nuffield Department of Clinical Laboratory Sciences, University of Oxford, Oxford, UK.

3.2.3 Cytotoxicity

3.2.3.1 Methods

The cytotoxicity of cisplatin, JM118, doxorubicin, 1C3 and Pt1C3 was determined in DLD-1 cells. Cisplatin and JM118 were prepared as 0.5 mM solutions in 100 mM KCl, doxorubicin as a 50 mM solution in DMSO, and 1C3 and Pt1C3 as 0.5 mM solutions in 40 % (v/v) 100 mM KCl and 60 % (v/v) DMF. The concentration of DMSO added to cells was maintained below 0.1 % (v/v), and DMF below 1 % (v/v).

Approximately 3 x 10^3 cells were seeded in each well of a 96-well plate in 100 µL of growth medium and allowed to adhere overnight. Compounds were added to the cells at concentrations ranging from 5 x 10^{-7} to 2 x 10^{-5} M, with the exception of doxorubicin, having a concentration range of 5 x 10^{-9} to 2 x 10^{-5} M. The total volume of medium in each well was 200 µL. Cells were incubated with the compounds for 2 hr or 4 hr (unless otherwise indicated) and subsequently left for a recovery phase of 72 hr in growth medium. Cytotoxicity was assessed using the MTT assay,\textsuperscript{156} whereby absorbance at 550 nm was measured in a SpectraMAX 250 plate reader (Molecular Devices). Cell viability was plotted as a function of compound concentration and the IC\textsubscript{50} values estimated by non-linear regression of the general dose-response curve.\textsuperscript{157} Viable cell number (cells remaining (% total)) was expressed as a percentage of that obtained in the absence of compound treatment for the highest concentration of drug tested.

3.2.3.2 Statistical and regression analyses

A minimum data set was obtained from at least three independent observations and values expressed as mean ± S.E.M. Statistical comparisons of mean values were performed using a one-way ANOVA with Bonferroni’s multiple comparison test and a
P-value <0.05 was considered significant. All non-linear regression analyses were generated using the GraphPad Prism3.0 program.

### 3.2.4 Platinum cellular uptake

The following complexes were examined for their platinum cellular uptake in A2780 cells: cisplatin, Pt1C3 and Pt2C3. The procedure is based upon that outlined previously by Hall et al.\(^{32}\)

Approximately 2 x 10^6 to 3 x 10^6 cells were seeded in cell culture dishes (60 mm\(^2\), Corning) in 5 mL growth medium and allowed to establish overnight. Cells were treated with platinum complexes at a concentration of 30 \(\mu\)M for time periods of 2, 6 and 24 hr. Following treatment, the cells were collected for analysis by removal of growth medium and disaggregation with trypsin (1 mL). The collected cells in PBS were centrifuged at 2500 rpm for 3 min, the supernatant removed from the cell pellet and replaced with fresh PBS. This procedure was repeated twice more to ensure removal of extracellular platinum. PBS (0.5 mL) was added to the final cell pellet, which was subsequently broken up by probe sonication (Ultrasonic Cell Disrupter, Misonix Incorporated, USA). Samples were stored at -20 \(^\circ\)C until analysis. All experiments were performed in triplicate.

Protein concentration was determined using the Biorad protein assay. The Biorad Protein Reagent was diluted 1 in 5 with double-distilled water. Diluted reagent (200 \(\mu\)L) was added to each well of a 96 well plate. Standards were measured in duplicate by adding 10 \(\mu\)L of the standard solutions (0 – 1.0 \(\mu\)g/mL) to the appropriate wells. Samples were measured in duplicate by adding 10 \(\mu\)L of diluted or undiluted samples to the appropriate wells such that its concentration could be interpolated from the standard curve. The protein/dye mixture was shaken and the absorbance measured at 620 nm using a microplate reader (SpectraCount, PACKARD).
Platinum concentrations were determined using graphite furnace atomic absorption spectrometry, with a Varian SpectrAA-20 Graphite Furnace Atomic Absorption Spectrometer, equipped with a GTA-96 Graphite Tube Atomiser and PSC-56 autosampling system.

Platinum concentrations were also determined using inductively coupled plasma mass spectrometry (ICPMS). Analyses were performed at the National Measurement Institute, Pymble, Australia.

### 3.2.5 SRIXE

The following complexes were examined for their platinum cellular distribution and uptake in A2780 cells: cisplatin, and Pt2C3. The procedure is based upon that outlined previously by Hall et al.\textsuperscript{37}

Approximately $5 \times 10^5$ A2780 cells were seeded in cell culture dishes (60 mm\textsuperscript{2}, Corning) in 5 mL growth medium and allowed to establish overnight. Medium was refreshed prior to addition of platinum solutions (cisplatin and Pt2C3) at a final concentration of 20 \(\mu\)M. Following the 24 hr incubation period, cells were harvested by removal of growth medium, and disaggregation with trypsin. Cells were thoroughly washed with PBS solution (pH 7.5, Sigma) three times by centrifuging (2500 rpm, 3 min). The supernatant was removed and the cells resuspended in ammonium acetate solution (200 mM). The cells were dehydrated by centrifuging in ammonium acetate solution incrementally replaced with alcohol (30 \%, 50 \%, 70 \%, 95 \%, 2 x 100 \%, 10 min). The cell pellet was infiltrated from 50 \% alcohol : 50 \% Spurr’s resin overnight, followed by 100 \% Spurr’s resin overnight. Pellets of the fixed cells (1 \(\mu\)m) were sectioned with a microtome.\textsuperscript{37, 158, 159}

Hard X-ray zone plate SRIXE experiments were performed on beam line 2-ID-D at the Advanced Photon Source (APS), Argonne National Laboratories, Argonne, Illinois, USA. Measurements were conducted under a He atmosphere and fluorescence
spectra were collected using a high purity Ge detector (Canberra). The sample was scanned and the integrated $K_{\alpha}$ or $L_{\beta}$ fluorescence signal of the elements of interest used to generate two dimensional maps of the sample. The elements of interest were Pt, Zn, P, S, Ca, Cl, Cu, Fe, K, and Ni.

### 3.2.6 Confocal fluorescence microscopy

#### 3.2.6.1 Instrumentation

A2780 cells were viewed using a BIO RAD Radiance Plus Confocal Microscope. Images were obtained using a 100x oil immersion objective (Nikon). The compounds 1C3, Pt-1C3, and doxorubicin were excited at 543 nm, and detected at $\lambda_{\text{em}} = 555 - 626$ nm except when SYTO21 was also present, in which case they were detected at $\lambda_{\text{em}} > 570$ nm. Stains were excited using $\lambda_{\text{ex}} = 488$ nm, and detected at $\lambda_{\text{em}} = 500-560$ nm. The compounds 2C3 and Pt2C3 were excited at 488 nm, and detected at $\lambda_{\text{em}} > 500$ nm.

DLD-1 cells were viewed using a Zeiss LSM510 confocal laser scanning microscope (Carl Zeiss, Welwyn Garden City, UK). Images were obtained using a 63x objective oil immersion objective. The compounds 1C3, Pt-1C3 and doxorubicin were excited at 543 nm, 76% laser strength, and detected at $\lambda_{\text{em}} > 560$ nm. Stains were excited using $\lambda_{\text{ex}} = 488$ nm, 10-15% laser strength, and detected at $\lambda_{\text{em}} = 505-530$ nm.

#### 3.2.6.2 Materials

Organelle specific probes were obtained from Molecular Probes – Invitrogen and stored in DMSO stock solutions at -20 °C. Nuclear staining was performed using SYTO 21 green fluorescent nucleic acid stain, mitochondrial staining was performed using MitoTracker Green FM stain, and lysosome staining was performed using LysoTracker Green DND-26 stain.
3.2.6.3 Methods

A2780 and DLD-1 cells were grown in 5 mL growth medium on glass coverslips in 6-well tissue culture plates. Cells were allowed to establish overnight, medium was removed and replaced with 1 mL of fresh medium. The concentration and incubation times for the compounds and stains are shown below in Table 3.1. The concentration of DMF added to the cells was maintained below 1.5% (v/v), and DMSO below 3.2% (v/v). Control samples contained only growth medium. Coverslips were washed thoroughly with PBS, mounted onto microscope slides and sealed with nail varnish prior to confocal fluorescence microscopy.

Table 3.1: The final compound and stain concentrations with which A2780 and DLD-1 cells were incubated. Incubation times are also shown. Note: SYTO21 = green fluorescent nucleic acid stain, MTG = MitoTracker Green, LTG = LysoTracker Green.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Compounds</th>
<th>SYTO21</th>
<th>MTG</th>
<th>LTG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time (hr)</td>
<td>Conc (µM)</td>
<td>Time (min)</td>
<td>Conc (µM)</td>
</tr>
<tr>
<td>A2780</td>
<td>1, 4, 24</td>
<td>12</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>DLD-1</td>
<td>4-5</td>
<td>12</td>
<td>120</td>
<td>5</td>
</tr>
</tbody>
</table>

3.2.7 DNA fluorescence titration

Small quantities of a stock solution of calf thymus DNA in phosphate buffer (aqueous solution of NaH₂PO₄, 5 mM, pH 7-8, adjusted with 3 M NaOH) were added to solutions of 1C3 and Pt1C3 in varying solvent systems (outlined below), such that the ratio of anthraquinone compound:DNA base pairs ranged from 1:2 to 1:100. Following each addition, solutions were mixed and fluorescence emission spectra recorded immediately.

The following solvent systems were used for the dissolution of 1C3 and Pt1C3:
Aminoalkylamino-anthraquinone compounds have been reported previously. The procedure reported herein for the synthesis and purification of 1C3 represents a significant improvement upon the reported procedures. Most notably, it does not utilise the time consuming and unreliable method of column chromatography, and yields of up to 90% have been routinely achieved. Side products are efficiently removed from the reaction mixture by the process of bubbling HCl gas through a suspension of the crude product, followed by subsequent extraction of the desired product in the free base form.

Gibson and coworkers have outlined the synthesis of analogues of Pt1C3. The procedure reported herein is a variation upon such procedures.

The limited solubility of Pt1C3 and 1C3 in aqueous solution restricted the stock solutions (0.5 mM) to consisting of 60% DMF and 40% 100 mM KCl. Addition of KCl prevents aquation of the chloro ligands of Pt1C3. Initial investigations of the cytotoxicity of the complexes involved 72 hr incubation, in the manner described previously. This procedure was subsequently abandoned due to the necrotic cell
damage produced by DMF (0.05 – 2 % v/v) during this prolonged exposure period. Incubation of cells with DMF for periods longer than 12 hr caused significant cytotoxicity (data not shown). Hence brief incubation periods (maximum of 4 hours), and low DMF concentrations (<1 % v/v) were chosen for these studies. It should be noted that these shorter incubation times are pharmacokinetically more realistic.

It was hypothesised that the anthraquinone moiety of the Pt1C3 complex may be an effective system to rapidly deliver the platinum moiety to DNA. One aspect of testing this hypothesis was determination of the efficacy of Pt1C3 compared to cisplatin, JM118, doxorubicin and 1C3. Cisplatin was chosen for comparison because of its proven anticancer efficacy and its status as the benchmark drug against which to compare the potency of novel platinum complexes. JM118 was chosen for its structural similarity to Pt1C3 (platinum(II) complex containing two chloro ligands, one ammine ligand, and one amine group that endows the complex with higher lipophilicity than cisplatin) though being devoid of an intercalating moiety. Hence comparison of JM118 to Pt1C3 should allow speculation as to the effect of the anthraquinone intercalator. Doxorubicin was chosen as an anthraquinone intercalator-based structure known to possess significant anticancer activity. 1C3 was chosen for its direct relation to Pt1C3, thus comparison should highlight the effects of the platinum moiety.

Each compound was tested for cytotoxicity in the DLD-1 colon cancer cell line. Figure 3.2 shows some example dose-response curves and Table 3.2 tabulates the cytotoxicity results for each of the compounds at various exposure times.
**Table 3.2**: Cytotoxicity of drugs in the DLD-1 cell line. Potency was estimated by non-linear regression analysis of the dose-response curve and all values represent the mean ± SEM of at least three independent observations.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Exposure time (hr)</th>
<th>Cells Remaining (% total)</th>
<th>Potency (IC$_{50}$) (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cisplatin</td>
<td>2</td>
<td>77±2</td>
<td>85±19</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>59±8</td>
<td>34±6</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>10±1</td>
<td>2.2±0.3</td>
</tr>
<tr>
<td>Pt1C3</td>
<td>2</td>
<td>38±5</td>
<td>19±4</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>25±7</td>
<td>11±1</td>
</tr>
<tr>
<td>1C3</td>
<td>2</td>
<td>67±8</td>
<td>25±2</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>48±6</td>
<td>21±6</td>
</tr>
<tr>
<td>JM118</td>
<td>2</td>
<td>29±15</td>
<td>16±7</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>20±8</td>
<td>5.9±2.9</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>2</td>
<td>18±9</td>
<td>0.30±0.08</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>16±9</td>
<td>0.17±0.04</td>
</tr>
</tbody>
</table>
Figure 3.2: Dose-dependent cytotoxicity produced in DLD-1 cells cultured as monolayers and exposed to the following: (a) cisplatin or JM118 for 4 hr; (b) doxorubicin for 4 hr or cisplatin for 24 hr; (c) 1C3 or Pt1C3 for 4 hr. The general dose response equation was fitted using non-linear least squares regression. Data points represent mean ± SEM of at least three independent observations.
It is interesting to compare the potency of cisplatin to that of the more hydrophobic derivative, JM118. A 4 hr exposure to cisplatin resulted in a viable cell count of 59 ± 8 % at the highest dose used (20 µM) and a corresponding potency of 34 ± 6 µM (Figure 3.2 (a), Table 3.2). In contrast, exposure to JM118 for the same period of time produced much greater cytotoxicity to the cells, with a viable cell count of only 20 ± 8 %, and corresponding potency of 5.9 ± 2.9 µM (Figure 3.2 (a), Table 3.2). However, exposing the cells to cisplatin for longer periods of time, such as 24 hr, produced much greater potency (2.2 ± 0.3 µM) and cytotoxicity in 90 % of the cell population. The equivalent 24 hr incubation with JM118 was not performed. It can be seen that JM118 exerts its toxic effects more rapidly than cisplatin, perhaps due to better cellular accumulation of JM118, a result of its higher lipophilicity.

The effect of 1C3 and Pt1C3 on cells was independent of exposure time. For example, 4 hr exposure to Pt1C3 produced cytotoxicity in 75 % of the cells, whilst 2 hr incubation caused a similar reduction in cell number. During the short incubation times used in this study, Pt1C3 displayed greater potency and extent of cell kill than cisplatin, although it was not significantly different than that of JM118 (P<0.05, Table 3.2). This suggests that 1C3 and Pt1C3 accumulate in DLD-1 cells more rapidly than cisplatin and perhaps are capable of overcoming the intrinsic resistance of colon cancer cells to cisplatin.

The effects of 1C3 and Pt1C3 on cells were not significantly different in terms of potency and extent of cytotoxicity. For example, at the highest possible concentration of 1C3 with a 4 hr exposure, the viable cell count was 48 ± 6 %, and the potency was 21 ± 6 µM, whilst Pt1C3 produced a viable cell count of 25 ± 7 % and a potency of 11 ± 1 µM for the equivalent exposure time. This suggests that the cytotoxicity of Pt1C3 is caused by the anthraquinone, rather than the platinum moiety.

Doxorubicin is a highly effective anticancer agent, displaying considerably greater potency than the other compounds tested in this study. Incubation of cells with doxorubicin for 2 or 4 hr produced similar effects, leaving a viable cell population of
less than 20 %, and potencies of $0.30 \pm 0.08 \mu M$ and $0.17 \pm 0.04 \mu M$, respectively. The results are consistent with a similar study performed by Hall et al., whereby monolayers of DLD-1 cells were exposed to a number of compounds, including doxorubicin, and cisplatin for 72 hr. The high potency of doxorubicin is indicative of a rapid and extensive intracellular accumulation of doxorubicin in DLD-1 colon cancer cells. This is consistent with doxorubicin acting through intercalation and subsequent local uncoiling of DNA;\textsuperscript{68} inhibition of topoisomerase-II activity;\textsuperscript{62} formation of active oxygen species that subsequently cause DNA strand breakage;\textsuperscript{68} and interaction with cell membranes.\textsuperscript{162, 163}

Gibson et al have previously studied platinum-anthraquinone complexes similar to Pt1C3. They found that complexes of this type consisting of an anthraquinone linked in a monodentate fashion to the platinum moiety displayed poor activity relative to cisplatin and similar complexes linked in a bidentate fashion.\textsuperscript{94} In contrast, the results of the current study suggest that compounds containing an intercalating moiety, such as Pt1C3, exert their cytotoxic activity more rapidly than cisplatin. In addition, doxorubicin is significantly more cytotoxic than the other compounds tested, even at shorter incubation times. Thus an intercalating moiety appears to be an efficient delivery vehicle by which to transport the platinum moiety rapidly into cells. Interestingly, JM118 displayed cytotoxic effects more rapidly than 1C3 or Pt1C3, in spite of the absence of an intercalating moiety.

### 3.3.3 Cellular distribution

#### 3.3.3.1 Platinum uptake

Comparison of the uptake of platinum anthraquinone complexes and cisplatin into cells should allow us to determine whether the intercalator causes the anticipated enhancement of platinum cellular uptake.
The uptake of cisplatin, Pt1C3 and Pt2C3 into A2780 cells, incubated with final concentrations of 30 µM for 2, 6 and 24 hr is shown in Figure 3.3. Platinum contents determined using GFAAS and ICPMS are shown.

Pt1C3 and Pt2C3 accumulated into A2780 cells to a similar extent. Their accumulation was more rapid than that of cisplatin. In addition, higher levels of platinum enter cells treated with the platinum anthraquinone complexes than those treated with cisplatin. This indicates that the intercalating moiety of the complexes acts to efficiently transport the platinum moiety into cells.

In this study, cisplatin displayed a linear correlation of uptake with respect to time ($R^2 = 0.992$). This is indicative of passive diffusion through the cell membrane, an established key mechanism of cisplatin uptake into cells. It is interesting to note that the platinum anthraquinone complexes do not seem to display a linear correlation, although subsequent studies with a larger number of time points would be required to confirm this observation. This suggests that the complexes are taken up by cells in a different manner. Previous studies indicate that the hydrophobicity of the non-leaving groups of a platinum complex affects the intracellular accumulation of the complex, with Pt-dach complexes showing approximately ten times higher
uptake than Pt-(NH$_3$)$_2$ complexes.$^{165}$ The presence of the anthraquinone group in the platinum complexes studied herein may be affecting their cellular uptake in a similar fashion.

For each complex, the uptake versus time plots (Figure 3.3) did not display a plateau within the time period investigated. This indicates that extra- and intracellular platinum did not reach equilibrium and that the uptake of the complexes is not saturable within 24 hr. Previous studies have established the non-saturable uptake of cisplatin.$^{164}$

Platinum cellular uptake was determined using GFAAS and ICPMS. The platinum contents obtained using these procedures were not significantly different from one another, as seen in Figure 3.3. ICPMS is considered to be more sensitive than GFAAS,$^{166, 167}$ although the current results suggest that the superior sensitivity of ICPMS is not needed for applications such as those outlined herein.

### 3.3.3.2 SRIXE

The platinum-anthraquinone complexes were designed to be rapidly transported to the cell nucleus. The SRIXE technique allows the distribution of the platinum moiety within cells to be monitored. Figure 3.4 shows SRIXE scans of thin-sectioned A2780 cells that were treated with cisplatin or Pt2C3 for 24 hr. The elemental maps display relative elemental concentrations according to the colour scale shown.
Figure 3.4: Elemental maps of Pt, P, S, Zn, Cl, K, Ca, Cu, respectively, obtained from sectioned A2780 cells treated with Pt(2C3), upper, and cisplatin, lower. The relative colour scale is also shown.

Zinc is often used as a marker for the nuclei of cells.\textsuperscript{37, 148} In the cisplatin treated cell (Figure 3.4), platinum is diffusely distributed throughout the cell, including the nucleus (as indicated by colocalisation with a concentrated area of zinc). This is consistent with the results of Hall et al who found that cisplatin localised in the nuclei of A2780 cells, with a lower concentration in the surrounding cytoplasm and no other distinct regions of localisation.\textsuperscript{37}

The Pt2C3 treated cell, shown in Figure 3.4, displays high levels of platinum localised within the cell. It is difficult to ascertain the colocalisation of the platinum map with that of zinc, due to the presence of a spot of unusually high zinc concentration (most likely a dust particle). Such spots effectively “drown out” the signal from the remainder of the cell, thus creating the false impression in the relative colour map of low elemental concentration. Other elements can be used to locate the nuclei of
cells. Potassium is known to be a major intracellular metal ion in living cells. It has previously been observed that potassium maps depict cellular boundaries, and that the nuclei of cells is distinguished by an intense region of potassium. The potassium map of the cell shown in Figure 3.4 reveals an area of higher potassium concentration, probably the nucleus of the cell, surrounded by a more diffuse region, most likely defining the cell boundary. Elemental maps of the other elements show high concentrations in this nuclear region. In particular, note the very high levels of platinum within this region and the good colocalisation of platinum and phosphorus maps. The results suggest that the platinum intercalator complex efficiently delivers platinum to the nucleus of the cell. It should be noted that, due to time restraints, only one Pt2C3 treated cell was scanned. This cell may not necessarily be representative of the entire cell population.

3.3.3.3 Confocal microscopy

The anthraquinone compounds used in the current study were designed to take the platinum moiety to the DNA rapidly as a result of the intercalative effect of the anthraquinone. The intrinsic fluorescence of the compounds allowed this hypothesis to be tested using confocal fluorescence microscopy, whereby their subcellular distribution was determined in DLD-1 and A2780 cells. In addition, this investigation may provide further information regarding the mechanism of action of the anthraquinone compounds. There was no discernible difference in the distribution of the compounds in the two cell lines and hence, for the most part, they will be discussed together.

3.3.3.3.1 Subcellular localisation of anthraquinone compounds

Pt1C3 and 1C3

The cellular distribution of 1C3 and Pt1C3 was found to be identical. Thus confocal images of 1C3 or Pt1C3 will be displayed herein (see figure labels). Figure 3.5 shows the dual staining results, obtained in the presence of Pt1C3 or 1C3 (incubated for 4-
5 hr) and the cell permeant nuclear stain SYTO21. Figure 3.5A and D show that the SYTO21 probe was confined to the nuclear compartment. In contrast, Figure 3.5B and E show that the anthraquinone compounds display a punctate distribution that, as indicated by the overlay in Figure 3.5C and F, is distinct from the nuclear region of the cells. It should be noted that shorter (<1 hour) and longer incubation times (>24 hours) revealed similar distribution patterns (results not shown). Hence the anthraquinone compounds do not localise in the nuclei of the cells.

**Figure 3.5:** Panels A – C are confocal images obtained from DLD-1 cells incubated in the presence of 1C3 or Pt1C3 for 4 – 5 hr, and the SYTO21 nuclear stain. Panels D – F are confocal images obtained from A2780 cells incubated in the presence of 1C3 for 4 – 5 hr, and the SYTO21 nuclear stain. Panels A and D display the localisation of the nuclear stain (the arrow in panel A indicates a typical cell nucleus); panels B and E display the localisation of 1C3 or Pt1C3, and panels C and F display the overlay of
the previous two panels. A yellow/orange colour in the latter overlay panels would be indicative of overlap and hence nuclear localisation.

The platinum complex may be degraded in the cellular environment. In such a situation, the fluorescent fragments observed in the confocal experiments may no longer contain platinum. This is consistent with the observed similarity in the confocal images obtained from cells treated with the anthraquinone ligand and its corresponding platinum complex. Further experiments (such as nuclear Pt DNA binding studies) would be required to ascertain whether the platinum moiety has been delivered to the nuclei of the cells.

Alternatively, fluorescence quenching in the presence of DNA may be occurring. DNA fluorescence titration experiments were performed to investigate this possibility for 1C3 and Pt1C3, see Appendix, Figures A1-A4 for results. The fluorescence intensity upon addition of DNA was found to be solvent dependent. Fluorescence quenching did not occur in the solvent systems containing only small volumes of DMF (and hence mimicking the confocal microscopy conditions) and hence is unable to account for the lack of fluorescence observed in the nuclei of the cells. This suggests that the platinum complex, or at least the fluorescent moiety being observed, does not enter the cell nuclei.

To identify the cellular organelles in which the compounds were accumulating, further double staining experiments were carried out. They may have been localising at mitochondrial DNA, rather than the expected nuclear DNA. Figure 3.6 shows the results of double staining experiments in the presence of a mitochondrial stain, MitoTracker Green, and 1C3 or Pt1C3 (4-5 hr incubation). The overlay panels, C and F, suggest that the compound and the probe are not present within the same regions of the cells. Hence the compounds do not localise in the mitochondria of the cells. Again, both longer and shorter incubation times produced similar results.
Figure 3.6: Panels A – C are confocal images obtained from DLD-1 cells incubated in the presence of 1C3 or Pt1C3 for 4 – 5 hr, and the MitoTracker Green mitochondrial stain. Panel D – F are confocal images obtained from A2780 cells incubated in the presence of 1C3 for 4 – 5 hr, and the MitoTracker Green mitochondrial stain. Panels A and D display the localisation of the mitochondrial stain (the arrow in panel A indicates some typical mitochondria), panels B and E display the localisation of 1C3 or Pt1C3, and panels C and F display the overlay of the previous two panels. A yellow/orange colour in the latter overlay panels would be indicative of overlap and hence mitochondrial localisation.

The punctate cellular accumulation patterns displayed by 1C3 and Pt1C3 may suggest uptake into the lysosomal compartments of the cells. Figure 3.7 shows the double staining experiments performed following 4-5 hr incubation of 1C3 or Pt1C3 with a lysosomal probe, LysoTracker Green. 3.7A and D show the punctate staining
pattern of the LysoTracker Green probe. Inspection of the overlay panels (Figure 3.7C and F) reveal colocalisation of the lysosome stain and the compound, as indicated by regions shown in yellow/orange. Both longer and shorter incubation times also produced similar results. Hence the compounds appear to be rapidly accumulating in the lysosomes of the cells.

Figure 3.7: Panels A – C are confocal images obtained from DLD-1 cells incubated in the presence of 1C3 or Pt1C3 for 4 – 5 hr, and the LysoTracker Green lysosomal stain. Panel D – F are confocal images obtained from A2780 cells incubated in the presence of Pt1C3 for 4 – 5 hr, and the LysoTracker Green lysosomal stain. Panels A and D display the localisation of the lysosomal stain (the arrow in panel A indicates some typical lysosomes), panels B and E display the localisation of 1C3 or Pt1C3, and panels C and F display the overlay of the previous two panels. A yellow/orange colour in the latter overlay panels would be indicative of overlap and hence lysosomal localisation.
It has been observed previously that the position at which the anthraquinone ring is
substituted significantly affects the activity of the platinum complex, with 1-
substituted complexes displaying better activity than the analogous 2-substituted
complexes.\textsuperscript{80, 94} To ascertain whether the substitution pattern on the anthraquinone
affects the cellular distribution of the compounds, 2-substituted anthraquinones,
2C3 and Pt2C3, were investigated using confocal microscopy.

Figure 3.8 shows the cellular distribution of 2C3 and Pt2C3 in A2780 cells following
4-5 hr incubation. Longer and shorter incubations were also performed, with similar
results. The compounds produce a predominantly punctate pattern of accumulation
within the cells, reminiscent of that displayed by 1C3 and Pt1C3. Also, in a spatial
sense, the regions of accumulation resemble the lysosomes of the cells. Note that
double staining experiments would be required to confirm this observation. However,
it is clear that these compounds do not enter the nuclei of the cells. In conclusion,
altering the substitution pattern of the anthraquinones does not significantly affect
their cellular distribution.
Doxorubicin

The distribution of doxorubicin in A2780 and DLD-1 cells was investigated in order to ascertain whether the lack of nuclear localisation observed in cells treated with 1C3, 2C3, Pt1C3 and Pt2C3 was a general feature of anthraquinones in these cell lines. Figure 3.9 indicates that doxorubicin localised almost exclusively to the nuclear compartment of DLD-1 cells. The equivalent images of A2780 cells incubated with doxorubicin and the nuclear stain are not shown, as the distribution pattern of doxorubicin was altered in the presence of the stain. Confocal images of doxorubicin on its own in A2780 cells closely resemble that shown in Figure 3.9B. Doxorubicin does not enter the mitochondria of DLD-1 or A2780 cells (Figure 3.10), although a small amount was seen in the lysosomes of the cells (Figure 3.11).
**Figure 3.9:** Confocal images obtained from DLD-1 cells incubated in the presence of doxorubicin for 4 – 5 hr, and the SYTO21 nuclear stain. Panel A displays the localisation of the nuclear stain, panel B displays the localisation of doxorubicin, and panel C displays the overlay of the previous two panels. The yellow/orange colour in the latter overlay panel is indicative of overlap and hence nuclear localisation.
Figure 3.10: Panels A – C are confocal images obtained from DLD-1 cells incubated in the presence of doxorubicin for 4 – 5 hr, and the MitoTracker Green mitochondrial stain. Panel D – F are confocal images obtained from A2780 cells incubated in the presence of doxorubicin for 4 – 5 hr, and the MitoTracker Green mitochondrial stain. Panels A and D display the localisation of the mitochondrial stain, panels B and E display the localisation of doxorubicin, and panels C and F display the overlay of the previous two panels.
Figure 3.11: Panels A – C are confocal images obtained from DLD-1 cells incubated in the presence of doxorubicin for 4 – 5 hr, and the LysoTracker Green lysosomal stain. Panel D – F are confocal images obtained from A2780 cells incubated in the presence of doxorubicin for 4 – 5 hr, and the LysoTracker Green lysosomal stain. Panels A and D display the localisation of the lysosomal stain, panels B and E display the localisation of doxorubicin, and panels C and F display the overlay of the previous two panels.

The cellular distribution of doxorubicin observed in the current study is consistent with previous literature reports. Doxorubicin is known to intercalate tightly into DNA, with previous studies showing a predominantly nuclear localisation of the drug in a number of drug sensitive cell lines. Cytoplasmic organelles play a major role in the intracellular distribution of doxorubicin and daunorubicin in multidrug resistant cell lines, effectively protecting the cells from the cytotoxic effects of the drugs.
3.3.3.2 Cellular processing of anthraquinone drugs

Reedijk et al have studied platinum anthraquinone complexes (closely related to those used herein, although with 1,4-substitution on the anthraquinone skeleton, and two platinum centres attached to each of the side chains).\textsuperscript{98-100} (also see Section 1.8 and Figure 1.17). In the parent A2780 ovarian cell line, the platinum complexes are more potent than cisplatin. The free ligands enter the cells more rapidly than the corresponding platinum complexes and seem to be processed differently by the cells. After 24 hr incubation with ligand, fluorescence was seen in the cytosol around the nucleus and the nucleoli within the nucleus, whereas after 24 hr incubation with platinum complexes, fluorescence localised in the lysosomes. The platinum complexes were shown to bind to DNA to a greater extent than cisplatin and the fluorescence of the complexes was significantly quenched in the presence of DNA. This suggests that the complexes entered the cell nuclei, in spite of the lack of fluorescence observed in this region.\textsuperscript{99}

In cisplatin resistant A2780 cells (A2780cisR), the cellular distribution pattern of the free anthraquinone ligands used by Reedijk et al was identical to that in the parent cells. However, the platinum complexes distribute differently in these cells, with localisation in the lysosomes almost immediately after incubation, and up to 24 hr later. This is consistent with the observed cross-resistance of the platinum complexes with cisplatin in the A2780cisR cell line, and provides a possible explanation for the lower activity of the platinum complexes in the resistant cell line compared to the sensitive cell line. The authors propose lysosomal encapsulation of platinum complexes as a resistance mechanism of the A2780cisR cell line, suggesting that the cells develop a greater number of lysosomal compartments and larger amounts of lysosomal enzymes. However, this resistance mechanism appears to be specific towards the platinum complexes. In addition, the platinum complexes were shown to be susceptible to glutathione deactivation in the A2780cisR cell line, although this appears to operate independently from the mechanism used by the cells to accumulate the drugs into lysosomes.\textsuperscript{98}
Studies of these dinuclear platinum anthraquinone complexes have also been performed in the U2-OS human osteosarcoma cell line, and its cisplatin resistant phenotype (U2-OS/Pt). Cellular processing and activity of the complexes and the free ligands was found to be similar in the two cell lines. The authors propose that the platinum complexes are capable of overcoming cisplatin resistance in the U2-OS/Pt cell line by escaping the DNA recognition and repair systems of the cells due to the formation of structurally unique DNA adducts (largely due to the presence of the intercalator). The platinum complexes and the ligands show rapid nuclear localisation, with the platinum complexes subsequently excreted from the cells by way of the Golgi apparatus, and the ligands excreted by the Golgi and the lysosomes. The authors propose that the platinum complexes are most likely protein bound when they enter the Golgi complex and are probably exocytosed by the mechanism used to transport secretory proteins from the Golgi to the external cell surface. On the other hand, the free ligands are unlikely to be covalently bound and are thus easily taken up by the lysosomes as a result of their basicity. Upon entry into the acidic lysosomes, they readily become protonated and are unlikely to diffuse back into the cytoplasm. It was also suggested that sequestration of the drugs into lysosomes is directly related to the pH of the lysosomes of a particular cell line. An unsuitable pH disrupts normal endocytosis, and may facilitate accumulation of drugs into the lysosomes. Hence the alkalinised lysosomes of A2780cisR cells relative to A2780 cells may be responsible for accumulation of the platinum complexes into the lysosomes of the resistant cell line. In contrast, U2-OS/Pt did not display enhanced lysosomal pH relative to the parent cell line, consistent with the observed lack of lysosomal accumulation of the platinum complexes in these cell lines.

In the current study, the free ligands (1C3 and 2C3) and platinum complexes (Pt1C3 and Pt2C3) showed similar cellular distribution and processing in the two parent cell lines studied (including the A2780 ovarian cancer cell line in common with that studied by Reedijk). All compounds appear to be rapidly sequestered by the lysosomes and excluded from entry into the nuclei of the cells, in contrast to the work of Reedijk, outlined above. The essential role played by the lysosomes in the processing of these compounds will be further discussed below.
Lysosomes are subcellular organelles responsible for the controlled intracellular digestion of macromolecules. Hydrolytic enzymes (acid hydrolases) are contained within the lysosomal membrane and the pH within the structures is maintained at approximately 5 for optimal enzyme activity. Lysosomes are also involved in the response of cells to drugs such as daunorubicin and doxorubicin. In particular, encapsulation of the drugs into acidic organelles has been implicated in cellular resistance. Drugs such as doxorubicin, daunorubicin, vincristine, vinblastine, and mitoxantrone are believed to become sequestered in these vesicles as a direct result of their weakly basic, lipophilic nature. These drugs have pKₐ values between approximately 7.4 and 8.4 and thus the majority of the drug molecules will be neutral at normal intracellular pH. Accordingly, they are able to freely penetrate the lipid bilayers of cytoplasmic organelles and vesicles. Upon entry into acidic compartments, such as the lysosomes, where the pH is substantially lower (pH 4-5), the drugs become protonated and thus less able to exit the organelle. A pH dependent equilibrium exists between the protonated and non-protonated forms of the drug. Resistant cancer cells tend to have a large pH difference between the cytoplasm and the lysosomes, and hence accumulation of drug into acidic compartments is favoured.

Lysosomes are known to be involved in the response of cells to platinum complexes. Previous studies have shown that platinum treatment may result in encapsulation of platinum within lysosomal compartments, a significant increase in the number of lysosomes, morphological changes in the lysosomes, and increases in the activity of lysosomal enzymes such as β-glucuronidase and acid phosphatase. In addition, there appears to be a connection between cellular resistance to cisplatin, and the lysosomes. Platinum sequestration into lysosomal and other acidic compartments has been proposed as a mechanism adopted by cells to extrude toxic compounds. The lysosomes also seem to play a role in regulating the sensitivity of cells towards cisplatin, and defects in the lysosomal function of cisplatin resistant cells have been observed. Alternatively, the
platinum complex may bind to proteins and subsequently be transported to the lysosomes for degradation\textsuperscript{100, 152}.

The anthraquinone ligands studied herein (1C3 and 2C3) are weakly basic and possess a protonatable primary amine group. As such, the mechanism by which they are sequestered into lysosomes is likely to be similar to that outlined above for doxorubicin and daunorubicin, whereby the drugs readily diffuse into acidic compartments, become protonated and effectively trapped within the vesicles\textsuperscript{175, 177, 179}. Given that parental cell lines (A2780 and DLD-1) were used in this study, significant pH gradients between the cytoplasm and lysosomes are less likely to exist, but can not be ruled out. The fact that the anthraquinone ligands studied by Reedijk and coworkers displayed no lysosomal sequestration in A2780 cells\textsuperscript{99} suggests that 1C3 and 2C3 possess structural features that are efficiently recognised by the cells and result in their lysosomal entrapment. It should also be noted that, contrary to the suggestions of Reedijk\textsuperscript{98}, these results indicate that lysosomal sequestration is not a cellular response specific towards platinum complexes.

The platinum anthraquinone complexes studied herein (Pt1C3 and Pt2C3) may be degraded in the cellular environment, rendering them subject to a similar lysosomal sequestration mechanism to that outlined above for weakly basic drugs. This is consistent with the observed similarity of the confocal images obtained from cells treated with the anthraquinone ligands and their corresponding platinum complexes. Alternatively, the platinum complexes may bind to proteins and subsequently enter the lysosomes for degradation. A similar mechanism has been suggested to account for the lysosomal sequestration of cisplatin analogues\textsuperscript{100, 152}. Again, the different processing of these platinum complexes compared to those used by Reedijk and coworkers in parental A2780 cells\textsuperscript{99} suggests that Pt1C3 and Pt2C3 possess structural features that enhance the process of lysosomal encapsulation.
3.4 Combined discussion

The cellular uptake of cisplatin is known to limit the effectiveness of this important chemotherapeutic agent, with decreased accumulation being implicated in cellular resistance to the drug.\textsuperscript{13-16} In an effort to enhance cellular accumulation and nuclear localisation of platinum, platinum anthraquinone complexes have been investigated. The intercalating properties of the anthraquinone group may potentially endow the platinum complex with the ability to generate novel DNA adducts, resulting in favourable cytotoxicity and evasion of DNA repair processes. In the following discussion, it will be assumed that the compounds studied herein behave similarly in DLD-1 and A2780 cells, as has been shown to be the case for their cellular distribution, determined by confocal microscopy.

The cytotoxicity of Pt1C3 was determined in DLD-1 cells. For the short incubation times investigated, Pt1C3 displayed potency higher than cisplatin, though similar to 1C3. Further examination, using confocal microscopy, indicated that Pt1C3 and 1C3, as well as their related counterparts, Pt2C3 and 2C3, were rapidly and efficiently sequestered into the lysosomal compartment of the cells. In fact, the platinum cellular uptake of these complexes was considerably higher than that of cisplatin. It has not been determined whether the platinum complexes remain intact in the cellular environment, and hence the entry of a degraded platinum species into the nuclei of the cells, and subsequent platinum DNA binding can not be ruled out. In fact, SRIXE imaging of A2780 cells treated with Pt2C3 indicate that large quantities of platinum efficiently enter the nuclei of the cells, suggesting degradation of the complexes, and encapsulation of the resulting fluorescent moiety into lysosomal compartments. Alternatively, the SRIXE images may be consistent with binding and fluorescence quenching of the platinum anthraquinone complex with a protein that is subsequently transported into the cell nuclei. Such proposed mechanisms may explain the higher cytotoxicity of the platinum anthraquinone complex relative to cisplatin, although they do not account for the equivalent potency of 1C3.
On the other hand, if the complexes remain intact within the cells, the similar cytotoxicity profiles and cellular distribution patterns of Pt1C3 and 1C3 suggest a common mechanism of drug toxicity. Lysosomal disruption as a mechanism of toxicity has been observed previously for platinum drugs.\textsuperscript{193, 194} It has been proposed that sequestration of drugs into lysosomes causes damage of the vesicles, leaking hydrolytic enzymes into the cytosol and subsequently leading to cell death.\textsuperscript{187} One study has suggested that the inhibition of lysosomal function caused by cisplatin may contribute to its nephrotoxicity.\textsuperscript{193} Whilst the current study suggests that the mechanism of lysosomal damage results in substantial cytotoxicity, were the complexes to have reached their expected DNA targets, greater cytotoxicity is likely to have ensued. Hence lysosomal trapping acts as a detoxification mechanism that may limit their effectiveness as anticancer agents, as discussed above in Section 3.3.3.3.2. In contrast, doxorubicin displayed cellular accumulation largely in the nuclear region of the cells, and a correspondingly high cytotoxic efficacy.

3.5 Conclusions

The platinum anthraquinone complexes studied herein are rapidly and efficiently taken up by cells. Upon entry, the complexes and their corresponding ligands are encapsulated by lysosomal cellular compartments and cause cytotoxicity to the cells by lysosomal disruption. Alternatively, the complexes are rapidly degraded in the cellular environment, whereby the resulting fluorescent moiety is sequestered by the lysosomes, and the remaining platinum moiety enters the nuclei, interacts with DNA, and produces DNA damage and subsequent apoptosis. An additional possibility is that some of the platinum anthraquinone complex binds to proteins, resulting in quenching of its fluorescence, and allowing the complex to be transported into the nuclei of the cells. Attachment of an anthraquinone intercalating moiety to a platinum complex has been shown to result in efficient cell uptake. However, the DNA targeting efficacy of the complexes appears to be highly dependent upon their structure. Further work is required to elucidate desirable structural features for incorporation into future drugs.
CHAPTER 4

Distribution of Platinum Complexes in Multicellular Tumour Spheroids
4.1 Introduction

4.1.1 The multicellular tumour spheroid model

Stabilised tumour cell lines grown in monolayer culture are commonly used to assess drug potency.\textsuperscript{195} Whilst this cell model has been useful, drugs often perform quite differently in \textit{in vivo} screening\textsuperscript{196, 197} suggesting that conventional monolayer \textit{in vitro} screening does not realistically reflect the potency of drugs in the clinic.\textsuperscript{160, 195, 196, 198-201} A more realistic model of the solid tumour environment is provided by multicellular tumour spheroids (see Figure 4.1). These were first used by Sutherland and associates in the early 1970s\textsuperscript{202-204} and provide a model intermediate in complexity between \textit{in vitro} monolayers and \textit{in vivo} tumours.\textsuperscript{195, 199, 205-207} Spheroids are characterised by well-defined geometrical arrangements, can be formed reproducibility in a controlled culture environment, are easy to control and manipulate, and are devoid of factors inherent to the host.\textsuperscript{205, 208-211}
Figure 4.1: Light optical image of a 5 µm section of a multicellular tumour spheroid consisting of DLD-1 cells and stained with Haematoxylin and Eosin. Outer regions show high cell density, whilst the central region consists of a necrotic core due to hypoxia.

The widespread use of spheroids to investigate drug action is largely due to their realistic mimicry of the abnormal microenvironments that develop in solid tumours as a result of inefficient vascularisation. Spheroids resemble such tumour nodules in their three-dimensional compact cellular organisation (cell-cell and cell-matrix interactions); growth dynamics (early exponential growth followed by a period of retarded growth); gradients of cell proliferation (development of necrotic areas in the core of the spheroids directly surrounded by quiescent cells and then proliferating cells on the exterior of the spheroid); and gradients of nutrients due to interstitial pressure and a lack of vascularisation (cells deprived of glucose, oxygen, metabolites, protons, hormones, growth factors and other substrates, possibly causing hypoxic spheroid cores).196, 198-200, 205, 210-213
Whilst spheroids provide a realistic model of solid tumours, it should be acknowledged that there are limitations to their usefulness. Certain investigations may warrant the use of *in vivo* systems, whereas others may require the simplicity of single cell systems. A further limitation is that spheroids are not able to be formed from some cell types.

4.1.2 Penetration and distribution of substances into tumour spheroids and tumour tissue

4.1.2.1 Tumour spheroids

A lack of penetration of cytotoxic drugs into tumours has been recognised as a significant issue for cancer chemotherapy. The spheroid model has been extensively utilised to study drug penetration qualitatively and semi-quantitatively. The rate and extent of drug penetration varies significantly between drugs, and a number of factors contribute to the variable penetration observed. Such factors are dependent on both the nature of the drug and the nature of the spheroids. It has been suggested that the size of the drug molecule, its lipophilicity, charge, polarity, pKₐ and reactivity are important determinants. Significant uptake and accumulation of drugs in the peripheral cell layers of spheroids is thought to limit the quantity of drug that is able to penetrate to deeper cell layers. On the other hand, substances that do not undergo significant binding tend to show rapid and efficient penetration. Properties of the spheroid, such as cell-cell interactions, intraspheroidal pH gradients, the amount and composition of the extracellular matrix surrounding the cells, or the characteristics of individual cells may also influence the extent of drug penetration into spheroids; a property collectively known as multicellular resistance.

Efficient spheroid penetration has been observed for many compounds, including cisplatin, carboplatin, tetraplatin, carmustine, bleomycin, fluorouracil, chlorambucil, mitomycin, and lomustine.
Nederman et al\textsuperscript{220} investigated spheroidal drug distributions of radiolabelled 5-fluorouracil and vinblastine using autoradiography. They found that 5-fluorouracil was able to penetrate the entire volume of glioma and thyroid cancer spheroids within a few minutes. In contrast, vinblastine penetrated the spheroids much less efficiently, showing predominantly peripheral accumulation, and requiring approximately 2 hours to penetrate efficiently through the spheroid. It has been suggested that the slower penetration of vinblastine may be due to its higher molecular weight.

In a separate study, 5-fluorouracil spheroidal penetration was determined indirectly. The study utilised a non-toxic fluorescent dye, Hoechst 33342, which is known to penetrate slowly and bind rapidly. Following exposure to the dye, spheroids were trypsinised to cause disaggregation to a single cell suspension. Cells were then sorted depending on their fluorescence intensities, and a drug response profile through the spheroid reconstructed. 5-fluorouracil was found to be toxic throughout the entire spheroid volume, consistent with penetration to the central region of the spheroids.\textsuperscript{221, 222}

The penetration of the anthracyclines, particularly doxorubicin (adriamycin), has been studied extensively. Doxorubicin is an inherently fluorescent molecule and as such its distribution in spheroids has often been studied using techniques such as fluorescence microscopy,\textsuperscript{219, 223} confocal microscopy\textsuperscript{224-226} and flow cytometry\textsuperscript{227, 228}. Doxorubicin was found predominantly in outer layers of the spheroids,\textsuperscript{215, 219, 221, 223, 224, 227} though this was less pronounced in smaller spheroids.\textsuperscript{225, 226} It has been suggested that this distribution pattern indicated slow drug penetration\textsuperscript{200} or rapid binding of drug to the outer cell layers. This may be due to its high affinity for biomolecules, thus shielding the inner cells from significant exposure to the compound,\textsuperscript{213, 217, 219, 229} or perhaps even preventing it.\textsuperscript{218} Researchers initially found it difficult to rationalise this observation with the known clinical efficacy of doxorubicin,\textsuperscript{229} but later studies have shown that doxorubicin is stored in cells and leaks from the cells after they die. Hence the drug slowly diffuses through the spheroid, resulting in delayed toxicity to more central cell layers.\textsuperscript{205, 213, 224, 229}
Platinum complexes have also been studied in spheroids. It is generally accepted that cisplatin and carboplatin are able to penetrate efficiently into the core of spheroids.\textsuperscript{227, 230} In the absence of a position at which a fluorescent or radioactive label can be readily attached, such conclusions have often been based upon indirect measurements considered to be consistent with thorough penetration of the complexes. For example, the potent cell killing effects of cisplatin in spheroids is assumed to be the result of penetration throughout the entire spheroid volume.\textsuperscript{227, 230} In contrast, using the Hoechst 33342 cell sorting technique (described above), cisplatin was found to have a more toxic effect on the outer leaflet of cells than internal cells, suggesting a drug concentration gradient into the spheroid.\textsuperscript{221} In a later study, radiolabelled tetraplatin was investigated using the cell sorting technique. It should be noted that drug retention, rather than drug exposure was measured in these studies, that is, intracellular drug that was able to withstand washings, trypsinisation, holding for assays, and mechanical shock during the cell sorting procedure. Simultaneous measurements of intracellular drug levels and toxicity at different depths demonstrated that at low concentrations, retention of the drug was greater in peripheral cells. However, at higher doses, drug retention was more uniform throughout the volume of the spheroids. At all doses, the drug was more toxic to the outermost cells than the inner cells. It was suggested that the cellular microenvironment of the spheroids plays a role in modulating drug toxicity.\textsuperscript{231}

A more recent study has investigated the behaviour of a novel porphyrin-platinum complex in spheroids. The native fluorescent properties of this complex were utilised to show a time dependent penetration into the spheroids, with fluorescence distributed throughout the entire spheroid after a 24 hour incubation.\textsuperscript{232}

\textbf{4.1.2.2 Tumour tissue}

Similar studies have been performed in tumours.\textsuperscript{233-236} Los \textit{et al}\textsuperscript{235, 236} studied the effects of cisplatin and carboplatin in rat tumours. Proton induced X-ray emission (PIXE) was used to quantitatively determine the distribution and concentration of platinum within the tumours. Cryostat sections (40 \textmu m thick) of tumour were excited
with a proton beam, the spectra of emitted X-rays collected, and the platinum content quantified using the Pt-L\(\beta\) line. They found that the highest platinum concentrations were at the periphery of cisplatin treated tumours, whereas carboplatin treated tumours displayed a more homogeneous platinum distribution, though at much lower concentrations than seen in their cisplatin treated counterparts. The distribution of platinum in organs such as kidney, liver, and brain have also been determined in both human\(^{237-239}\) and animal subjects\(^{240}\) treated with cisplatin.

The relationship between penetration and binding of substances in spheroid or tumour tissue has not been firmly established. Binding in the peripheral regions of a spheroid may be mistaken for poor drug penetration, even though the drug may actually be penetrating to inner regions but only binding significantly in outer layers.\(^{195, 218}\) The results may be dependent upon the sample preparation procedure; conventional histological procedures have been shown to wash away the majority of the test substance, probably leaving only that which was covalently bound to cells or extracellular matrix.\(^{195, 218, 220}\) An alternative ‘dry’ histological procedure has been developed to ensure that the bound and unbound fractions of the drug are preserved and detectable.\(^{195, 241}\) The method involves rapid freezing of the spheroids in liquid propane-propene mixture, followed by freeze drying, vapour fixation in a chamber saturated with paraformic aldehyde, wax embedding using xylene, and dry sectioning.

There are a variety of techniques available to study distributions of fluorescent drugs, with early studies often involving fluorescent microscopy of cryosections. Prior to embedding and sectioning, the spheroids are removed from the drug-containing medium and either fixed,\(^{223}\) washed briefly,\(^{227}\) or embedded immediately with no washing.\(^{219}\) This may result in wash out of unbound cytoplasmic fluorescence or penetration of the fluorescent compound into cellular compartments that were previously inaccessible.\(^{225}\) The ‘dry’ histological procedure outlined above can also be used, though it is time consuming and requires the use of dead tissue. Confocal microscopy provides a means by which live tissue can be examined in a noninvasive fashion. Drug distribution throughout the spheroid can be analysed in optical
sections rather than physical sections, providing a realistic picture with few artefacts originating from the sample preparation procedure.

### 4.1.2.3 Synchrotron techniques

The above discussion highlighted the means by which penetration and distribution of substances in spheroids or tumours have been studied previously, namely, fluorescence microscopy, radiolabelling/autoradiography, cell killing as a function of depth into a spheroid, fluorescence activated cell sorting (using Hoechst 33342) and proton induced X-ray emission (PIXE). Whilst these techniques are useful, there are disadvantages associated with their use. Drug concentrations are difficult to quantify using fluorescence microscopy or autoradiography, and it is difficult to ascertain whether drug metabolites or the parent drug are being imaged.\(^{195, 200, 235}\) Cell killing and sorting techniques provide only indirect evidence of drug penetration.\(^{200}\) On the other hand, the PIXE technique allows for quantitative determination of platinum and other elements. Whilst the parent platinum drug is likely to be metabolised in the tumour environment, the platinum moiety is known to be the biologically active part of most platinum drugs. Thus monitoring the distribution of platinum provides useful information.

The techniques available to probe the internal elemental distribution of a sample in the micrometre/sub-micrometre scale are limited. They include X-ray fluorescence tomography (XRFT), energy filtered transmission electron microscopy (EFTEM) tomography, focused ion beam (FIB) tomography, scanning transmission ion microscopy (STIM), and PIXE tomography. Many of these tomographic methods are destructive (EFTEM, FIB, PIXE), are limited by the size of the objects (EFTEM), or require prior knowledge of the samples (PIXE).\(^{242}\)

X-ray fluorescence tomography (XRFT) involves detection of photons at energies characteristic for each chemical element within a sample following excitation of the sample with a high flux of X-rays (usually produced by a synchrotron). The large penetration depth of the hard X-rays allows information concerning the internal
spatial distribution of elements to be obtained without the need for destructive sample preparation methods such as sectioning.\textsuperscript{243-245} It also takes advantage of the high sensitivity and resolution obtainable using the synchrotron radiation induced X-ray emission (SRIXE) technique. However, its use is restricted to samples within a certain size range and matrix composition, and the range of elements that can be analysed is limited. Such restrictions stem from the fact that the fluorescent X-rays must be sufficiently energetic to emerge from the sample without undergoing significant absorption during their exit journey.\textsuperscript{242}

During a typical XRFT experiment, the sample is translated through the X-ray beam, brought back to its starting position, rotated, and then the translation repeated. Fluorescence is acquired by a detector placed at $90^0$ to the direction of the incident beam. X-ray spectra are collected at each point in the scan and after a complete rotation ($180^0$), a two dimensional image of the elemental distribution in a virtual slice through the sample can be obtained. Repetition of the procedure along the rotation axis, with the separation between slices equal to the transverse size of the beam, can provide a complete three dimensional elemental image of the entire sample, given sufficient time.\textsuperscript{242-245} Figure 4.2 shows the setup used for the tomography experiments outlined in this chapter.
Recently, X-ray fluorescence microtomography (XRFT with resolution in the micrometre range) has been used to study the internal elemental distributions of a variety of samples. For example, the distribution of arsenic and iron in common wetland plant roots has been studied. Arsenic and iron were found to be concentrated on the exterior of the roots and there was little arsenic present in the interior. This suggested that such wetland plants are capable of sequestering arsenic from their surroundings and may have a significant role to play in the cycling of arsenic in contaminated environments.$^{246}$

In a separate study, X-ray fluorescence microtomography was used to investigate the distribution of cadmium in single fly ash particles. The concentration of cadmium and other heavy trace elements was found to be highest in the interior of the particles. As such, it was suggested that cadmium would be unable to leach out of the particles.
easily and thus, it was proposed that the environmental impact of ash disposal would be minimal.247

X-ray fluorescence microtomography provides an ideal technique with which to study platinum distributions in tumour spheroids. Destructive sample preparation techniques can be avoided, as the spheroid sample is analysed whole without the need for sectioning. The biologically active part of the drug, the platinum moiety, is imaged directly and several elements can be analysed simultaneously, thus providing additional information about the sample. In addition, spheroid size can be optimised for an individual experimental set up, such that absorption effects and data collection times are minimised, and elements of interest are easily detected.

Tumour spheroids have been studied previously using X-ray fluorescence microtomography.248 Zinc and copper were found to be homogeneously distributed throughout the spheroid tissue, regardless of the immunotoxin with which they had been treated. However, spheroid samples were mounted inside quartz capillaries in polyacrylamide gel for analysis and as such, contaminants present in the capillary presented difficulties in elemental detection.

Jonson et al have developed an X-ray fluorescence technique whereby platinum concentrations in human patients can be determined in vivo.237, 249 A modified X-ray therapy tube was used to produce the photons required to ionise platinum atoms, and the characteristic Pt Kα1 and Kα2 X-rays were then detected. This has been used to quantify platinum concentrations in kidneys and brain tumours of patients treated with cisplatin. The maximum concentration of platinum in brain tumours was reached approximately 5-15 hr following intravenous injection, and the maximum uptake varied between 14-40 µg/g. The maximum uptake into kidneys occurred 3-4 hr after intravenous cisplatin injection.

Platinum distribution in spheroid samples can also be analysed by the closely related technique known as SRIXE. This technique has been outlined in Chapter 3 and will
also be used herein. In contrast to microtomography, sectioned samples are required to study spheroids using SRIXE, and as such, sample preparation may be somewhat destructive in nature and may cause alterations in element distributions. Accordingly, the results of micro-SRIXE and microtomography experiments will be compared to one another and to other independent studies to gain a consensus concerning the distribution of platinum in spheroids.

Liu et al used SRIXE to investigate the distribution of platinum in the liver of normal mice that had been treated with cisplatin.240 Using a beam size of 150 x 150 µm², and a 200 µm thick liver slice, they found that platinum concentration was distributed non-homogeneously throughout the liver tissue.

This chapter presents the results of a study of the platinum distribution in multicellular tumour spheroids treated with cisplatin and three platinum(IV) drugs. In particular, we aimed to ascertain any differences in the ability of such drugs to penetrate and bind within the central regions of spheroids. Given the similarities between the tumour spheroid model and solid tumours nodules in vivo, such a study may provide a useful indication of the behaviour of these platinum drugs in tumour systems. The study utilised the synchrotron techniques of X-ray fluorescence microtomography, micro-SRIXE and micro-XANES.

4.2 Methods

4.2.1 Complexes examined

The following complexes were examined: cis-[PtCl₂(NH₃)₂], cis-[PtCl₄(NH₃)₂], cis,trans,cis-[PtCl₂(OAc)₂(NH₃)₂], cis,trans,cis-[PtCl₂(OH)₂(NH₃)₂], cis,trans-[PtCl₂(OH)₂¹⁴C-en], cis,trans-[PtCl₂(OH)₂¹⁴C-en]. Each complex was stored at -20 °C as a 0.5 mM stock solution in 100 mM KCl.
CHAPTER 4: Distribution of Platinum Complexes in Multicellular Tumour Spheroids

4.2.2 Tumour spheroid production

DLD-1 human colon carcinoma cells were obtained from Dr Roger Phillips (University of Bradford, UK), maintained as monolayers in RPMI-1640 medium with Glutamax, 25 mM HEPES and supplemented with 10% (v/v) fetal calf serum, penicillin (100 IU/mL) and streptomycin (100 mg/mL) (supplements were obtained from Invitrogen, Paisley, UK).

Tumour spheroids of DLD-1 cells were grown by seeding approximately $2 \times 10^7$ cells into 150 mL of RPMI-1640 medium in a 250 mL spinner flask (Techne Ltd., Cambridge, UK). The flask was placed in an incubator (37 °C, 5 % CO$_2$) and spun. Growth was assessed by measurement of the diameter of spheroid samples using a calibrated eye-piece graticule (100 points per 10 mm, Pyser-SGI, UK) of an inverted phase contrast microscope. Tumour spheroids were cultured until they were approximately 400 μm in diameter (typically 2 days).

4.2.3 Treatment of tumour spheroids with platinum complexes

96-well culture plates were base-coated with 100 μL of 0.78 % (w/v) agarose in RPMI-1640 medium (without fetal calf serum). After the agarose set, an additional 100 μL of RPMI-1640 medium, supplemented as above, was added to each well. One spheroid was then added to each well. Platinum complexes were diluted in cell culture medium and 100 μL added to each well, giving the desired final concentration of 50 μM or 200 μM.

Subsequent treatment methods differed depending upon the analysis technique, and will be outlined in the appropriate section below.
4.2.4 Synchrotron techniques of spheroid analysis

Spheroids were analysed by a number of synchrotron techniques, as outlined in the subsequent sections below, and summarised in Table 4.1.

**Table 4.1:** Summary of some experimental details of the synchrotron techniques used to study platinum treated spheroids at the Advanced Photon Source (APS) and the European Synchrotron Radiation Facility (ESRF).

<table>
<thead>
<tr>
<th>Synchrotron</th>
<th>APS</th>
<th>ESRF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beam line</td>
<td>13-ID-C</td>
<td>2-ID-D, 2-ID-E</td>
</tr>
<tr>
<td>Technique</td>
<td>µ-tomography</td>
<td>µ-SRIXE</td>
</tr>
<tr>
<td>Spheroid samples</td>
<td>whole; formalin fixed</td>
<td>sectioned; cryo embedded or formalin fixed/paraffin embedded</td>
</tr>
<tr>
<td>Platinum complexes</td>
<td>cis, Cl4 (50µM, 24hr), OAc, OH (200µM, 24hr), control</td>
<td>cis, OH (50µM, 24hr), control</td>
</tr>
<tr>
<td>Scan size</td>
<td>whole sample, up to 400µm across</td>
<td>25 x 25 or 30 x 30µm (ie. 625µm² or 900µm²)</td>
</tr>
</tbody>
</table>

4.2.4.1 **Micro-Tomography**

Following 8 or 24 hr incubations with the platinum complexes (cis-[PtCl₂(NH₃)₂], cis-[PtCl₄(NH₃)₂], cis,trans-[PtCl₂(OAc)₂(NH₃)₂], cis,trans-[PtCl₂(OH)₂(NH₃)₂]), growth medium was removed and spheroids were washed with 10 mL of cold PBS.
The spheroids were fixed and stored in neutral buffered formalin fixative (BDH, UK) until analysis.

Experiments were performed on beamline 13-ID-C at the Advanced Photon Source (APS), Argonne National Laboratories, Argonne, Illinois, USA. Whole spheroids were analysed using a 13.45 keV monochromatic X-ray beam focussed to a 4 - 5 µm spot. Fluorescence was detected using a Vortex EX silicon drift energy dispersive detector (SII NanoTechnology Inc., formerly Radiant Detector Technologies, Inc.). The elements analysed were Pt, Zn, Cu, Fe, Ni, detected using the Kα fluorescence lines for each element except for platinum where the Pt-Lβ line was used to avoid the interference of Pt-Lα by Zn-Kβ. In cases where the platinum concentrations were low, the Pt-Lα peak was used and a correction for the Zn-Kβ contribution was determined using the observed Zn-Kα/Zn-Kβ ratio for a spectrum with negligible platinum.

Individual spheroids were removed from formalin immediately prior to analysis. Epoxy resin (Fast Set Epoxy, MG Chemicals) was used to attach the spheroid to the end of a glass fibre (attached to a wooden applicator stick). The wooden applicator stick was then mounted into a goniometer head and aligned to the sample stage rotation axis using the manual x-y goniometer head screws. The experimental setup has been shown above in Figure 4.2. Sample alignment was assessed by performing line scans at 0°, 90°, and 180° and determining whether the scans approximately overlaid one another. The scan z range was chosen such that the full range of the three scans was covered. The angle range of each scan was 0 – 180°. Each step was typically 1 or 2°, 0.5 or 1 second per step and 4 or 5 µm step size.

Data was acquired on two separate occasions. There was some overlap between the sample types analysed on these occasions, enabling comparison of the results obtained from both. At least two spheroids of each type of treatment were analysed.

The “translation/rotation” data collection scheme resulted in 2D “sinograms” (intensity on a “position-angle” plot) for each element. 2D element images were then
computationally reconstructed using fast Fourier transform based Gridrec software developed by Brookhaven National Laboratory,\textsuperscript{250} which was controlled by the IDL programming language (Research Systems, Inc.) yielding images of the cross-sectional internal metal distributions. Elemental contents were quantified by comparison to the NIST SRM 1833 thin film standard which contained K, Ti, Fe, Zn and Pb at known concentrations (\(\sim 10 \, \mu g/cm^2\)). The sensitivities for Ni-K\(\alpha\) and Cu-K\(\alpha\) were obtained by interpolating the Fe-K\(\alpha\) and Zn-K\(\alpha\) values. The sensitivities for the Pt lines were obtained by making small corrections to the measured Pb line sensitivities, using the sensitivity prediction mode of the NRLXRF program.\textsuperscript{251} This procedure yielded elemental images in \(\mu g/cm^2\). Images in element weight fraction (e.g. ppm) were computed by dividing the \(\mu g/cm^2\) value in each pixel by the beam height and assuming the density of each pixel was that of water (i.e. 1 g/cm\(^3\)). The detection limit for Pt was near 10 ppm.

For each elemental tomogram, a mean concentration for the object was determined by averaging the concentration in the object pixels ignoring obvious “hot spots” on the exterior surfaces of some of the objects. In addition, attempts were made to produce radial distribution profiles for each tomogram, though this was somewhat difficult because the spheroids often did not have an ideal spherical shape. The basic procedure was to determine the concentration profile through the centre of the object at 1 degree intervals. Since the near surface distribution was of the most interest, the central portion of each profile was expanded or contracted so that each ray had the same total length. These were then averaged to produce a single concentration profile.

\textbf{4.2.4.2 Micro-SRIXE}

Following 24 hr incubation with Pt complexes (\textit{cis}-[PtCl\(_2\)(NH\(_3\))\(_2\)] 50 \(\mu\)M, \textit{cis},\textit{trans},\textit{cis}-[PtCl\(_2\)(OH)\(_2\)(NH\(_3\))\(_2\)] 50 \(\mu\)M or 200 \(\mu\)M) growth medium was removed and spheroids were washed with 10 mL of cold PBS.
Paraffin embedded samples were prepared by fixing spheroids in neutral buffered formalin fixative (10 mL) for at least 30 min. The spheroids were transferred to casting moulds, allowed to settle, and excess formalin was removed. The spheroids were then embedded in melted 2 % (w/v) agarose / 4 % (v/v) formalin (in milliQ water) and allowed to set. The samples were then removed from the casting moulds, placed in a tissue culture cassette (RA Lamb, Eastbourne, UK) and processed according to routine histopathology procedures (Histopathology Department, John Radcliffe Hospital, Oxford, UK), involving several washes of varying dilutions of ethanol and xylene. Processed samples were then embedded in paraffin wax, sections of 20 µm were cut using a microtome and placed onto either nickel electron microscope grids (for analysis at APS), or onto doubly formvar-coated teflon supports (1.5 cm x 2 cm, 0.3 mm thick, with 4 holes of 3 mm diameter down one side, for analysis at ESRF).

Cryosections were prepared by removing excess PBS and embedding the spheroids in OCT embedding medium. Samples were then frozen in liquid nitrogen and stored at -80 °C. A cryo-microtome (maintained at approximately -20 °C) was used to cut 20 µm sections, which were placed directly onto formvar-coated nickel electron microscope grids (for analysis at APS).

Experiments were performed on beamline ID22 at the European Synchrotron Radiation Facility (ESRF), Grenoble, France. Fluorescence spectra were collected using a single element Si(Li) detector, placed approximately 20 mm from the sample. Two dimensional maps corresponding to the integrated Kα fluorescence signal of an element of interest were collected by scanning the sample. The elements analysed were P, S, Cl, K, Ca, Fe, Ni, Cu, Zn. Pt was analysed using the Lα and Lβ fluorescence lines. Elemental contents were quantified by comparison to the SRM 1832 and 1833 thin polymer film standards (NBS/NIST, Gaithersburg, MD, USA) using the assumption for a thin film target. Sectioned spheroids (20 µm thick) were analysed using a 13 keV monochromatic X-ray beam focused to a 1.8 µm x 4.5 µm (vertical x horizontal) spot. Samples were mounted and suitable spheroid sections located by
viewing the sample with a video-zoom microscope. Scan areas were chosen such that representative portions of the inner and outer regions of the spheroid section were incorporated. PyMca*, an ESRF code, was used for fitting the acquired spectra. This allowed the background contribution to be removed, facilitated analysis of overlapping peaks, and accounted for the eventual escape peaks. X-ray lines were fitted using the Hypermet function and a model for background fitting was chosen as a 10th order polynomial for an exponential background model. Computed X-ray line intensities were normalized to the value of the incident photon flux.

Data analysis was performed by choosing regions of interest (ROIs) within each scan. Such ROIs typically included one ROI encompassing the majority of the scan area (“whole spheroid”), another encompassing central regions of the spheroid (“centre”), and another encompassing an edge region of the spheroid (“edge”) (30 – 60 µm into the spheroid from the edge, chosen so as to include any intense copper edge regions). In addition, small regions of high elemental content were also selected. The average fluorescence spectra from each ROI were fitted using the PyMca X-ray fluorescence fitting program. Elemental concentrations (µg cm\(^{-2}\)) were calculated from peak areas using peak area:concentration ratios determined from NIST thin-film standards (SRM 1832 and 1833), and then converted to µg cm\(^{-3}\) by accounting for the known section thicknesses. Such calculations neglected secondary fluorescence processes and assumed that the standards and the sample were similar to one another. The Pt-L\(\alpha\) fluorescence line was used to quantify platinum content in a similar fashion to that outlined by Ilinski et al.,\(^{252}\) using fluorescence line cross sections (at 13 keV) of Zn-K\(\alpha\) = 43.089 cm\(^2\)g\(^{-1}\), and Pt-L\(\alpha\) = 18.208 cm\(^2\)g\(^{-1}\).\(^{253}\) Elemental content of ROIs were compared to that of the “whole spheroid” ROI, and the zinc content of the ROI.

Hard X-ray zone-plate-based experiments were performed on beamline 2-ID-D and 2-ID-E at the Advanced Photon Source (APS), Argonne National Laboratories, Argonne, Illinois, USA. All measurements were conducted under a He atmosphere in order to

* http://www.esrf.fr/computing/bliss/downloads/pymca/PyMCA.pdf
eliminate the Ar K-shell fluorescence signal and to significantly reduce absorption of low energy X-rays by air and thus to improve the signal to noise ratio for detection of low-Z elements. The sample was raster-scanned through the focussed x-ray beam, and at each scan position the entire fluorescence spectrum was collected using a single element ultra LEGe detector at 2-ID-D, and an energy dispersive three element ultra LEGe (low energy germanium) detector (Canberra, Meriden, CT, USA) at 2-ID-E. Two dimensional distributions of elements of interest were generated from the acquired dataset by mapping the fluorescence corresponding to the integrated Kα or Lα lines, using either ROI based spectral filtering, or peak fitting using modified Gaussians on a per pixel basis. Elemental concentrations (µg cm\(^{-2}\)) were calculated by comparison of sample counts to counts from NIST thin-film standards (SRM 1832 and 1833), and subsequent conversion to µg cm\(^{-3}\) by accounting for the known section thicknesses. Sectioned spheroids (20 µm thick) were analysed using a 13.4 keV monochromatic X-ray beam focussed to a spot size of approximately 0.5 x 0.3 µm at 2-ID-D and 0.8 x 0.4 µm at 2-ID-E. Suitable spheroid sections were initially located by off-line viewing of the sample with a light microscope (Leica DMXRE) and noting the location using the finder grid reference. The sample was then mounted and the appropriate reference area located by remotely viewing an X-ray transmission image of the sample using a scintillator screen and a video camera. Short point spectra and rough 11x11 µm scans were acquired to ascertain whether a particular sample area contained biological elements, sufficient Pt and low levels of Ni. Micro-SRIXE maps were obtained from scan areas of 25 x 25 µm or 30 x 30 µm with 0.5 or 0.6 µm step size, and 1 second acquisition time per step.

Data analysis was performed by dividing the scans up into groups, based upon the complex with which the spheroids had been treated (cis, OH, or control), the technique used to prepare the samples (paraffin or cryo embedding), and the region of the spheroid scanned (edge or centre). The elements of interest were P, S, Cl, K, Ca, Fe, Ni, Cu, Zn, Pt. The elemental content of each scan, and the average and standard deviation of each group was determined. A one-way ANOVA test was used to statistically compare the mean values of the data sets (P value < 0.05 was
considered significant). Caution was used when interpreting the results of these analyses, given the small size of the data sets within each group. The correlation coefficients (R\(^2\) values) of platinum with each of the elements of interest were calculated for each scan in a similar fashion to that described previously\(^{240}\) and compared to the correlation coefficient of phosphorus to sulfur in the scan of interest. This was then used to provide an indication of the elements that correlated well with platinum. Cluster analysis (k-means cluster analysis, equal weights, maximum of 4 clusters) was performed on each of the scans using MAPS v1.2.21, 19\(^{th}\) October 2005 (Vogt, de Jong).\(^{255}\) The following groups of elements were used to perform cluster analysis on each of the scans: P/S, P/S/Pt, Zn/Ca/S/Pt, Zn/Pt, Ca/Pt, K/Pt. Clusters were compared qualitatively by visual inspection, and quantitatively by comparing their size, location and platinum content.

### 4.2.4.3 Micro-XANES

Micro-XANES spectra were collected on beamline 2-ID-D at the Advanced Photon Source (APS) at the Argonne National Laboratories, Argonne, Illinois, USA. Pt L\(\text{II}\) edge micro-XANES spectra were collected from 13.235 – 13.335 keV using formalin/paraffin embedded and cryo spheroid sections that were treated with cis,trans,cis-[PtCl\(_2\)(OH)\(_2\)(NH\(_3\))\(_2\)] or cis-[PtCl\(_2\)(NH\(_3\))\(_2\)]. Points of sufficiently high Pt content (approximately 150 counts per second for Pt(II) and 20 – 60 counts per second for Pt(IV)) were identified by performing point scans on areas towards the edge or the centre of the spheroids. Acquisition times of 10 seconds (Pt(II) and Pt(IV)-treated paraffin embedded sections) or 40 seconds (Pt(IV)-treated cryo sections) were used. Changes in incident intensity were corrected for by averaging the downstream and upstream ion chamber signals and dividing the platinum signal at each point by this value. Multiple spectra obtained at a single point were averaged. The spectra displayed herein were smoothed using 3-point adjacent averaging, and a/b ratios were calculated from the smoothed spectra.
### 4.2.5 Wash out experiments

Following 24 hr incubation with radiolabelled platinum complexes (\([\text{PtCl}_2(\text{14C-en})]\), specific activity 37 Curies/mol, or cis,trans-[\text{PtCl}_2(\text{OH})_2(\text{14C-en})], specific activity 6.4 Curies/mol) at approximately 20 \(\mu\)M concentration, growth medium was removed and spheroids (approximately 60) were washed with 10 mL of cold PBS.

Following removal of PBS, spheroids were processed by one of the methods outlined below:

a) Placed directly into scintillation fluid (4 mL) ("Ready protein liquid scintillation cocktail for protein, peptides, and nucleic acids", Beckman Coulter, High Wycombe, UK), OR  
b) Placed in neutral buffered formalin for at least 2.5 hr, removed, and placed in scintillation fluid (4 mL), OR  
c) Placed in neutral buffered formalin fixative (10 mL) for approximately 1 hr and processed similarly to that outlined above. Briefly, spheroids were transferred to casting moulds, embedded in hot 2 % (w/v) agarose / 4 % (v/v) formalin (in milliQ water) and allowed to set. The samples were then processed according to routine histopathology procedures. Processed samples were placed in scintillation fluid (4 mL).

Spheroids remained in scintillation fluid for at least 4 hr prior to counting.

### 4.3 Results and Discussion

#### 4.3.1 Whole Spheroids: Micro-Tomography

##### 4.3.1.1 Platinum distribution in spheroids

Platinum treated (and control) whole spheroid samples were analysed using X-ray fluorescence microtomography on beamline 13-ID-C at the Advanced Photon Source.
(APS). The spheroids analysed were nominally 400 µm in diameter, chosen such that self-absorption by the sample for the elements of interest and data collection times would be minimised. Figure 4.3 shows an optical microscope image of a typical spheroid mounted for analysis. Such images aided the choice of the vertical position at which to obtain a slice through the sample. A schematic representation of the sample and detector setup for these experiments has been shown above in Figure 4.2 (Section 4.1.2.3).

Figure 4.3: An optical image of a typical spheroid sample mounted for analysis, showing a spheroid approximately 400 µm in diameter.

Microtomography provides a means by which images of elemental distribution in virtual slices through the spheroid can be obtained rapidly. This gives a broad overview of relative concentrations through a slice, ideal for our purposes. The main aim of this work was to compare the ability of cisplatin and its platinum(IV) analogues to penetrate into tumour spheroids.

Figure 4.4 shows the platinum distribution in spheroids treated with cisplatin after 8 and 24 hour incubations. It was found that the platinum distribution patterns after 8 and 24 hour incubation times with cisplatin (50 µM) and the OH platinum(IV)
complex (200 μM) were indistinguishable from one another, with some surface enrichment of platinum and uniform distribution in the interior of the spheroid. In addition, there was no significant difference in the average platinum content or distribution in spheroids treated with cisplatin for 8 hours or 24 hours. As such, 24 hour incubation times were the focus of subsequent investigations.

![Figure 4.4: Platinum maps of virtual slices through DLD-1 tumour spheroids treated with 50 μM cisplatin for A: 8 hours; and C: 24 hours. B (8 hours) and D (24 hours) show the corresponding average profiles of platinum concentration through the centre of the object. Note that the fixation process often causes shrinkage of spheroids.](image)

**Figure 4.4:** Platinum maps of virtual slices through DLD-1 tumour spheroids treated with 50 μM cisplatin for A: 8 hours; and C: 24 hours. B (8 hours) and D (24 hours) show the corresponding average profiles of platinum concentration through the centre of the object. Note that the fixation process often causes shrinkage of spheroids.201
Each of the platinum complexes studied displayed similar distribution patterns in spheroids following 24 hour incubation, with a surface enrichment of platinum and uniform distribution deeper within the samples. The exterior platinum content was approximately two times higher than the interior platinum content for each of the samples. Figure 4.5 shows representative examples of the platinum distribution in spheroids treated with each of the platinum complexes for 24 hours. Control spheroids treated in an identical fashion, but to which no platinum complexes were added, contained average platinum of less than 10 ppm, and thus it was not meaningful to produce their platinum profiles.
Figure 4.5: Platinum distribution and the corresponding average concentration profiles through representative spheroids treated with cisplatin: 50 μM, 24 hours (A, B); Cl4: 50 μM, 24 hours (C, D); OAc: 200 μM, 24 hours (E, F); OH: 50 μM, 24 hours (G, H); control (I, J). Threshold values were set for some samples in order to minimise distortions in the images as a result of spots of high elemental content. Platinum
content of the control spheroids was too low (below 10 ppm) to allow a platinum profile to be generated.

These results indicate that cisplatin and the platinum(IV) complexes are capable of penetration into the central regions of the spheroid. This is consistent with similar previous studies, which found that cisplatin, carboplatin, and tetraplatin penetrated efficiently into spheroids following incubation times of up to 2 hours. A porphyrin-platinum complex was found to penetrate throughout spheroids within 24 hours. Studies performed in in vivo peritoneal rat tumours found that the periphery of the tumours had higher platinum concentrations than the centre, with a concentration gradient towards the centre of the tumour following intraperitoneal administration of cisplatin. In contrast, following equimolar treatment with carboplatin, only low platinum concentrations were observed on the periphery of the tumours, and platinum was undetectable in the centre of the tumour. When dosed at significantly higher concentrations of carboplatin, platinum distribution throughout the tumour was fairly homogeneous, though still at low concentrations.

It is important that the current results be viewed in light of the procedures used to prepare the spheroid samples. Fixation and storage of spheroids in neutral buffered formalin may wash out some platinum from the spheroids. This possibility was investigated using radiolabelled platinum(II) and platinum(IV) complexes to provide an indication of the extent of washout. These experiments are described in detail in Section 4.3.4. It was shown that the platinum content of the spheroids decreased following fixation in formalin compared to spheroids that did not undergo the fixation process. Most of the platinum that washed out of the spheroids is likely to not have been bound to macromolecules such as DNA and proteins, thus leaving only bound platinum and perhaps some traces of unbound platinum. It is assumed that bound platinum species would not be washed out, or that such washout would only occur under much harsher conditions.
The microtomography results are likely to primarily reveal the distribution of platinum species bound to macromolecules. As such, information regarding the penetration of complexes into the spheroids can only be inferred, since we were unable to quantify the concentration of platinum that reached the inner regions of the spheroids whilst remaining unbound. The microtomography experiments suggest that the platinum complexes penetrate and bind to the central regions of the spheroids following 8 and 24 hour incubation. However, the total concentration of platinum that reached the central or peripheral regions of the spheroids cannot be determined using these techniques. The results indicate that a significant quantity of the complex reacts in the peripheral, actively dividing cells, leaving only a small amount to penetrate deeper. Perhaps higher doses of complex are required to enhance penetration into the central regions of the spheroid. Previous studies with tetraplatin have shown that with higher drug doses, retention of tetraplatin becomes more uniform throughout the spheroid, though still with slightly higher concentrations in the outer cell layers.

It is worth speculating as to the implications of the current results for the situation in real tumour systems. For a tumour to be effectively treated, high concentrations of drug should reach all cells within the tumour. The results presented herein suggest that the platinum complexes should be capable of penetration into the poorly vascularised regions of tumours (typically <1 mm, and thus of similar size to the spheroids studied herein). Inert platinum complexes may perhaps hold the key to improving the in vivo efficacy of platinum drugs. High concentrations of such drugs should be capable of penetrating beyond the outer cell layers of spheroids or tumours without undergoing significant reaction. Hence the anticancer activity exerted upon central cell layers would be enhanced.

The concentrations of platinum, zinc and copper in each of the slices were quantified relative to the SRM 1833 standard (a thin film standard, containing known concentrations of particular elements). Figure 4.6 shows the mean platinum concentration in each of the samples. Platinum content of the samples ranged from 50 ppm to 150 ppm, with the exception of one of the OAc treated samples (mean Pt
content 400 ppm), which is believed to be an anomaly. This sample also contained high levels of zinc and copper. As noted above, there was no significant difference in the platinum content of spheroids treated with cisplatin (50 µM) for 8 or 24 hours (mean 110 ppm, standard deviation 30, 6 samples). In addition, there was no significant difference in the platinum content of the spheroids, regardless of the platinum complexes with which they were treated. The mean platinum content of spheroids treated with OH (200 µM) for 24 hours was found to be 80 ppm (standard deviation 50, 4 samples). Spheroids treated with OAc (200 µM) for 24 hours had platinum content of 300 ppm, standard deviation 200 (2 samples, including the unusually high value), whilst those treated with Cl4 (50 µM) for 24 hours had 90 ppm (standard deviation 10, 2 samples). Control samples had platinum content less than 10 ppm (2 samples). Further work would be required to obtain a statistically significant data set for the determination of accurate values of elemental content of the samples. However, this was not the primary aim of the current research.
CHAPTER 4: Distribution of Platinum Complexes in Multicellular Tumour Spheroids

Figure 4.6: Plot of the mean platinum concentrations of slices through spheroids treated with various platinum complexes: cis 50 µM for 8 or 24 hr (3 of 6 samples); OH 200 µM for 24 hr; OAc 200 µM for 24 hr; Cl4 50 µM for 24 hr.

The OH and OAc complexes were dosed at a concentration four times higher than that used for cisplatin and Cl4. Microtomography scans performed during the first period of beam time investigated spheroids that had been treated with 50 µM OH for 8 hours or 24 hours. The platinum content of such spheroids was found to be very low, as shown in Figure 4.7, and did not allow for an accurate determination of platinum concentration in the spheroid slices. In contrast, the same treatment concentration of cisplatin (50 µM) was found to be sufficient to obtain good quality maps of the distribution of platinum in the spheroids. Hence it was decided that subsequent experiments would be performed with higher concentrations of OH and that this was likely to be necessary for the OAc complex. The dosing concentration of the Cl4 complex was the same as used for cisplatin, given that previous studies within our research group indicated that the Cl4 complex is rapidly reduced to...
cisplatin.\textsuperscript{34} Hence it is likely to behave in a similar manner to cisplatin in the spheroid environment. Given that under the conditions of the current study, platinum spheroid concentrations were independent of the complex used, cisplatin and Cl\textsubscript{4} appear to be taken up and bound in the spheroids more efficiently than OAc and OH. 

Figure 4.7: Platinum distribution through slices of spheroids treated with OH, A: 50 \(\mu\)M, 8 hours; B: 50 \(\mu\)M, 24 hours; C: 200 \(\mu\)M, 24 hours.

Several of the spheroids were found to contain “hot spots” of platinum. Platinum contents up to approximately 30 times higher than the mean platinum sample content were found in such spots. The spots often also contained zinc and/or nickel. They were usually evident from the initial line scans across the sample and avoided by altering the vertical position of the scan. Any full scans that included hot spot areas were corrected by adjusting the threshold intensity of the image. The origin of such hot spots was not identified.

4.3.1.2 Distribution of other elements in spheroids

Information concerning several elements of interest can be obtained from a single X-ray fluorescence microtomography experiment. The selection of elements is restricted by atomic number, with lighter elements such as calcium and potassium suffering from significant self absorption. For the present analysis, the elements iron, nickel, copper, zinc, and platinum were monitored, with the focus being on the latter
three. Figure 4.8 shows the reconstructed elemental images of a representative sample.

![Elemental distribution of a virtual slice through a spheroid that had been treated with cisplatin for 8 hours.](image)

**Figure 4.8:** Elemental distribution of a virtual slice through a spheroid that had been treated with cisplatin for 8 hours.

Iron is not seen in the sample but is present in high concentrations in the glass fibre and/or the epoxy resin used to mount the sample (Figure 4.8, image D). Iron is known to be a major constituent of biological systems. Its apparent absence in the spheroid samples is likely due to its much larger concentration in the fibre/epoxy, saturating the image. Liu *et al.*\(^{240}\) have previously reported a correlation between the spatial distribution of iron and platinum, iron and copper, and iron and zinc in the liver of mice treated with cisplatin. In contrast, no correlation was reported by Mutsaers *et al.*\(^{234}\) between iron and platinum in the peritoneal tumour tissue of rats after intravenous administration of cisplatin. Such correlation information for iron could not be obtained from the current work for the reasons mentioned above. The
subsequent micro-SRIXE work, outlined in Section 4.3.2 is better suited to these purposes.

Nickel appears to be present in higher concentrations at the periphery compared to the interior regions of the spheroids (Figure 4.8, image E). Zinc and copper also appear to be present in these materials, though at lower concentrations.

Zinc was found to be distributed homogeneously throughout the spheroids, as expected. The zinc content of the spheroids was not altered by treatment with platinum complexes. In addition, zinc content did not differ depending upon the platinum complex with which the spheroids were treated. The mean zinc content of all spheroids studied was 70 ppm (standard deviation 50, 16 samples). These findings are consistent with the previous X-ray fluorescence microtomography study, by Burattini et al., which found that zinc was homogeneously distributed throughout spheroid samples, independent of how they were treated. In some samples, such as that shown in Figure 4.8, slightly lower concentrations of zinc were observed in peripheral regions of the samples. One study has shown an inverse correlation between intracellular zinc and platinum content in cisplatin sensitive and resistant PC-9 lung carcinoma cells. Whilst it appears that a similar phenomenon may be occurring in the outer cell layers of the spheroids studied herein (such that the outer cells have enhanced platinum levels but diminished zinc levels), it can not account for the fact that the control spheroid samples also appear to have diminished levels of zinc in their outer cell layers.

The distribution of copper in the spheroid samples was also analysed. The samples scanned during the first set of experiments displayed copper distributions similar to that shown in Figure 4.8 (image C), and Figure 4.9 (image A), with copper concentrated in a narrow region at the periphery of the spheroids. However, in samples measured during the second set of experiments, this effect was not as pronounced. Approximately half of the samples studied during this time showed a slight increase in copper concentration at the outer region of the spheroids, though to a much lesser extent than that seen previously. The remainder of the samples had
uniform copper distributions throughout the volume of the spheroids. Figure 4.9 shows the copper distribution in some representative spheroid samples, highlighting the observations mentioned.

**Figure 4.9:** Copper distribution in representative spheroid samples with the corresponding plots of distribution through the samples. A/B show a narrow region of very intense copper concentration; C/D show a similar effect, though it is less pronounced; and E/F show a uniform distribution throughout the spheroid.

The possibility of these differences originating from the instrumental setup has been ruled out and thus we must take it to be a consequence of the samples themselves. The procedures used to prepare the samples on the two separate occasions were identical. Quantification of copper revealed no significant variation between samples, regardless of the platinum complex with which they were treated, or the beam time during which the analysis was performed. Values ranged from 30 to 500 ppm, with a mean of 200 ppm, standard deviation 100. Possible causes of the observed copper distribution within the samples are outlined in Section 4.3.5. Burattini et al reported that the distribution of copper within spheroids was essentially homogeneous.
However, they had difficulties in distinguishing the spheroidal copper from the copper in the quartz in which the spheroid samples were contained for analysis.\textsuperscript{248}

### 4.3.2 Sectioned Spheroids: Micro-SRIXE

#### 4.3.2.1 Analysis performed at ESRF

Micro-SRIXE was performed on selected regions within slices (20 \( \mu m \)) of spheroids treated with 50 \( \mu M \) cisplatin or 200 \( \mu M \) OH for 24 hours. Scan areas were chosen to incorporate representative regions at the interior and exterior of spheroid sections. The experiments were performed on beam line ID22 at the European Synchrotron Radiation Facility (ESRF), France. The aim of this work was to further investigate the platinum distribution patterns observed with micro-tomography with higher resolution and different sample preparation procedures. Note that 20 \( \mu m \) sections were used for all of the studies to ensure sufficient platinum signal from the samples. See Appendix, Figure A5 for a representative microscope image of one of the spheroid sections that was analysed.

The following figures show representative images of the elemental distribution in spheroids treated with cisplatin (Figure 4.10), OH (200 \( \mu M \), Figure 4.11, and 50 \( \mu M \), Figure 4.12), or untreated (Figure 4.13).
Figure 4.10: Fitted images of the elemental distributions in a segment of a cisplatin treated spheroid (24 hr, 50 μM, scan 79). Images obtained by using the Pt-L\(\alpha\) and Pt-L\(\beta\) lines of the fluorescence spectrum are shown. The scale on the axes represents the number of pixels, where each pixel is 3 x 2 μm (horizontal x vertical). The images show relative elemental concentrations, using the colour scale shown, which ranges from blue, representing low levels, to red, representing high elemental levels.
Figure 4.11: Fitted images of the elemental distribution in a segment of an OH treated spheroid (24 hr, 200 µM, scan 88). Images obtained by using the Pt-Lα and Pt-Lβ lines of the fluorescence spectrum are shown. The scale shown on the axes represents the number of pixels, where each pixel is 3 x 2 µm (horizontal x vertical). The images show relative elemental concentrations, using the colour scale shown, which ranges from blue, representing low levels, to red, representing high elemental levels.
Figure 4.12: Fitted images of the elemental distribution in a slice of an OH treated spheroid (24 hr, 50 μM, scan 83), taken through the centre of a spheroid section from one edge to the other. Images obtained by using the Pt-Lα and Pt-Lβ lines of the fluorescence spectrum are shown. The scale shown on the axes represents the number of pixels, where each pixel is 3 x 2 μm (horizontal x vertical). The images show relative elemental concentrations, using the colour scale shown, which ranges from blue, representing low levels, to red, representing high elemental levels.
**Figure 4.13:** Fitted images of the elemental distributions in a segment of a control spheroid (scan 108). Images obtained by using the Pt-Lα and Pt-Lβ lines of the fluorescence spectrum are shown. Platinum was undetectable using the Pt-Lα and Pt-Lβ line of the fluorescence spectrum. The scale shown on the axes represents the number of pixels, where each pixel is 3 x 2 μm (horizontal x vertical). The images show relative elemental concentrations, using the colour scale shown, which ranges from blue, representing low levels, to red, representing high elemental levels.

Both of the platinum complexes displayed similar distribution patterns in spheroids following 24 hour incubation, with a uniform distribution throughout the volume of the samples and little variation between platinum levels at the inner and outer regions of the spheroids. This can be seen qualitatively in Figures 4.10, 4.11, 4.12.
above, and quantitatively in Figures 4.14, 4.15, 4.16 below. In each scan, regions of interest (ROIs) incorporating edge or central areas of the spheroids were chosen. The average elemental content of the regions was quantified and plotted in several formats. The absolute elemental contents of the ROI regions were averaged and plotted, as shown below in Figure 4.14 for platinum. In addition, elemental content of an edge or central spheroid region was calculated relative to the content of an ROI representative of the whole spheroid and expressed as a percentage (with a value close to 100 indicating an average elemental content similar to that of the whole spheroid, see Figure 4.15 for plots obtained from cisplatin treated spheroids and Figure 4.16 for OH treated spheroids). Similar plots, though of elemental ratios relative to zinc and compared to the whole spheroid, were also generated (see Appendix, Figure A8 for cisplatin treated spheroids and Figure A9 for OH treated spheroids).
Figure 4.14: Average platinum content (µg cm⁻³) of scans obtained from spheroid sections (20 µm thickness). Scans were grouped according to the complex with which the spheroids had been treated (cis 50 µM, OH 200 µM, control (con)), and the region of the spheroid from which the scan was obtained (edge, centre). The errors shown represent the standard deviation of the data sets.
Figure 4.15: The elemental content of edge or central regions of cisplatin treated spheroids relative to a region representative of the entire spheroid. Values are expressed as percentages, where a value of 100 is indicative of an elemental content identical to that of the whole spheroid.
Figure 4.16: The elemental content of edge or central regions of OH treated spheroids relative to a region representative of the entire spheroid. Values are expressed as percentages, where a value of 100 is indicative of an elemental content identical to that of the whole spheroid.

Spheroids were dosed with OH at two different concentrations (50 and 200 µM, Figure 4.12 and 4.11, respectively). The platinum signal was much lower from spheroids treated with 50 µM OH, with an average platinum content of approximately 12 µg cm⁻³. On the other hand, the average platinum content of spheroids treated with 200 µM OH was 73 ± 26 µg cm⁻³ (see Figure 4.14). Such a difference was also evident in the Pt-Lα images and thus the focus of subsequent investigations was the 200 µM OH dosed samples. In spheroids treated with 50 µM cisplatin, the average platinum content was 86 ± 5 µg cm⁻³. Thus platinum uptake of spheroids treated with 50 µM cisplatin was similar to that of those treated with 200 µM OH. These
results are consistent with the tomography work, and suggest that cisplatin is taken up and bound within spheroids more efficiently than OH.

In general, most elements were uniformly distributed throughout the spheroid samples, as seen in Figures 4.15, 4.16 above (cisplatin and OH treated spheroids, respectively), Figure 4.17 below, and Appendix Figure A10 (control spheroids). This is consistent with the results of Burattini et al.248 Also shown below is a plot of the average copper content of spheroid regions. Equivalent plots for zinc, iron, calcium and potassium are shown in Appendix Figure A11-A14, respectively).

**Figure 4.17:** The elemental content of edge or central regions of control spheroids relative to a region representative of the entire spheroid. Values are expressed as percentages, where a value of 100 is indicative of an elemental content identical to that of the whole spheroid.
Figure 4.18: Average copper content ($\mu$g cm$^{-3}$) of scans obtained from spheroid sections (20 $\mu$m thickness). Scans were grouped according to the complex with which the spheroids had been treated (cis, OH, control (con)), and the region of the spheroid from which the scan was obtained (edge, centre). The errors shown represent the standard deviation of the data sets.

The copper distribution of the samples was consistent with that observed in the tomography work. This suggests that histological processing has little effect on copper distribution within the samples, consistent with previous work that monitored the copper content of rat liver following formalin fixation for up to a week.$^{257}$ Many of the spheroids had a surface enrichment of copper, with lower concentrations in the central regions of the spheroids (for example, see Figure 4.10, 4.13, Figure 4.17 (scan 108) and Figure 4.15 (scan 81)). Some of the spheroids displayed a relatively uniform copper distribution in all regions of the sample (for example, see Figure 4.12, Figure 4.15 (scan 80) and Figure 4.16 (scan 86)), whilst others had surface
enrichment with some large regions of concentrated copper in the centre of the spheroids (see Figure 4.11). Copper distribution patterns were independent of the complex with which the spheroids had been treated, though copper surface enrichments seem to be less pronounced in OH treated spheroid samples. Possible reasons for such copper distributions are discussed in Section 4.3.5.

In many of the samples, small areas of relatively high platinum concentration were seen (up to approximately 30 x 30 µm in size). The average elemental content of these regions was calculated relative to the content of a region encapsulating the majority of the spheroid. As percentages, the platinum contents of these regions varied between approximately 130 % - 290 %. Some of these spots were also elevated in the content of other elements (>130 % relative to the content of the “whole spheroid”), such as zinc, copper, iron and calcium. However, there was no apparent trend dictating the elements elevated in any particular spot. In some scans, spots of other elements such as zinc, copper, iron and calcium, distinct from the platinum spots, were also seen. The resolution of the scans limits the information that can be obtained concerning elemental distributions on a cellular scale and as such, regions of elevated elemental content can not be conclusively defined as cells. It may be that the formalin/paraffin procedure has caused some elemental redistributions to occur within the samples (also see discussions below in Sections 4.3.2.2.3 and 4.3.5).

4.3.2.2 Analysis performed at APS

High resolution micro-SRIXE was performed on selected regions within slices of spheroids treated with 50 µM cisplatin or 50 µM OH for 24 hours. The experiments were performed on beam lines 2ID-D and 2ID-E at the Advanced Photon Source (APS), USA. The aim of this work was to determine whether platinum within spheroids localised intracellularly or extracellularly, the elements with which it colocalised in the spheroid environment, and any differences in the elemental content of the inner and outer regions of the spheroids. Formalin fixed/paraffin embedded and cryo
embedded samples were compared in this context. Appendix Figure A6 shows a representative microscope image of a cryo embedded spheroid section, and Figure A7 shows a representative formalin fixed/paraffin embedded spheroid section.

4.3.2.2.1 Elemental content

The elemental content of each scan area was quantified. Scans of similar types were grouped together and the average elemental content of each group determined. Plots of average elemental content are displayed in this section. In addition, plots of the ratio of each element relative to zinc are also shown. Zinc was chosen for this purpose because of its uniform distribution throughout the spheroid samples and as such it should provide an indication of the amount of biological material present within each section, thus reducing the effect of any errors in section thickness. Also, there is literature precedent for the calculation of ratios relative to zinc.\textsuperscript{258, 259} One such study found that the Cu/Zn ratio was increased in larger tumours, though changes in individual trace element concentrations was not as accurately associated with tumour growth.\textsuperscript{258}

It should be noted that scans within one scan group were often obtained from only a small number of spheroid sections and thus we can not be certain that the results are representative of all spheroids treated in the same manner.

Figure 4.19 shows the average platinum content and Figure 4.20 the average Pt:Zn ratio of each of the scan groups.
Figure 4.19: Average platinum content (µg cm⁻³) of scans obtained from spheroid sections (20 µm thickness). Scans were grouped according to the complex with which the spheroids had been treated (cis, OH, control (Con)), the procedure used to prepare the samples (cryo (cr), formalin (f)), and the region of the spheroid from which the scan was obtained (edge, centre). The number of scans (n) used to calculate the averages are also shown. The errors shown represent the standard deviation of the data sets.
Several trends emerge from this data:

- The platinum content and Pt:Zn ratio of the control (untreated) samples was small, and did not vary with sample preparation techniques or scan regions.
- There was no significant difference in the platinum levels or Pt:Zn ratio at the edge or the centre of the spheroids, regardless of the manner in which the spheroids were treated.
- The platinum content of the cisplatin treated spheroids appears to have been affected by the sample preparation procedure, being significantly lower in the...
cryo embedded samples. However, the Pt:Zn ratio does not show this trend, with similar ratios for all cisplatin treated samples.

- The platinum content and Pt:Zn ratio of the OH treated spheroids was not affected by sample preparation procedures, with similar platinum levels in all samples.
- Platinum levels in cisplatin treated spheroids (formalin samples) are higher than those in OH treated spheroids. Pt:Zn ratios in cisplatin treated spheroids are higher than in OH treated spheroids.

Cryo embedding procedures may cause redistribution of elements within the samples,\textsuperscript{260} whilst formalin fixation procedures often result in wash out of some elements (as will be discussed further in Section 4.3.4). Hence the lower platinum content of the cisplatin treated cryo embedded samples relative to the cisplatin treated formalin samples was quite unexpected. However, given that variations between scans in a group were relatively small, and that sample size was sufficiently large (n=6 for cryo, n=11 for formalin), we conclude that the observed differences are real. This trend was absent from the Pt:Zn ratio data. Pt:Zn ratios in all cisplatin treated samples were similar to one another, and ratios in OH treated samples were also similar to one another, but lower than the cis treated samples. The different picture portrayed by the absolute platinum levels and Pt:Zn ratios could be due to the low zinc content of the cis cryo samples compared to the cis formalin samples (as shown below in Figure 4.21). This may be the result of spheroid shrinkage during the fixation process,\textsuperscript{201} resulting in higher density of material within the formalin samples. Alternatively, it could be a consequence of the cryo treatment of the samples, or the spheroid section from which all scans of the cis cryo group were obtained may have been thinner than 20 µm due to errors in sectioning.

Zinc is known to be a major intracellular element and hence it is useful to study the zinc content and distribution of the spheroid samples. Figure 4.21 shows the zinc content of the scan groups.
Figure 4.21: Average zinc content (µg cm⁻³) of scans obtained from spheroid sections (20 µm thickness). Scans were grouped according to the complex with which the spheroids had been treated (cis, OH, control (Con)), the procedure used to prepare the samples (cryo (cr), formalin (f)), and the region of the spheroid from which the scan was obtained (edge, centre). The number of scans (n) used to calculate the averages are also shown. The errors shown represent the standard deviation of the data sets.

Several trends emerge from this data:

- The zinc content was similar across all samples, with a few exceptions noted below.
- There was no significant difference in the zinc levels at the edge or the centre of the spheroids, regardless of the manner in which the samples were treated. The exception is the OH/cryo samples, having slightly higher zinc levels at the edge than the centre.
The zinc content of the cisplatin treated spheroids may have been affected by the sample preparation procedure, being lower in the cryo samples than the formalin samples. The zinc content of such cisplatin/cryo spheroids is lower than that of the control cryo samples. As mentioned above, this could be explained by the cis cryo samples being thinner than expected.

The zinc content of the OH/cryo spheroids is higher than that of the control/cryo spheroids.

Previous studies have shown that tightly bound elements, such as zinc, tend to be well retained in samples that had been formalin fixed and paraffin embedded.\cite{257, 261} Such observations are consistent with the current study.

Figure 4.22 shows the phosphorus content and Figure 4.23 shows the average P:Zn ratio of the scan groups. Figure 4.24 shows the sulfur content and Figure 4.25 shows the average S:Zn ratio of the scan groups.
### Figure 4.22: Average phosphorus content (µg cm⁻³) of scans obtained from spheroid sections (20 µm thickness).

Scans were grouped according to the complex with which the spheroids had been treated (cis, OH, control (Con)), the procedure used to prepare the samples (cryo (cr), formalin (f)), and the region of the spheroid from which the scan was obtained (edge, centre). The number of scans (n) used to calculate the averages are also shown. The errors shown represent the standard deviation of the data sets.
Figure 4.23: Average P:Zn ratios of scans obtained from spheroid sections (20 µm thickness). Scans were grouped according to the complex with which the spheroids had been treated (cis, OH, control (Con)), the procedure used to prepare the samples (cryo (cr), formalin (f)), and the region of the spheroid from which the scan was obtained (edge, centre). The errors shown represent the standard deviation of the data sets.
**Figure 4.24:** Average sulfur content (µg cm⁻³) of scans obtained from spheroid sections (20 µm thickness). Scans were grouped according to the complex with which the spheroids had been treated (cis, OH, control (Con)), the procedure used to prepare the samples (cryo (cr), formalin (f)), and the region of the spheroid from which the scan was obtained (edge, centre). The number of scans (n) used to calculate the averages are also shown. The errors shown represent the standard deviation of the data sets.
Figure 4.25: Average S:Zn ratios of scans obtained from spheroid sections (20 μm thickness). Scans were grouped according to the complex with which the spheroids had been treated (cis, OH, control (Con)), the procedure used to prepare the samples (cryo (cr), formalin (f)), and the region of the spheroid from which the scan was obtained (edge, centre). The errors shown represent the standard deviation of the data sets.

The trends in phosphorus and sulfur content will be discussed together due to their similarity:

- There was no significant difference in the phosphorus/sulfur levels or P:Zn/S:Zn ratios at the edge or the centre of the spheroids, regardless of the manner in which the samples were treated.
- Phosphorus/sulfur levels and P:Zn/S:Zn ratios were similar in all control samples.
OH formalin spheroids have lower phosphorus/sulfur content than OH cryo spheroids, with the former having similar levels to the controls, and the latter having higher levels than the controls. This trend was absent from the P:Zn/S:Zn ratios.

The phosphorus/sulfur content of the cisplatin cryo spheroids was lower than that in the cisplatin formalin spheroids. In addition, the phosphorus/sulfur content of such cisplatin cryo spheroids was lower than that of the control cryo samples. This trend was not present in the P:Zn/S:Zn ratio plot, and in fact, the P:Zn/S:Zn ratio of the cis cryo spheroids was higher than that in the cis formalin samples. This may be the result of artefacts in the sectioning of the different sample types.

Minor variations in phosphorus and sulfur content have been observed in the current study, depending upon the sample preparation method. This is consistent with the results of Vogt et al.\textsuperscript{261} who found phosphorus and sulfur to be well retained in paraffin embedded samples and cryo embedded samples. In contrast, another study found that phosphorus and sulfur were better retained in cryo fixed sections than chemically fixed ones.\textsuperscript{262} The P:Zn/S:Zn ratios have evened out many of these variations, again consistent with a thinner cis cryo spheroid section, or a more dense cis formalin section.

Figure 4.26 shows the average potassium content and Figure 4.27 shows the average K:Zn ratio of the scan groups.
Figure 4.26: Average potassium content (µg cm\(^{-3}\)) of scans obtained from spheroid sections (20 µm thickness). Scans were grouped according to the complex with which the spheroids had been treated (cis, OH, control (Con)), the procedure used to prepare the samples (cryo (cr), formalin (f)), and the region of the spheroid from which the scan was obtained (edge, centre). The number of scans (n) used to calculate the averages are also shown. The errors shown represent the standard deviation of the data sets.
The trends emerging from this data are as follows:

- There was no significant difference in the potassium levels or K:Zn ratios at the edge or the centre of the spheroids, regardless of the manner in which the samples were treated.
- Potassium levels were similar in all control samples. However, K:Zn ratios were significantly lower in control formalin samples than control cryo samples.
- Potassium levels and K:Zn ratios of all formalin samples were similar to one another.
- Potassium levels and K:Zn ratios of all cryo samples were similar to one another.
Potassium levels vary considerably, depending upon the sample preparation procedure, with the highest levels being found in cryo embedded samples. Potassium is an easily diffusible element and as such may be redistributed and/or washed out during sample preparation procedures. The concentration of potassium was found to decrease in paraffin embedded samples compared to cryo samples, in accord with the results of the current study. This trend was further exaggerated in the K:Zn ratios.

Figure 4.28 shows the average calcium content and Figure 4.29 shows the average Ca:Zn ratio of the scan groups.
**Figure 4.28:** Average calcium content (µg cm⁻³) of scans obtained from spheroid sections (20 µm thickness). Scans were grouped according to the complex with which the spheroids had been treated (cis, OH, control (Con)), the procedure used to prepare the samples (cryo (cr), formalin (f)), and the region of the spheroid from which the scan was obtained (edge, centre). The number of scans (n) used to calculate the averages are also shown. The errors shown represent the standard deviation of the data sets.
Figure 4.29: Average Ca:Zn ratios of scans obtained from spheroid sections (20 \( \mu \text{m} \) thickness). Scans were grouped according to the complex with which the spheroids had been treated (cis, OH, control (Con)), the procedure used to prepare the samples (cryo (cr), formalin (f)), and the region of the spheroid from which the scan was obtained (edge, centre). The errors shown represent the standard deviation of the data sets.

A number of trends emerge from this data:

- There was no significant difference in the calcium levels or Ca:Zn ratios at the edge or the centre of the spheroids, regardless of the manner in which the samples were treated.
- Calcium levels and Ca:Zn ratios in formalin samples were similar to one another.
- Calcium levels and Ca:Zn ratios in cryo samples were similar to one another.
The calcium content and Ca:Zn ratio of formalin samples tended to be higher than that of cryo samples.

Calcium levels and Ca:Zn ratios are highly dependent upon the preparation procedure, with formalin samples displaying higher levels of calcium and higher Ca:Zn ratios than cryo samples. These results appear to be in contrast to previous studies that found calcium to be better retained in cryo-fixed sections than chemically fixed sections.\textsuperscript{262,263} However, the possibility that calcium was introduced into the samples during the formalin treatment can not be ruled out.

Figure 4.30 shows the average copper content and Figure 4.31 shows the average Cu:Zn ratio of the scan groups.
Figure 4.30: Average copper content ($\mu g \text{ cm}^{-3}$) of scans obtained from spheroid sections (20 $\mu m$ thickness). Scans were grouped according to the complex with which the spheroids had been treated (cis, OH, control (Con)), the procedure used to prepare the samples (cryo (cr), formalin (f)), and the region of the spheroid from which the scan was obtained (edge, centre). The number of scans (n) used to calculate the averages are also shown. The errors shown represent the standard deviation of the data sets.
Several trends emerge from this data:

- Copper levels and Cu:Zn ratios were similar within all samples.
- There was no significant difference in the copper levels or Cu:Zn ratios at the edge or the centre of the spheroids, regardless of the manner in which the samples were treated.
Previous studies have shown that copper tends to be well retained in samples that were formalin fixed and paraffin embedded.\cite{257, 261} Such observations are consistent with the current study.

The results of Section 4.3.1.2 and 4.3.2.1 showed that many of the spheroid samples had copper enrichment in peripheral cell layers. At first glance, it appears that such a trend is not observed in the current analysis. However, the results displayed in Figure 4.30 show average copper levels, and edge copper enrichments in individual spheroid sections may not be obvious. Comparison of copper levels in scans obtained from inner and outer regions of individual spheroid sections (results not shown) revealed that a small number of spheroid sections had some surface enrichment of copper relative to the central regions. In addition, such copper distribution patterns were not dependent upon how the samples were treated (in terms of the complex with which they were treated or the sample preparation procedure). Thus each of the techniques used to quantify copper distributions in platinum treated spheroid samples gave consistent results.

Similar plots showing the average chlorine, iron, and nickel elemental contents and Cl:Zn, Fe:Zn and Ni:Zn ratios are shown in Appendix Figures A15-A20. Of particular note is the lower chlorine and iron contents of the cis/cryo samples compared to the cis/formalin samples. However it should be noted that it is only the iron levels in the cis/cryo samples that are significantly different (lower) than the levels of the cryo controls. The plots of Fe:Zn ratios eliminate this trend, showing similar Fe:Zn ratios in all samples. Nickel contents are of use in determining whether a scan was collected from a region in close proximity to a nickel grid bar. Table 4.2 summarises some of the general trends seen in the elemental content graphs.
Table 4.2: Summary of the trends in elemental content of the spheroids. Note: s = similar; d = different; l = lower; h = higher; y = yes; n = no; * = some dependence, refer to main text for further details.

<table>
<thead>
<tr>
<th>Element (x)</th>
<th>Pt</th>
<th>Zn</th>
<th>P</th>
<th>S</th>
<th>K</th>
<th>Ca</th>
<th>Cu</th>
<th>Cl</th>
<th>Fe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Edge and centre content / x:Zn ratios</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>s</td>
</tr>
<tr>
<td>Levels in controls (cr compared to formalin)</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>d</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>s</td>
</tr>
<tr>
<td>Levels in cis cr compared to cis f samples</td>
<td>l</td>
<td>l</td>
<td>l</td>
<td>h</td>
<td>l</td>
<td>l</td>
<td>l</td>
<td>l</td>
<td>l</td>
</tr>
<tr>
<td>Compared to cryo controls</td>
<td>h</td>
<td>l</td>
<td>l</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>l</td>
</tr>
<tr>
<td>x:Zn ratios in cis cr compared to cis f</td>
<td>s</td>
<td>-</td>
<td>h</td>
<td>h</td>
<td>l</td>
<td>s</td>
<td>h</td>
<td>s</td>
<td>s</td>
</tr>
<tr>
<td>Compared to cryo controls</td>
<td>h</td>
<td>-</td>
<td>s</td>
<td>s</td>
<td>h</td>
<td>s</td>
<td>s</td>
<td>h</td>
<td>s</td>
</tr>
<tr>
<td>Dependence on preparation procedures?</td>
<td>n</td>
<td>n</td>
<td>*</td>
<td>y</td>
<td>y</td>
<td>n</td>
<td>*</td>
<td>n</td>
<td></td>
</tr>
</tbody>
</table>

Cisplatin treated cryo samples had lower platinum, sulfur, phosphorus, zinc and iron concentrations than cisplatin treated formalin samples or control cryo samples (with the exception of platinum). These elements, perhaps with the exception of platinum, tend to be tightly bound within biological samples, even in paraffin embedded samples. Perhaps the cryo embedding procedure damaged cell membranes, thus causing leakage of certain elements from the spheroids (see discussion in Section 4.3.4). Another possible explanation is that the cisplatin cryo spheroid section from which all of the scans were obtained was actually thinner than 20 µm, perhaps due to errors associated with the cryo microtome. Alternatively, the formalin fixation process may have caused shrinkage of the spheroids, consistent with previous studies, resulting in a higher density of the formalin sections relative to the cryo sections. Plots of the ratio of elemental content to zinc content are consistent with the latter two explanations. Such a calculation provides a normalisation to the amount of biological material present within the section. In these plots, cisplatin treated spheroid samples showed either similar ratios or higher ratios in cis cryo samples compared to cis formalin samples, in contrast to the trend noted in the elemental content plots.
4.3.2.2.2 Correlation coefficients

The correlation coefficients \( R^2 \) (RSQ) values of platinum with the elements of interest were determined, relative to the RSQ value of phosphorus to sulfur, for each scan. It was thought that this would reveal the elements, if any, that were closely correlated with platinum. It was assumed that the RSQ value of P to S would represent a good correlation within a particular scan. This assumption was based upon a previous study that found P and S to vary little depending upon sample preparation procedures,\(^{261}\) and the results of the current study that found P and S to vary in a similar fashion between sample types (see Figures 4.22, 4.24 above).

Correlation coefficients varied significantly between sample types, and between individual scans. No general trends were evident and thus values for all scans were averaged to provide an approximate indication of the correlation of each element with platinum. The average values and standard deviations are shown in Table 4.3. Appendix Figure A21 shows the variation in correlation coefficients between scans.

**Table 4.3:** Average correlation coefficients of elements to platinum, relative to the phosphorus to sulfur RSQ for each scan obtained from cisplatin and OH treated spheroid samples (%). A value of 100 indicates a correlation coefficient equal to that of P to S. In the upper set of values, OH f centre values have been excluded (due to an unusually high value, as evident from Appendix Figure A21). In the lower set of values, OH f values have been included.

<table>
<thead>
<tr>
<th></th>
<th>Zn</th>
<th>S</th>
<th>P</th>
<th>Ca</th>
<th>Cl</th>
<th>Cu</th>
<th>Fe</th>
<th>K</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average RSQ</td>
<td>50</td>
<td>90</td>
<td>70</td>
<td>50</td>
<td>60</td>
<td>30</td>
<td>30</td>
<td>100</td>
</tr>
<tr>
<td>Stdev</td>
<td>40</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>40</td>
<td>50</td>
<td>30</td>
<td>160</td>
</tr>
<tr>
<td>Average RSQ</td>
<td>200</td>
<td>160</td>
<td>90</td>
<td>200</td>
<td>90</td>
<td>80</td>
<td>120</td>
<td>130</td>
</tr>
<tr>
<td>Stdev</td>
<td>430</td>
<td>210</td>
<td>80</td>
<td>430</td>
<td>80</td>
<td>140</td>
<td>260</td>
<td>160</td>
</tr>
</tbody>
</table>
Chapter 4: Distribution of Platinum Complexes in Multicellular Tumour Spheroids

The large variations in values suggest that correlation coefficients do not provide useful information concerning the elements with which platinum is well correlated in platinum treated spheroid samples. With larger data sets, the value of this technique may increase.

4.3.2.2.3 Cluster analysis

Cluster analysis was performed to ascertain the location of platinum; whether intracellular or extracellular, within the spheroids. The elements on which the analysis was performed were chosen according to the following criteria: high correlation coefficients with platinum (Zn, S, Ca, K, see Section 4.3.2.2.2); known high intracellular concentrations and/or precedent for use as an intracellular marker in previous biological synchrotron studies (Zn, P, S, K); or levels that were dependent upon sample preparation techniques (Ca, K, see Section 4.3.2.2.1). The cluster analyses performed on each elemental map, and the motivations behind each of them are outlined below:

- **P/S:** The SRIXE work outlined in Chapter 3 showed high intracellular levels of these elements. In addition, Vogt et al found little variation in phosphorus and sulfur levels in samples prepared using a variety of techniques, an observation similar to that described above in Section 4.3.2.2.1. P/S clusters within an elemental map were used to define cells. These clusters were compared to those listed below to ascertain the localisation of platinum within spheroids. Comparisons were performed qualitatively by visual inspection of their shape and other features, and quantitatively by comparing their size, location, and platinum content.
- **P/S/Pt:** Similarity of P/S/Pt to P/S clusters was taken to indicate intracellular platinum localisation.
- **Zn/Pt:** Colocalisation of zinc with platinum has previously been used to demonstrate nuclear accumulation of platinum complexes. Thus zinc is another useful intracellular marker. In addition, the work of Section 4.3.2.2.1 indicated
that zinc was not significantly affected by sample preparation procedures, maintaining similar levels in most samples. The location of zinc within an elemental map was used as an additional marker for cellular material. Hence comparison of Zn/Pt clusters to zinc locations provides a further indication of the localisation of platinum. Further, comparison of Zn/Pt clusters to the above mentioned clusters should also assist in this.

- **K/Pt:** Potassium meets all of the criteria mentioned above, with a relatively high correlation coefficient with platinum, high intracellular concentrations, precedent for use as an intracellular marker in biological synchrotron studies, and concentrations dependent upon preparation techniques. The location of potassium within cryo samples can be used to identify cellular regions and thus intracellular localisation of platinum within such spheroids may be ascertained by comparison of K/Pt clusters to other cluster types. It may also provide an indication of how potassium redistributes within samples depending upon preparation techniques.

- **Ca/Pt:** Determination of such clusters was based upon the correlation coefficient of calcium with platinum, and the strong dependence of calcium levels on preparation technique (see Section 4.3.2.2.2 and 4.3.2.2.1, respectively).

- **Zn/Ca/S/Pt:** Zinc, calcium and sulfur were quite well correlated with platinum (Section 4.3.2.2.2) and thus Zn/Ca/S/Pt clusters were identified for comparison to the other types.

We have used cluster analysis to classify pixels in a scan into groups that are similar in content of the elements of choice. Cluster analysis of the above combinations of elements was performed on each elemental map. For example, when analysing the P/S clusters, the MAPS program numbers each P/S cluster (maximum of 4) according to its size, with the largest cluster being assigned as ROI 0, and the smallest ROI 3. Subsequent clusters (P/S/Pt, Zn/Pt, etc) were compared to one another quantitatively in terms of the size, location and platinum content of ROI 0, 1, 2, 3, and qualitatively in terms of their shape (on a visual basis). Distinct structures common to several cluster types were taken to indicate cellular regions. Attempts were made to identify trends within scan groups. No apparent differences were
identified between scans obtained at the edge or centre of the spheroids and hence subsequent discussions will not distinguish these scan types from one another.

In general, most scans from the platinum treated spheroid samples had similar P/S, P/S/Pt and Zn/Pt clusters. This is suggestive of an intracellular localisation of platinum in the spheroids. The size and resolution of the scans should allow for identification of single cells within the spheroids. However, it was difficult to distinguish cellular structures within most scans, or to ascertain whether cryo or formalin procedures better retained the chemical integrity of the samples. A larger number of scans would be required to confirm any distinction between the two sample preparation procedures in this respect. Many of the scans had diffuse distributions of the elements and few distinct structures were evident in the maps of their cluster analyses. In the SRIXE work of Chapter 3, Spurr’s resin was used to embed the single cell samples that were subsequently sectioned (1 μm). Elemental maps of these samples displayed distinct regions that could easily be identified as cells. The same technique of embedding in Spurr’s resin has been used in the preparation of thin sections (1 μm) of platinum treated mouse tumours.\textsuperscript{264, 265} High resolution 20 x 20 μm scans displayed a number of cells, particularly evident in the zinc, calcium and phosphorus maps. Distinct cellular regions of this type were largely absent from the spheroid SRIXE scans of the current work, most likely a consequence of the relatively harsh sample preparation conditions.\textsuperscript{266} Figures 4.32 - 4.36 show elemental maps of spheroid samples that displayed some distinct regions perhaps consistent with cellular structures.
**Figure 4.32:** Elemental maps (above) and cluster analysis maps (below) obtained from a spheroid that had been treated with cisplatin, then formalin fixed and paraffin embedded. The relative colour scale for the elemental maps is shown, with black representing the lowest concentration and red the highest. In the cluster analysis maps, pixels of the same colour belong to one distinct cluster, where red = ROI 0, green = ROI 1, blue = ROI 2, turquoise = ROI 3, in order of increasing cluster size. Distinct structures are seen, particularly in the phosphorus and sulfur maps, and the P/S and P/S/Pt cluster maps, perhaps indicative of cellular structures. Scan is 30.5 x 30.5 µm in size, and a 10 µm scale bar is also shown.
Figure 4.33: Elemental maps (above) and cluster analysis maps (below) obtained from a spheroid that had been treated with cisplatin, then formalin fixed and paraffin embedded. The relative colour scale for the elemental maps is shown, with black representing the lowest concentration and red the highest. In the cluster analysis maps, pixels of the same colour belong to one distinct cluster. Distinct structures are seen, particularly in the P/S, P/S/Pt, Zn/Pt and Zn/Ca/S/Pt cluster maps, perhaps indicative of cellular structures. Scan is 30.5 x 30.5 µm in size, and a 10 µm scale bar is also shown.
Figure 4.34: Elemental maps (above) and cluster analysis maps (below) obtained from a spheroid that had been treated with cisplatin, then cryo embedded. The relative colour scale for the elemental maps is shown, with black representing the lowest concentration and red the highest. In the cluster analysis maps, pixels of the same colour belong to one distinct cluster. Distinct structures are seen in most of the cluster maps and many of the elemental maps, perhaps indicative of cellular structures. Scan is 25.5 x 25.5 µm in size, and a 10 µm scale bar is also shown.
**Figure 4.35:** Elemental maps (above) and cluster analysis maps (below) obtained from a spheroid that had been treated with OH, then cryo embedded. The relative colour scale for the elemental maps is shown, with black representing the lowest concentration and red the highest. In the cluster analysis maps, pixels of the same colour belong to one distinct cluster. Distinct structures are seen in most of the cluster maps, perhaps indicative of cellular structures. Scan is 30.5 x 30.5 µm in size, and a 10 µm scale bar is also shown.
Figure 4.36: Elemental maps (above) and cluster analysis maps (below) obtained from a spheroid that had been treated with OH, then cryo embedded. The relative colour scale for the elemental maps is shown, with black representing the lowest concentration and red the highest. In the cluster analysis maps, pixels of the same colour belong to one distinct cluster. Distinct structures are seen in many of the cluster maps, and some of the elemental maps, perhaps indicative of cellular structures. Scan is 25.5 x 25.5 µm in size, and a 10 µm scale bar is also shown.

K/Pt and Ca/Pt clusters showed significant variation between preparation procedures. The K/Pt clusters in cryo embedded spheroids were quite similar to the other elemental clusters within the same scan, whereas in formalin fixed spheroids, K/Pt clusters tended to be visually different from the other elemental clusters within
a scan. In scans from cryo embedded samples, Ca/Pt clusters were different to the other cluster types within the same scan, whilst scans from formalin fixed spheroids had similar Ca/Pt clusters compared to the other elemental cluster types within a scan. These results are consistent with the work outlined in Section 4.3.2.2.1 that showed the potassium and calcium content of the spheroids to vary depending upon the procedure used to prepare the samples, with calcium levels being considerably higher in formalin samples than cryo samples, and potassium levels being higher in cryo than formalin samples. In addition, calcium tended to be localised in discrete regions of the formalin spheroids, though more diffusely distributed in cryo samples. The identity of these discrete regions is unknown. The results also confirm that potassium is an easily diffusible element that is likely to be washed out during formalin fixation/paraffin embedding procedures, consistent with the previous work of Vogt et al.\textsuperscript{261} On the other hand, calcium seems to consistently be lost during cryo embedding procedures, or perhaps gained during formalin fixation procedures. The processes by which this occurs are not known. Perhaps damage of cell membranes allows calcium to leak into or out of the spheroids.

In summary, cluster analysis has indicated that most of the platinum within cisplatin and OH treated spheroids is intracellular in both the central and peripheral regions of the spheroids. This observation appears to be independent of the procedure that had been used to prepare the spheroids for analysis. However, the sample preparation procedures treat the samples harshly and result in elemental redistribution and loss. Future work of a similar nature should involve a larger scan set and/or Spurr’s resin embedding or a similarly gentle preparation technique.

### 4.3.3 Micro-XANES

Pt LII edge XANES spectra were collected at selected points within 20 µm slices of spheroids treated with 50 µM OH or 50 µM cisplatin for 24 hours. The experiments were performed on beam line 2ID-D at the Advanced Photon Source (APS) using the samples analysed as outlined in Section 4.3.2.2. The aim of this work was to
determine the oxidation state of the platinum(IV) species (OH) at the periphery of the spheroids as compared to the central regions of the spheroids.

Figure 4.37 shows the normalised XANES spectra obtained in spheroid sections. Table 4.4 shows the peak height ratios (a/b) for each of the spectra. Note that the spectra are Pt LII edge XANES, whereas the previous XANES work of the Hambley group, including that outlined in Chapter 2, were obtained at the Pt LIII edge. Pt LII edge XANES was used in the current study due to the relative ease of adjusting the experimental setup from use with micro-SRIXE. The peak height ratio of these spectra may not necessarily fall within the ranges that have been determined for platinum(II) and platinum(IV) complexes using Pt LIII edge XANES. Any changes are expected to be relatively small, given the similarity in the electronic transitions that are excited in each case (LIII edge corresponds to excitation from the \(p_{3/2}\) orbital, LII edge corresponds to excitation from the \(p_{1/2}\) orbital). Further experiments are required to verify this assumption.

![Figure 4.37](image)

**Figure 4.37:** Pt LII XANES spectra obtained from regions at the edge or centre of 20 \(\mu\)m spheroid sections. Spheroids were treated with 50 \(\mu\)M cis (cisplatin) or OH for 24 hours, “f” denotes a spheroid that was formalin fixed and paraffin embedded, whilst “cr” denotes a spheroid that was cryo embedded. An identical sample number
indicates that the spectra originated from different spheroids within the same slice, whilst an identical sample number and letter indicates that the spectra were obtained from different areas within the same spheroid section. Acquisition times are also indicated for each of the scans.

Table 4.4: The ratio of the peak height (parameter a) to the post-edge minima (parameter b) for XANES spectra shown in Figure 4.37.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Complex</th>
<th>Region</th>
<th>Treatment</th>
<th>a/b ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>OH</td>
<td>edge</td>
<td>f</td>
<td>1.46</td>
</tr>
<tr>
<td>1A</td>
<td>OH</td>
<td>centre</td>
<td>f</td>
<td>1.52</td>
</tr>
<tr>
<td>1B</td>
<td>OH</td>
<td>centre</td>
<td>f</td>
<td>1.43</td>
</tr>
<tr>
<td>2</td>
<td>cis</td>
<td>centre</td>
<td>f</td>
<td>1.50</td>
</tr>
<tr>
<td>3A</td>
<td>OH</td>
<td>edge</td>
<td>cr</td>
<td>1.63</td>
</tr>
<tr>
<td>3A</td>
<td>OH</td>
<td>centre</td>
<td>cr</td>
<td>1.62</td>
</tr>
</tbody>
</table>

The XANES spectrum obtained from a region in the centre of a spheroid treated with cisplatin for 24 hours (sample 2) is a suitable “standard” against which to compare the spectra of OH treated spheroids. In addition, it enables comparison of the a/b ratio previously determined for platinum complexes at the Pt LIII edge (platinum(II) a/b = 1.52 ± 0.08; platinum(IV) a/b = 2.51 ± 0.13).36

From Figure 4.37 it can be seen that all spectra have a qualitatively similar shape to that of sample 2 (cisplatin), indicating that the platinum in the spheroids is in the platinum(II) oxidation state. The ratios shown in Table 4.4 provide an indication of the variation between spectra. It should be emphasised that the spectra originated from very small regions (the size of the beam) and as such may not be representative of the bulk sample.

The average peak height ratio for the OH spectra was 1.53 ± 0.09. Sample 1A centre and 1B centre provide an indication of the experimental variation (0.09), given that their spectra were obtained from regions that should contain approximately the same
CHAPTER 4: Distribution of Platinum Complexes in Multicellular Tumour Spheroids

proportion of platinum(II)/platinum(IV). Given that the ratio for the cisplatin treated sample (sample 2, 1.50) lies within this range, it is reasonable to assume that platinum is present as platinum(II) within the central and peripheral regions of the spheroids.

Micro-XANES has been used previously within our research group where spectra were obtained at points of high platinum concentration within single cells that had been treated with cisplatin, Cl4, OAc or OH, though using a different energy to the studies described above. The results, with the exception of those for OH, were consistent with the presence of platinum(II) species within the cells. The spectra obtained in a cell that had been treated with OH more closely resembled a platinum(IV) XANES spectra. This result was in contrast to XANES experiments in bulk cell samples whereby it was found that all of the platinum had been reduced to platinum(II) within cells treated with OH in an identical fashion. The latter technique was deemed more reliable, given that it averaged over a large number of cells, and that the noise levels in the micro-XANES spectra caused uncertainty in the results.

4.3.4 Wash out experiments

Conventional histological procedures are known to wash away much of the test substance from spheroid samples and are likely to leave only that covalently bound to cells or extracellular matrix. The work outlined in this chapter has utilised a variety of sample preparation procedures. A washout study was performed to gain a preliminary indication of the extent to which the platinum test substances were retained by the spheroids following subjection to such procedures. Radiolabelled platinum complexes ([PtCl2(14C-en)] and cis,trans-[PtCl2(OH)2(14C-en)]) were used in this study. These complexes are closely related to cisplatin and OH and are thus suitable model complexes to use. It should be noted that the results reported herein are preliminary in nature and as such, the data should be taken to indicate general, rather than quantitative trends. Repeats of the experiments are required to obtain statistically significant data sets.
Figure 4.38 shows the concentration of radioactive substance remaining in the spheroid samples at various stages of the sample preparation procedure. It can be seen that the fixation process causes some loss of test substance. Subsequent processing of the samples causes further loss.
Figure 4.38: Average concentration of $[\text{PtCl}_2^{(14}\text{C-en})]$ and cis,trans-$[\text{PtCl}_2(\text{OH})_2^{(14}\text{C-en})]$ remaining in spheroids after 24 hour incubation and various sample processing procedures. “Medium” indicates the initial dosing concentration; “Treatment” indicates the concentration of platinum complex taken up by the spheroids during the incubation; “Fixing” indicates the concentration of platinum complex remaining in the spheroids following fixation in neutral buffered formalin for at least 2.5 hr; “Processing” indicates the concentration of platinum complex remaining in the spheroid following routine histopathology processing procedures (involving several washes of ethanol and xylene). Note that the results shown for the platinum(II) treated spheroids were averaged from two independent measurements, whilst those shown for the platinum(IV) treated spheroids were averaged from three independent measurements.

These results are consistent with previous studies that found similar histological procedures to cause wash out of test substances.\textsuperscript{195, 218, 220} The rate of element loss from a sample during such procedures is known to be dependent upon specimen size, processing temperature, and fixative.\textsuperscript{263} In addition, the procedures may promote leakage of some cytoplasmic components, aggregation artefacts, and possibly structural rearrangements over the course of the slow chemical fixation process.\textsuperscript{266, 267} The work of this chapter should be viewed in light of the washout studies outlined above. Spheroid samples analysed herein have largely been prepared by formalin fixation followed by paraffin embedding, involving histological processing in an identical fashion to the procedures used for the “Processing” samples (see Figure 4.38 and Section 4.2.4.2). Spheroid samples for microtomography analysis were prepared by the fixation and storage of whole spheroids in formalin. This procedure has also been shown to cause some loss of platinum (see Figure 4.38, “Fixing”). In light of these results, the analyses presented in this chapter of platinum distribution through a spheroid volume do not necessarily indicate the ability of a platinum complex to penetrate into the central area of a spheroid. Such information can only be inferred since the majority of the unbound platinum species is likely to have been washed away during the preparation
procedures. Rather, these results are more likely to indicate the regions at which the substances were able to irreversibly bind.

It should be noted that a small number of samples were prepared by cryo embedding, involving a brief PBS wash, followed by embedding in OCT embedding medium, rapid freezing and subsequent sectioning. Such a procedure should result in smaller loss of platinum than from the formalin/paraffin samples. Cryo embedded samples have been shown to maintain close to accurate elemental concentrations, although the structural preservation of the samples is questionable.\textsuperscript{261} The formation of ice crystals during the procedure may damage structures such as membranes, and perhaps cause enrichment of electrolytes on ice crystal boundaries, resulting in a redistribution of the elements within the cells.\textsuperscript{260, 266, 268, 269} Similar damage is likely to have occurred in the samples studied herein. SRIXE analysis of cryo and formalin/paraffin samples (Section 4.3.2.2) suggested that significant redistribution of elements had occurred within the spheroids following these treatments. To avoid such damage, alterations to the cryo preparation procedure used in the current work should be considered. Such changes may include rapid freezing in isopentane (cooled in liquid nitrogen), rather than straight liquid nitrogen; sectioning at lower temperatures; and freeze drying, rather than air drying of the resultant thin sections.\textsuperscript{260} Other possible techniques have been developed, and compared to conventional chemical fixation procedures (such as formalin fixation/paraffin embedding).\textsuperscript{262, 266, 269-271} Whilst there are numerous advantages to using cryo procedures, significant expertise is required.\textsuperscript{266} Hence formalin/paraffin samples represent the majority of those analysed herein. This was also partly motivated by the fact that previous work had shown paraffin embedded samples to maintain their structural integrity and the concentrations of tightly bound elements, though with some washout of more easily diffusible elements.\textsuperscript{261}

4.3.5 Combined discussion

It is a requirement of effective solid tumour treatment that the anticancer agent gains access to all viable cells within a tumour at sufficient concentrations to cause
cell death.\textsuperscript{214, 272} However, poor drug penetration is often an obstacle to the treatment of solid tumours. Multicellular tumour spheroids have proven to be a useful model for studying drug penetration, whereby the drugs face similar barriers in penetrating throughout the entire spheroid volume as they do the tumour volume. In the current work, DLD-1 spheroids have been used to investigate the penetration and distribution of cisplatin and several of its platinum(IV) analogues. Included within this study was a comparison of a number of procedures commonly used to prepare biological samples for analysis. A variety of mainly synchrotron techniques have formed the basis for such studies. Each of the techniques differ, mainly in the resolution, and hence the information able to be gleaned from each of them. The techniques ranged from micro-tomography, whereby a low resolution overview of the elemental distribution in an entire slice through a spheroid was obtained without the need for physical sectioning; to micro-SRIXE, whereby high resolution scans on a cellular scale were obtained from sectioned spheroid samples. Micro-XANES was also used to monitor the oxidation state of the platinum species at various points within the spheroid volume.

Cisplatin and the platinum(IV) analogues have been shown to be capable of entering the spheroid environment and penetrating through to the central regions. The techniques used herein were semi-quantitative and involved direct monitoring of platinum locations within the spheroids. This is in contrast to the largely indirect measurements that have been used previously to ascertain penetration of platinum complexes into spheroids.\textsuperscript{221, 227, 230-232}

The distribution of platinum seen within the samples varied depending upon the technique used to prepare the spheroids and/or the technique used to analyse the spheroids. Microtomography (Section 4.3.1) suggested an enrichment of platinum in peripheral cell layers of the spheroids, with a lower, uniform distribution in the inner layers (approximately half the platinum concentration of that in the outer layers). This distribution pattern was consistently observed in all platinum treated spheroid samples. On the other hand, micro-SRIXE analysis of the spheroids (see Section 4.3.2.1 and 4.3.2.2) showed no significant difference in the platinum content of the
inner and outer regions of the spheroids. The differences in the results may be a consequence of the procedures used to prepare the samples, all of which were shown to result in loss of elements from the spheroids. Formalin fixation and subsequent storage (such as that used for preparation of spheroids for microtomography analysis) resulted in washout of platinum from the spheroids (see Figure 4.38, Section 4.3.4). The vast majority of this platinum is likely to have been unbound. Thus the microtomography images most likely show the distribution of bound platinum species. Histological processing, paraffin embedding and sectioning (such as was used to prepare samples for micro-SRIXE) resulted in additional loss of platinum from the spheroids. This may have involved the loss of all unbound platinum, as well as many bound platinum species, in a similar fashion to that observed previously.\textsuperscript{195, 218, 220} Thus as a result of such losses, the distribution of platinum observed in such samples appears to be uniform, with the only remaining platinum species those that were tightly bound and able to withstand the preparation procedures.

Hence it seems that formalin fixation and storage of the spheroids for microtomography analysis is the most favourable of the procedures used herein to analyse platinum treated spheroids. However, only information concerning the distribution of bound platinum species can be gained and this does not reflect the ability of the complexes to penetrate into the spheroid samples. In addition, only broad, low resolution scans can be performed on such samples, and there are restrictions as to the size of the spheroids and the elements that are able to analysed. Should higher resolution, smaller scans be required to obtain elemental information on a cellular level, alternative preparation procedures should be attempted, such as Spurr’s resin embedding (used previously for SRIXE analysis of platinum treated mouse tumours).\textsuperscript{264} This seems to be a more gentle procedure than those used herein that better maintains elemental distributions within the samples. Alternatively, ‘dry’ histological procedures that preserve bound and unbound forms of drug, such as that outlined by Nederman et al,\textsuperscript{195, 241} may also be useful.
Micro-XANES was used to determine the oxidation state of the platinum species present at the inner and outer regions of spheroids that had been treated with the platinum(IV) dihydroxo complex (OH). This analysis indicated no differences in oxidation state at the centre and edge of such spheroids, being largely platinum(II) throughout. In line with the above discussion, the analysis only provides information regarding the oxidation state of bound platinum species within the spheroids. The platinum(IV) dihydroxo complex (OH) is the most difficult to reduce of the platinum(IV) complexes studied herein and hence it is assumed that observations in spheroids treated with the Cl4 or OAc complexes would be similar.

The distribution of copper within the spheroids was also found to be of particular interest, with many samples displaying an enrichment of copper in the peripheral cell layers. Previous studies have shown that copper (and zinc) are not washed out during the formalin fixation process. In formulating possible explanations for the observed copper distributions, we assume the following:

- Copper washout during histological processing was minimal.
- Processing did not cause redistribution of copper within the spheroids.
- The procedures used to prepare the samples on separate occasions were the same. As such, this is unable to explain the different copper distributions observed in the microtomography samples during the two separate synchrotron visits (see Section 4.3.1.2).
- Differences in instrumental setup can not account for the variations in copper distributions that were observed.

Copper is known to play a vital role in tumour biology. Copper levels in many cancers are higher than that of normal tissue, and the localisation of copper differs between tissue types; with cytoplasmic accumulation in normal tissue, and intra- and perinuclear localisation in tumour tissue. Copper plays a role in the early stages of tumour angiogenesis; that is, in the growth of a tumour’s blood supply. Tumours up to approximately 1-2 mm in diameter are able to obtain the nutrients necessary for growth by passive diffusion from surrounding blood vessels, however, further growth requires angiogenesis. Copper activates endogenous angiogenic factors,
such as vascular endothelial growth factor (VEGF), that play a central role in stimulating the angiogenic process, though the molecular mechanisms by which copper regulates this process remain unknown. Regions of VEGF expression in spheroids are dependent upon the nutrient supply to the cells. When spheroids were grown under normal conditions, such that the central core was necrotic, VEGF was upregulated in the inner hypoxic cells. On the other hand, spheroid growth under low glucose conditions resulted in VEGF upregulation in peripheral cells. We propose the following explanation for the spheroidal copper distributions observed in the current investigation: redistribution of copper within the spheroids may take place in response to nutrient deficiencies (such as oxygen or glucose), and copper accumulation is likely to occur in regions of VEGF expression. The extent of copper redistribution is likely to be dependent upon spheroid size, cell phase, growth conditions, and may also vary between individual spheroids. Hence such a process may account for the copper distributions observed in the spheroids of the current investigation. Further studies are required to confirm this hypothesis and should involve determination of the areas of VEGF upregulation in spheroids grown under conditions identical to those used in the current studies.

The work outlined in this chapter may have implications for platinum chemotherapy in solid tumours. The development of relatively unreactive platinum(IV) complexes (less reactive than OH) may help to improve the effectiveness of platinum therapies. Such complexes should have an enhanced ability to penetrate into the central regions of tumours and/or spheroids without undergoing significant reactions or reduction in peripheral cell layers. Thus higher drug concentrations are able to reach all areas of the tumour, resulting in a more effective treatment. Multicellular tumour spheroids should play a major role in assessment of the efficacy of such novel platinum complexes and synchrotron techniques similar to those outlined herein may assist in such evaluations.
CHAPTER 5

Conclusions
The behaviour of several platinum complexes in biological systems has been assessed. XANES spectroscopy was used to monitor the interaction of cisplatin and some of its platinum(IV) analogues with DNA. It was found that cisplatin undergoes ligand changes during incubation with DNA, suggestive of aquation and subsequent reaction with DNA. The platinum(IV) complexes were not reduced over the course of the DNA incubation. Any reaction with DNA may have been either direct or platinum(II) catalysed. In particular, there was some evidence that this had occurred with the tetrachloro platinum(IV) complex, suggested by changes in its coordination sphere. Alternatively, these changes may reflect aquation of the complex.

The behaviour of platinum(II) anthraquinone complexes and their corresponding ligands was investigated in tumour cells using several techniques. Cytotoxicity and cell uptake studies have shown that the platinum complexes rapidly enter cells, though their toxicity was not significantly different than that of the corresponding ligand. This may be because both the complex and the ligand are sequestered to the lysosomal compartments of the cells, as suggested by confocal microscopy. Alternatively, this observed cellular distribution may be the result of degradation of the platinum complex and subsequent entry of a fluorescent species of some description into the lysosomes, accompanied by entry of a platinum species into the cell nuclei. A further possibility is that the platinum complex binds to certain proteins that cause the quenching of its fluorescence, as well as allowing transport into the cell nuclei. The latter two possibilities are consistent with micro-SRIXE analysis of single cells treated with a platinum anthraquinone complex, showing that platinum has entered the nuclei of the cells. These studies have indicated that the presence of the anthraquinone moiety endows the platinum complexes with favourable cellular uptake properties. However, the DNA targeting efficacy of such complexes appears questionable.

The distribution of platinum(IV) cisplatin analogues was studied in multicellular tumour spheroids using a number of synchrotron techniques. Microtomography is an ideal technique by which to study platinum distributions within platinum treated spheroid samples. The technique is non-destructive in nature and provides a broad
overview of elemental distributions within the samples. Platinum was present within all regions of the spheroids, with higher concentrations in the outer cells. In contrast, micro-SRIXE techniques revealed an essentially uniform distribution of platinum throughout the samples, of which the majority was present as platinum(II) (as indicated by micro-XANES). These apparent differences are likely to be a consequence of the procedures used for spheroid preparation. Such procedures have been shown to result in loss of some platinum species from the spheroids. Regardless of the anomalies, it is clear that the platinum complexes are capable of penetration throughout the entire spheroid volume. However, more inert platinum(IV) species may be capable of penetrating the spheroid volume at higher concentrations, and thus such species are worth investigating in the pursuit of more effective platinum anticancer agents.
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Appendix
Figure A.1: The resulting fluorescence spectra upon titration of small quantities of calf thymus DNA (in phosphate buffer) to a 20 mM solution of 1C3 in 2.4 % DMF, 1.6 % 100 mM KCl, and phosphate buffer solution. The ratios shown are that of 1C3:DNA base pairs. See Section 3.2.7 for full experimental details.
**Figure A.2:** The resulting fluorescence spectra upon titration of small quantities of calf thymus DNA (in phosphate buffer) to a 20 mM solution of 1C3 (in phosphate buffer). The ratios shown are that of 1C3:DNA base pairs. See Section 3.2.7 for full experimental details.

**Figure A.3:** The resulting fluorescence spectra upon titration of small quantities of calf thymus DNA (in phosphate buffer) to a 20 mM solution of Pt1C3 in 2.4 % DMF, 1.6 % 100 mM KCl, and phosphate buffer solution. The ratios shown are that of Pt1C3:DNA base pairs. See Section 3.2.7 for full experimental details.
Figure A.4: The resulting fluorescence spectra upon titration of small quantities of calf thymus DNA (in phosphate buffer) to a 20 mM solution of Pt1C3 in 60 % (v/v) DMF, 40 % (v/v) 100 mM KCl/5 mM phosphate buffer solution. The ratios shown are that of Pt1C3:DNA base pairs. See Section 3.2.7 for full experimental details.

Figure A.5: Light microscope image (x10) of an cisplatin treated spheroid that was formalin fixed/paraffin embedded, and sectioned at 20 µm. The analysis of such samples was performed at ESRF and has been outlined in Section 4.2.4.2 and 4.3.2.1.
Figure A.6: Light microscope image (x10) of a cisplatin treated spheroid that was cryo embedded and sectioned at 20 µm. The analysis of such samples was performed at APS and has been outlined in Section 4.2.4.2 and 4.3.2.2.

Figure A.7: Light microscope image (x4) of an OH treated spheroid that was formalin fixed/paraffin embedded, and sectioned at 20 µm. The analysis of such samples was performed at APS and has been outlined in Section 4.2.4.2 and 4.3.2.2.
**Figure A.8:** The elemental content as a ratio of zinc content of edge or central regions of cisplatin treated spheroids relative to a region representative of the entire spheroid. Values are expressed as percentages, where a value of 100 is indicative of an elemental content to zinc ratio identical to that of the whole spheroid. Spheroids were analysed at ESRF (see Section 4.2.4.2 and 4.3.2.1).
Figure A.9: The elemental content as a ratio of zinc content of edge or central regions of OH treated spheroids relative to a region representative of the entire spheroid. Values are expressed as percentages, where a value of 100 is indicative of an elemental content to zinc ratio identical to that of the whole spheroid. Spheroids were analysed at ESRF (see Section 4.2.4.2 and 4.3.2.1).
Figure A.10: The elemental content as a ratio of zinc content of edge or central regions of control spheroids relative to a region representative of the entire spheroid. Values are expressed as percentages, where a value of 100 is indicative of an elemental content to zinc ratio identical to that of the whole spheroid. Spheroids were analysed at ESRF (see Section 4.2.4.2 and 4.3.2.1).
Figure A.11: Average zinc content (µg cm\(^{-3}\)) of scans obtained from spheroid sections (20 µm thickness). Scans were grouped according to the complex with which the spheroids had been treated (cis, OH, control (con)), and the region of the spheroid from which the scan was obtained (edge, centre). The errors shown represent the standard deviation of the data sets. Spheroids were analysed at ESRF (see Section 4.2.4.2 and 4.3.2.1).
Figure A.12: Average iron content ($\mu$g cm$^{-3}$) of scans obtained from spheroid sections (20 $\mu$m thickness). Scans were grouped according to the complex with which the spheroids had been treated (cis, OH, control (con)), and the region of the spheroid from which the scan was obtained (edge, centre). The errors shown represent the standard deviation of the data sets. Spheroids were analysed at ESRF (see Section 4.2.4.2 and 4.3.2.1).
Figure A.13: Average calcium content (µg cm\(^{-3}\)) of scans obtained from spheroid sections (20 µm thickness). Scans were grouped according to the complex with which the spheroids had been treated (cis, OH, control (con)), and the region of the spheroid from which the scan was obtained (edge, centre). The errors shown represent the standard deviation of the data sets. Spheroids were analysed at ESRF (see Section 4.2.4.2 and 4.3.2.1).
**Figure A.14:** Average potassium content (µg cm⁻³) of scans obtained from spheroid sections (20 µm thickness). Scans were grouped according to the complex with which the spheroids had been treated (cis, OH, control (con)), and the region of the spheroid from which the scan was obtained (edge, centre). The errors shown represent the standard deviation of the data sets. Spheroids were analysed at ESRF (see Section 4.2.4.2 and 4.3.2.1).
Figure A.15: Average chlorine content (µg cm\(^{-3}\)) of scans obtained from spheroid sections (20 µm thickness). Scans were grouped according to the complex with which the spheroids had been treated (cis, OH, control (Con)), the procedure used to prepare the samples (cryo (cr), formalin (f)), and the region of the spheroid from which the scan was obtained (edge, centre). The number of scans (n) used to calculate the averages are also shown. The errors shown represent the standard deviation of the data sets. Spheroids were analysed at APS (see Section 4.2.4.2 and 4.3.2.2.1).
Figure A.16: Average Cl:Zn ratios of scans obtained from spheroid sections (20 \( \mu \text{m} \) thickness). Scans were grouped according to the complex with which the spheroids had been treated (cis, OH, control (Con)), the procedure used to prepare the samples (cryo (cr), formalin (f)), and the region of the spheroid from which the scan was obtained (edge, centre). The errors shown represent the standard deviation of the data sets. Spheroids were analysed at APS (see Section 4.2.4.2 and 4.3.2.2.1).
Figure A.17: Average iron content (µg cm\(^{-3}\)) of scans obtained from spheroid sections (20 µm thickness). Scans were grouped according to the complex with which the spheroids had been treated (cis, OH, control (Con)), the procedure used to prepare the samples (cryo (cr), formalin (f)), and the region of the spheroid from which the scan was obtained (edge, centre). The number of scans (n) used to calculate the averages are also shown. The errors shown represent the standard deviation of the data sets. Spheroids were analysed at APS (see Section 4.2.4.2 and 4.3.2.2.1).
Figure A.18: Average Fe:Zn ratios of scans obtained from spheroid sections (20 µm thickness). Scans were grouped according to the complex with which the spheroids had been treated (cis, OH, control (Con)), the procedure used to prepare the samples (cryo (cr), formalin (f)), and the region of the spheroid from which the scan was obtained (edge, centre). The errors shown represent the standard deviation of the data sets. Spheroids were analysed at APS (see Section 4.2.4.2 and 4.3.2.2.1).
Figure A.19: Average nickel content (µg cm\(^{-3}\)) of scans obtained from spheroid sections (20 µm thickness). Scans were grouped according to the complex with which the spheroids had been treated (cis, OH, control (Con)), the procedure used to prepare the samples (cryo (cr), formalin (f)), and the region of the spheroid from which the scan was obtained (edge, centre). The number of scans (n) used to calculate the averages are also shown. The errors shown represent the standard deviation of the data sets. Spheroids were analysed at APS (see Section 4.2.4.2 and 4.3.2.2.1).
Figure A.20: Average Ni:Zn ratios of scans obtained from spheroid sections (20 µm thickness). Scans were grouped according to the complex with which the spheroids had been treated (cis, OH, control (Con)), the procedure used to prepare the samples (cryo (cr), formalin (f)), and the region of the spheroid from which the scan was obtained (edge, centre). The errors shown represent the standard deviation of the data sets. Spheroids were analysed at APS (see Section 4.2.4.2 and 4.3.2.2.1).
Figure A.21: Correlation coefficients of elements (as shown) to platinum, relative to P to S RSQ for each treatment type in formalin or cryo embedded spheroid samples treated with cisplatin or OH. Values close to 100 indicate good correlation with platinum. Spheroids were analysed at APS (see Section 4.2.4.2 and 4.3.2.2.2).
A.2 Publications during candidature

