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INVESTIGATING PEPTIDE CONJUGATION FOR THE SELECTIVE ACTIVATION OF CYTOTOXIC METAL COMPLEXES IN SOLID TUMOURS

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Abstract

There are various strategies in the treatment of cancer including surgical excision, radiotherapy, chemotherapy and immunotherapy. Chemotherapy offers the distinct advantage of systemic distribution, with the potential to reach primary tumours as well as metastases. A significant drawback of the vast majority of chemotherapeutic agents is their lack of specificity for cancer cells as well as poor tumour penetration, meaning that cells distal to blood vessels receive an inadequate dose and remain viable. There is an imperative need for the development of anti-tumour drugs which exhibit good tumour penetration and distribution with minimal systemic toxicity. Tumour activated prodrugs (TAPs) are one of the main strategies now employed in antitumour drug development, and involve administering an inert, less toxic form of a particular drug which is then selectively transformed into its toxic version by the body in the vicinity of the tumour.\(^1\)

In this study, TAPs which contain a matrix-metalloproteinase II (MMP-2) specific peptide cleavage sequence were synthesised. There is significant evidence that there is increased expression of MMP-1, -2, -3, -7, -9, -13, -14 in both primary tumours and metastases, and that there is a positive correlation between high levels of MMP expression and poor patient prognosis.\(^2\)

In this work, recombinant DNA techniques were used to visualise the expression of MMP-2 and MMP-9 \textit{in vitro}. Plasmid constructs in which MMP-2 and MMP-9 were fused to the AmCyan fluorescent protein gene were generated for insertion into a mammalian transfection vector. Transfection into three cell lines that exhibited different levels of MMP-2 and MMP-9 expression was evaluated by measuring the fluorescence emission in the 480 – 550 nm range. The results demonstrated potential for monitoring MMP expression and secretion in monolayer and 3D cell culture by transfection with MMP-fluorescent protein plasmid vectors. However, employing AmCyan as the fluorescent protein resulted in an unexpected interaction with the riboflavin component of cell culture media whereby fluorescence emission could not be observed in the 500 - 550 nm range as expected. The use of other fluorescent proteins should also be pursued, as well as the use of cell-culture media which is riboflavin-free.

In order to evaluate the potential of targeting MMP-2 overexpression in solid tumours for selective activation of a TAP, a series of model fluorophore-peptide substrates were investigated in cell monolayers and 3D multicellular tumour spheroids. The target compound
DDDK(FITC)DIPVSLRSK(RhB) (4.8) contained a tetra-aspartate (DDDD) uptake-blocking group designed to prevent influx of the compound into the cell prior to activation by MMP-2 cleavage. Additionally the fluorescein isothiocyanate and rhodamine B fluorophores were attached to the peptide on opposing sites of the MMP-2 cleavage site, facilitating visualisation of the intact peptide as well as the post-cleavage fragments.

LC-MS studies showed that in the absence of the uptake-blocking group K(FITC)DIPVSLRSK(RhB) (4.5) is almost entirely cleaved into its respective fragments K(FITC)DIPVS (4.6) and LRKS(RhB) (4.7) after 24 hours. LC-MS results also showed that the uptake-blocking group slows cleavage of the peptide, a trait which was also observed in fluorescence confocal microscopy.

K(FITC)DIPVSLRSK(RhB) (4.5) and DDDDK(FITC)DIPVSLRSK(RhB) (4.8) were tested in DLD-1 cell monolayers in the absence of MMP-2 activity. After 4 hours K(FITC)DIPVSLRSK(RhB) (4.5) was found to have entered the cells intact and undergone a small degree of cleavage, while DDDDK(FITC)DIPVSLRSK(RhB) (4.8) did not enter cells intact, and instead only a very minor amount of intracellular fluorescence in the red channel was visible, indicative of non-specific cleavage. In the presence of MMP-2, less uptake of the intact K(FITC)DIPVSLRSK(RhB) (4.5) peptide was observed and instead discreet localised fluorescence was observed in the red and green channels, suggesting that cleavage was occurring in the extracellular space. This result was also observed, but to a lesser degree, for DDDDK(FITC)DIPVSLRSK(RhB) (4.8).

These compounds were then also tested in multicellular tumour spheroid models, where the intact peptide K(FITC)DIPVSLRSK(RhB) (4.5) underwent cleavage in the surrounding media, resulting in sequestration of the LRSK(RhB) (4.7) fragment by the outermost cells, preventing it from penetrating further into the spheroid. Contrarily, the slower cleavage of DDDDK(FITC)DIPVSLRSK(RhB) (4.8) improved the distribution of fluorescence in the red channel, having allowed the intact peptide to diffuse further into the spheroid before cleavage and cellular uptake of the LRSK(RhB) (4.7) fragment. These results confirmed the importance of the uptake-blocking group for ensuring delivery of the payload to the less accessible regions of solid tumour.

Overall, this work demonstrated the potential of using an MMP-2 specific cleavage sequence for the selective delivery of chemotherapeutic agents to solid tumours, and as such a series of cytotoxin-peptide substrates containing the MMP-2 specific cleavage sequence were synthesised. The cytotoxic compounds investigated were the platinum(IV) complex cis, cis,
trans-acetato[(1R,2R)-cyclohexane-1,2-diamine-N,N’succinatoxalatoplatinum(IV)] and ruthenium(II) complex [(4-methyl-4’-carboxy-2,2’-bipyridine)bis(4,4’-di-tert-butyl-2,2’-bipyridine)]ruthenium(II) hexafluorophosphate.

The cytotoxic properties of the platinum(IV)-peptide conjugates were tested, with none of the peptide-Pt(IV) conjugates possessing IC\(_{50}\) values similar to the free platinum(II) precursor. The intact DIPVSLRSK(Pt) (5.8) peptide is slightly less toxic than its post-cleavage fragment LRSK(Pt) (5.7) in the absence of MMP-2, but the IC\(_{50}\) of DIPVSLRSK(Pt) (5.8) is almost identical to LRSK(Pt) (5.7) in the presence of MMP-2, suggesting that MMP-2 is contributing to the activation and subsequent cytotoxicity of the compound. In both cell lines, the conjugate which contains the uptake-blocking group DDDDGDIPVSLRSK(Pt) (5.9) is not cytotoxic, proving that incorporation of the tetra-aspartate moiety can modify the activity of these compounds.

Platinum accumulation studies in cell monolayers did not show a significant difference between the levels of intracellular platinum following incubation with LRSK(Pt) (5.7), DIPVSLRSK(Pt) (5.8), and DDDDGDIPVSLRSK(Pt) (5.9). However, no platinum was detected in the hypoxic/necrotic regions or periphery of spheroids treated with LRSK(Pt) (5.7) and DDDDGDIPVSLRSK(Pt) (5.9) when analysed by SRIXE mapping.

After observing the poor spheroid penetration of the hydrophilic platinum(IV)-peptide compounds, the substitution of the cytotoxic platinum(IV) complex for a ruthenium(II)-bipyridyl complex saw an improvement in spheroid penetration. Peptide conjugation of the ruthenium(II) complex improved the cellular uptake of the ruthenium(II) and fluorescence confocal microscopy of LRSK(Ru) (5.10) showed that the compound was localised in the cytoplasm, while little of the free complex [Ru(tBu\(_2\)bpy)\(_2\)(HOOC-4’-CH\(_3\)bpy)]\(_2\)PF\(_6\) (5.6) was observed inside cells. This increase in uptake is likely to have contributed to the increased cytotoxicity of the compound, reducing the IC\(_{50}\) value by a factor of 2.
Statement of Student Contribution

This project was carried out under the supervision of Professor Trevor Hambley at the University of Sydney. Some of the work was also carried out under the supervision of Professor Nils Metzler-Nolte at the Ruhr Universitat Bochum, Germany. All of the work presented in this thesis is original and undertaken by myself unless stated otherwise. All images and figures were generated by myself, unless otherwise referenced. I would like to acknowledge Professor Trevor Hambley and Dr Nicole Bryce for their assistance in proof-reading and editing of this thesis.

[Pt(OAz)(OAc)(ox)(chxn)] and [Pt(OAz)(OCH₃)(ox)(chxn)] were kindly provided by Dr Jenny Zhang. The cell lines were maintained by Dr Nicole Bryce and Mr Byung Juen Kim. The live cell imaging experiment was carried out with assistance from Dr Nicole Bryce. All people mentioned are at the University of Sydney.

ICP-MS analysis was carried out at the University of Technology, Sydney with the guidance and assistance of Dr Christine Austin.

I certify that this thesis contains work carried out by myself except where otherwise acknowledged.

Signed:
“‘Pooh?’

‘Yes Piglet?’

‘I’ve been thinking...’

‘That’s a very good habit to get into, Piglet.’”
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Firstly, my greatest thank you goes to my supervisor, Professor Trevor Hambley, for giving me the opportunity to join the Hambley group in the first place. I am so glad that I took the chance, as I can say without doubt that I have amassed more knowledge in my PhD years than all the years of education that came before. Your guidance, optimism and unique ideas have been invaluable during the course of this occasionally challenging project. Also, thank you for giving me the opportunity to travel - to Germany (twice!), Greece and Canada – which contributed greatly to my entire PhD experience and taught me a great deal about where life can take you. On a more personal note, thank you for your patience, kindness and support when life decided to throw a few curve balls in my direction, I am so grateful.

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<td>two-dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>three-dimensional</td>
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<tr>
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<td>antibiotic antimycotic</td>
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<tr>
<td>ADC</td>
<td>antibody-drug conjugate</td>
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<tr>
<td>AmCyan</td>
<td>AmCyan fluorescent protein</td>
</tr>
<tr>
<td>APMA</td>
<td>$p$-aminophenylmercuric acetate</td>
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<tr>
<td>a.u.</td>
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<td>adenosine triphosphate</td>
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<td>DIPEA</td>
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<td>Dubelco’s Modified Eagle’s Medium</td>
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<td>dpq</td>
<td>dipyrido[3,2-$f$;2',3'-$h$]-quinoxaline</td>
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<td>DRIFTS</td>
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<td>DTT</td>
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<td>$E_p$</td>
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Chapter 1

INTRODUCTION
1.1 Cancer and chemotherapy

Despite improvements in awareness, prevention and treatment, cancer is still a major cause of death worldwide with 7.6 million people dying from the disease in 2008 (WHO). In 2013, about 124,910 Australians are expected to be diagnosed with cancer, with an estimated 149,990 expected to be diagnosed in 2020\(^1\).

There are various strategies in the treatment of cancer including surgical excision, radiotherapy, chemotherapy and immunotherapy. The optimum course of treatment is dependent on the type, size and progressive stage of the cancer. Chemotherapy offers the distinct advantage of systemic distribution, with the potential to reach primary tumours as well as metastases, but a significant drawback of the vast majority of chemotherapeutic agents is their lack of specificity for cancer cells which leads to a variety of non specific interactions in the body. Many chemotherapeutic agents also exhibit poor tumour penetration, meaning that cells distal to blood vessels do not receive an adequate dose and consequently remain viable.

To this end, there is an imperative need for the development of anti-tumour drugs which exhibit good tumour penetration and distribution, favourable pharmacokinetic profiles, limited toxicity to healthy tissue and minimal residual disease.

1.2 Solid tumours

Solid tumours result from disruption of the factors which promote homeostatic regulation and proliferation of cells\(^2\), and may be benign or malignant (cancerous). Solid tumour based cancers are extremely common and were responsible for 87.5% of all cancer cases in Australia in 2005\(^3\). These tumourigenic masses of cells essentially transform into their own organ, exhibiting markedly different physiological, chemical and biological characteristics to the surrounding healthy tissue.
Figure 1.1 depicts the predominant tumour regions and their definable features, as will be discussed further in this chapter. Essentially, these regions are self-establishing based on the distance of the cells from the nearest blood, and therefore oxygen and nutrient, supply. Closest to the blood vessel there exists a zone of viable, rapidly proliferating cells which are the greatest contributors to tumour growth. Moving outwards, approximately 3-4 cell diameters from the blood vessel (30-40 µm) there is a quiescent region, where although the cells are not actively proliferating they remain viable. It is in this zone that cells will tend to upregulate specific biomolecules to maintain their survival, and these changes in protein and enzyme expression can be exploited as chemotherapeutic targets. Cells which are located >100 µm from a blood vessel generally experience hypoxia, which will be discussed in section 1.2.1.3. These cells tend to have more aggressive and drug resistant phenotypes and therefore are amongst the most difficult to target and treat effectively. The cells which exist furthest away from any vascular supply (>200 µM) are regarded as necrotic, and typically exist at the core of solid tumours and 3D in vitro spheroid models.

1.2.1 The tumour microenvironment

Marked differences exist between healthy tissue and solid tumours at a structural, cellular and molecular level. These characteristic differences give rise to challenges in achieving effective treatment of solid tumours, but are also capable of being exploited to selectively deliver or
activate chemotherapeutic agents and as such present attractive targets in the rational design of future drugs.

1.2.1.1 Tumour vasculature

The processes of angiogenesis and vasculogenesis in healthy tissue give rise to hierarchically structured vessels capable of delivering oxygen and micronutrients to all cells, which are typically within 3-4 cell diameters (70 µm) of a blood source. Widely regarded as the underlying cause of the unique tumour microenvironment, the vascular network in solid tumours is arranged erratically and irregularly, containing arterio-venous shunts, blind ends, a lack of smooth muscle or enervation and incomplete endothelial linings and basement membranes. This results in a languid and variable blood flow, and localised leakiness of the blood vessels through widening of the fenestrae in the range of 100 to 600 nm. The poor, and in some cases non-existent lymphatic system present in tumours also contributes in part to the extracellular accumulation of drugs resulting in what is known as the enhanced permeability and retention (EPR) effect. This presents an opportunity for size-selective accumulation of therapeutic agents.

1.2.1.2 Acidic pH

The poor vascular and lymphatic networks also in part contribute to chemical anomalies in solid tumours. It has been well established that the extracellular pH of tumour tissue is significantly lower than that of surrounding healthy tissue, lying within the range of 5.4-7.4. The morphological characteristics of pH levels in mice brains have been recently imaged using magnetic resonance imaging, revealing a marked difference between the interstitial pHs of healthy and solid tumour tissue: 7.3 and 6.0 respectively. The acidic environment was originally attributed to the accumulation of lactic acid as a product of glycolytic respiration in hypoxic regions. However, more recent work has demonstrated that the hydrolysis of ATP in oxidative respiration has a significant effect on interstitial pH level due to the build up of CO2 and carbonic acid by-products which are not removed efficiently by the inadequate vascular and lymphatic networks and increased interstitial pressure. The intracellular pH of tumour cells is known to be slightly above neutral, consistent with normal healthy cells. The interstitial pH gradient existing between healthy and tumour tissue can be exploited for tumour therapy by designing agents which are inert at a normal interstitial pH of 7.4 but are activated by protonation in acidic surroundings.
1.2.1.3 Tumour hypoxia

Tumour hypoxia refers to the state of diminished oxygen concentration (typically <3 µM) found within solid tumours, and often presents along with localised regions of anoxia and necrosis\(^\text{20}\) such as in the necrotic core. The dominating factors contributing to chronic tumour hypoxia are the poor and irregular development of vascular tissue and the increased interstitial pressure within the tumour. Transient hypoxia can also result from temporary shutdown of blood vessels\(^\text{7}\), and as a result both the inter- and intra-cellular regions of tumours experience hypoxic stress.

1.2.1.4 Biomolecular changes

The altered physiology and chemistry of solid tumours is also associated with extraordinary changes in the biological characteristics of the cells and tissue. It is well established that tumour cells express characteristic antigens on their surface\(^\text{21}\), and that the various processes involved in tumour progression lead to the upregulation and suppression of particular biomolecules. Aggressive tumour cells often increase their expression of cathepsins, matrix metalloproteinases and plasminogen activators\(^\text{21}\) in order to perform the cellular functions required by metastasis and angiogenesis including: degradation of basement membrane and extracellular matrix (ECM) and the activation of enzymes, growth factors, and other proteases as part of the metastatic cascade.

1.3 Treatment of solid tumours

1.3.1 Current problems

In order for an anticancer drug to be effective, it needs to be able to reach all tumour cells in a sufficient dosage in order to exert its pharmacological effect and induce cell death, while minimising toxicity to the surrounding healthy tissue. The inherent complexities in the physiological and chemical composition of solid tumours, such as those mentioned previously, act as both an impediment as well as a prospective means of achieving improved delivery of chemotherapeutics.

One contributory factor towards tumours receiving insufficient levels of chemotherapeutics is that many anticancer agents are unable to diffuse far enough away from blood vessels into the quiescent and necrotic regions of tumours. This is not improved by the inadequate
vascularisation of tumour tissue, which leads to non-uniform drug localisation with high concentrations around the blood vessels and tumour periphery and regions distal to the blood supply receiving no dose at all as the intermittent and erratic flow through the blood vessels can also lead to the micro-regional cessation of any blood supply. This feature of chemotherapy has been extensively documented in the literature, but Primeau et al. have perhaps demonstrated this most effectively by fluorescence microscopy of the anticancer drug doxorubicin relative to fluorescently stained blood vessels and regions of tumour hypoxia as shown in Figure 1.2.

![Figure 1.2](image-url) Perivascular distribution of the anti-cancer drug doxorubicin (blue), relative to blood vessels (red) and hypoxic regions (green) in 16C mammary adenocarcinomas. Scale bar represents 100 µm. Image taken from Primeau et al.23.

The hypoxic cells which exist in the quiescent viable zone are the greatest contributors to tumour malignancy and are the extremely difficult to treat for several reasons. Firstly, the decreasing ratio of proliferating to non-proliferating cells in increasingly hypoxic areas of a tumour limits the effectiveness of many traditional chemotherapeutics which are mainly effective against rapidly proliferating cells. Also, drugs which function by generating DNA-damaging free radicals only when in the presence of oxygen are also less effective in these hypoxic regions where the cells can survive. Oxygen-deprived cells also exhibit resistance to radiotherapy which is similarly reliant on the generation of oxygen radicals. Hypoxic cells tend to be inherently resistant to chemotherapy due to the upregulation of genes implicated in drug resistance, including the transcription factor Hypoxia Inducible Factor (HIF)-1. HIFs in general are involved in the regulation of proliferation, angiogenesis, and metastasis. HIF-1
regulates the multi-drug resistance transporter p-glycoprotein\textsuperscript{28} as well as apoptotic mechanisms, thereby reducing the effectiveness of therapeutic agents which act by inducing apoptosis. Hypoxic cells also show inducible expression of thiol containing molecules such as thioredoxin, glutathione and metallothionin in response to oxidative stress. These cellular thiols are capable of detoxifying chemotherapeutics, and enabling resistance towards reactive oxygen species (ROS)\textsuperscript{29,30}. For these reasons, high levels of tumour hypoxia in the quiescent cell region correspond with poor prognosis for cancer patients\textsuperscript{31} and are why new antitumour drugs are focused on enhancing penetration into this tumour region and overcoming the mechanisms of resistance.

One of the most significant problems in the treatment of solid tumours is that the cytotoxic properties of many antitumour agents, while being highly effective towards cancerous cells, are not exclusive in their target of action and thus also affect healthy cells. Drugs which undergo significant levels of cellular uptake and accumulation in healthy cells following administration are unlikely to reach the tumour in high enough concentrations to be effective. This lack of specificity leads to significant levels of systemic toxicity in the patient and ultimately limits the therapeutic efficacy of the drug.

### 1.3.2 Current Strategies

The impetus in recent anticancer drug design has largely been towards addressing the problem of tumour selectivity. This has been based on the rationale that non-specific cytotoxicity is most likely to occur as the compound is en route to the tumour, prior to any degree of metabolism. Drug development has also worked towards addressing the challenging dichotomy between increasing drug accumulation in order to reach a cytotoxic concentration and improving drug penetration into the solid tumour which requires controlled cellular uptake in the rapidly proliferating and quiescent tumour regions.

#### 1.3.2.1 Tumour Activated Prodrugs

Prodrugs are one of the main strategies now employed in antitumour drug development, and involve administering an inert, less toxic form of a particular drug which is then transformed into its toxic version by the body in the vicinity of the tumour. The use of prodrugs is not confined to tumour chemotherapy; they have also been employed in the development of therapeutics for the treatment of blood cancers such a leukaemia, chronic inflammation, and HIV. Compounds which demonstrate a high degree of selectivity for the cells and tissues of interest can potentially be administered in smaller doses and less frequently. This, in
combination with reduced side effects due to a more specific mode of action, can in theory drastically reduce the systemic toxicity of a particular drug. However, in the case of many of these prodrug compounds – both organic and inorganic – investigated to date, these expected benefits have rarely translated into in vivo outcomes\textsuperscript{32,33}.

In the treatment of solid tumours, this class of compounds are referred to as tumour activated prodrugs (TAPs). One proposed model for their selective mechanism is shown in Figure 1.3.

![Figure 1.3 Schematic of trigger-linker-effector model.](image)

These three domains all have distinct roles, and must remain an intact unit until within close vicinity of the tumour. Upon reaching the tumour site, the trigger unit should undergo metabolism by a tumour-specific mechanism. The linker component, designed to deactivate the effector, should then transmit this change to the effector and therefore activate the drug\textsuperscript{34}.

TAPs which target the normoxic-hypoxic dichotomy in cancer therapy have the potential to select for tumour tissue over healthy tissue and as such minimise problems regarding systemic toxicity and acquired resistance. In normal cells, reduction by endogenous reductases or another reducing agent is reversible due to reoxidation by transfer of the free electron to available O\textsubscript{2}. However, in a hypoxic environment reoxidation is less likely and the labile form of the drug accumulates. In the case of metal complexes, this manifests as the reduction of the
metal centre from a higher oxidation state to a lower one, which can involve the release of at least one attached ligand. There are two groups of hypoxia activated metal complexes: those which lose ligands to yield an active complex or metal ion (a), and those which act as chaperones for delivery of active ligand upon reduction (b). In the case of (b), the leaving ligand(s) exert their own cytotoxic effects on tumour cells following reduction and employ the metal centre merely as a carrier and deactivator. These metal chaperones tether biologically active molecules and as a result can alter the pharmacokinetics, biodistribution, and biotransformation of the original compounds. Some of the foremost examples of this strategy include the synthesis of Co(III) complexes of nitrogen mustards by Ware and co-workers. Coordinating the mustard ligand to the Co(III) centre through the electron lone pair on the nitrogen atom mutes the toxicity of the mustard as the nucleophilic lone pair is unavailable and gives rise to hypoxia selective toxicity. As a result, the Co(III) mustard complexes were up to 20 times more toxic in hypoxic cells than healthy normoxic cells.

Along with irreversible reduction, TAPs can also be activated by a number of other mechanisms which take advantage of unique features of tumours. One of these involves modification of the drug compound, whether by cleavage or transformation, by enzymes which are selectively expressed or overexpressed in the tumour region. However, targeting these enzymes does not necessarily localize the drug to the cell surface, as is the case when targeting tumour specific antigens. This is another method which has been widely attempted by tethering small cytotoxic molecules to antibodies, via a deactivating linker component which is cleaved following endocytosis such that the drugs are activated when internalised, referred to as antibody-drug conjugates (ADCs). This strategy requires that the antibody immunoreactivity is preserved such that the antibody-antigen binding affinity is retained. It is also important that the potency of the drug is not reduced by conjugation; however, inactivation upon conjugation can be desirable providing that the drug can be reactivated upon reentering cells as it limits toxicity to non-targeted cells. Furthermore, the ratio of drug to antibody in the immunoconjugate should ideally be as high as possible, to maximise potency by delivering a greater number of drug molecules per immunoconjugate.
Based on these principles, Gao and co-workers have synthesised Herceptin-Pt(II) binding complexes for targeted breast cancer therapy\(^{39}\), as shown in Figure 1.4. Herceptin is a humanised monoclonal antibody which is overexpressed in 15-30% of breast cancer cases and binds to the Her2/neu protein\(^{40}\). The Herceptin antibody was tethered to ten Pt(II) complex units via covalent interactions. This allowed for the delivery of ten cytotoxic agents per single antibody in an attempt to improve therapeutic potency, particularly in cases where receptor expression was low. In vitro studies showed that the Herceptin-Pt(II) compounds displayed a high degree of selectivity for Her2/neu overexpressing cancer cells over normal cells. The apoptotic potential of the antibody-drug compounds was found to be limited by the level of receptor expression, as doubling the dose of Herceptin-10[PtCl\(_2\)(L)] (where \(L = 2,2'-(1R,2R)\)-cyclohexane-1,2-diylbis(azanediyl)bis(methylene)bis(4-methylphenol)) resulted in only a minor increase in the proportion of apoptotic cells in the Her2/neu overexpressing SK-BR-3 cell line. This study is indicative of the potential of antibody-metal complex therapeutic strategies, as the inherent selectivity of the antibody is coupled to metal complexes with various mechanisms of action.

1.3.2.2 Improving drug distribution in solid tumours

Another strategy employed in the attempt to more improve the efficacy of chemotherapeutic agents is designing compounds which are able to infiltrate all regions of solid tumours which, as already discussed, have a markedly different pathophysiology to healthy tissue that can obstruct and limit drug diffusion from blood vessels into the less accessible regions of the
tumour, such as the quiescent zone and necrotic core. While a significant proportion of anticancer drug development has focused on optimising cellular accumulation, recent investigations such as those undertaken by Bryce et al.\textsuperscript{41} have suggested that increasing cellular accumulation and increasing tumour penetration may in fact be two conflicting goals.

Drug distribution in tissue is the result of three processes – supply, flux and consumption. The latter two occur somewhat simultaneously, and are influenced by the characteristics of the tumour tissue as well as the physicochemical properties of the drug. Flux, the movement of the drug through tissue, can occur via extra- or trans-cellular pathways and this is dependent largely on the lipophilicity of the drug. More water soluble molecules tend to travel paracellularly via the ECM while more lipophilic compounds will traverse the cell-membrane and adopt a trans-cellular route. The movement of the compounds will be hindered by their interactions with both extra- and intra-cellular components as well as the cell membrane\textsuperscript{42}, including protein binding, sequestration and cellular metabolism; collectively these constitute what is known as the consumption process. While consumption can increase the net tissue levels of the drug, it restricts the passage of drug diffusion to the cells in the quiescent tumour zone.

Several strategies have been proposed for enhancing the tumour penetration of anticancer agents, such as regulating and normalising the vascular networks of tumours,\textsuperscript{43} co-administration with a pH-modifying agent\textsuperscript{44} or cellular uptake inhibitor\textsuperscript{45}, pre-treatment to lower cell density via induction of apoptosis\textsuperscript{46,47}, and raising the endosomal/lysosomal pH to reduce sequestration\textsuperscript{48}. Altering the physicochemical properties of drug molecules directly may also potentially be able to modify the flux and consumption behaviour, and therefore increase the diffusion, into the tumour.

It has been shown that molecular weight and size will determine the method of intratumoural diffusion, with smaller molecules diffusing into the interstitial space following the oncotic pressure gradients, with larger molecules relying on a convectional fluid pathway\textsuperscript{49}. Exploiting the EPR effect (section 1.2) for the delivery of macromolecules can allow for significant improvements in uniform drug distribution in higher concentrations, such as in the case of liposomal\textsuperscript{50} and polymer\textsuperscript{51,52} conjugates of the popular anticancer drug doxorubicin. In fact, the success of PEG-liposomal doxorubicin has led to its clinical use (Doxil®, Caelyx®) in Japan, the US and Europe.
Free doxorubicin has been typically limited by its poor therapeutic index due to insufficient concentrations on the intact drug reaching the tumour site. The weakly basic amine groups present on doxorubicin and the anthracenedione derivative mitoxantrone, another widely used chemotherapeutic agent, are membrane permeable in their neutral form, but less so when protonated in the acidic compartments of cells such as endosomes. This results in their sequestration, reducing the intracellular concentration of the available drug and depending on the rate of endosomal release into the ECM, extracellularly for transport to distant cells. This ultimately limits the extent of tumour penetration of these compounds.

Conversely, weakly acidic drugs such as methotrexate and 5-fluorouracil have been shown to penetrate more rapidly through multi-cellular layers (MCLs) than their basic counterparts, achieving diffusion levels of 30% and 40% respectively compared to that of the free cell system. This may be a result of deprotonation in normoxic extracellular surroundings, with a pH of 7.2, and the anionic charge inhibiting cellular uptake and facilitating paracellular diffusion of the drugs. In the acidic tumour environment, protonation of the weakly acidic groups and a return to neutrality will promote cellular permeability.

Lipophilicity is another property which can modify the extravascular rate of diffusion in tumours as well as cellular uptake. Lipid soluble compounds tend to have high volumes of distribution and diffuse quickly throughout tumours as they are able to adopt a transcellular route as they pass through cell membranes more easily than water soluble compounds. Pruijn et al investigated the effects of increasing lipophilicity on a series of tirapazamine analogues with either methyl-, methanesulfonyl-, chloro-, trifluoromethyl- or diethylamino-substituents on the aromatic ring. It was found that lipophilic compounds (logP > 1.5) had ca. 15-fold higher level of diffusion through MCLs than hydrophilic compounds (logP < -1).

Ultimately, drug diffusion and cellular uptake are difficult to predict, and improvements to both factors will not necessarily confer a greater therapeutic effect. The transport of drug molecules between and into cells is largely dependent on the cell-type and, more specifically, the transporters found on their cell surface. Work by Tunggal et al has shown that diffusion of the imaging agent $^{99m}$Tc-sestamibi and doxorubicin to distal cells was promoted by the presence of the P-glycoprotein membrane-based efflux pump. In cells where expression of P-pg was inhibited, drug penetration through the MCL was lowered, as a result of cellular uptake and subsequent retention which meant that transcellular diffusion was limited.
1.3.3 Tumour model systems

In order to gain a comprehensive understanding of the behaviour of newly developed compounds, in vitro tumour models are required. These cell-based models range from the relatively simplistic to the more complex, and are routinely used for screening potential drug candidates before proceeding to in vivo models such as xenografts and animal studies and then potentially to clinical trials.

1.3.3.1 Monolayer cell culture

Monolayer cell cultures are the most widely used two dimensional (2D) tumour model and are an invaluable tool for investigating the cytotoxic properties, cellular accumulation and mechanism of action of anticancer compounds\(^{42}\), as well as various aspects of tumour biology\(^{54}\). While these 2D tumour models are useful for rapid, high throughput screening of new drugs against multiple cell lines, they are inherently limited by their lack of 3D structure and host tissue microenvironment\(^{55}\) which leads to markedly different cellular properties compared to the primary tumour from which they were derived. Cells grown in monolayer formation are also exposed to equal concentrations of oxygen, nutrients and dosed drugs, which does not accurately represent the complex pathophysiology of the non-uniform tumour microenvironment.

1.3.3.2 Multi-Cellular Tumour Spheroids

In order to reproduce the multicellular complexity of solid tumours in vitro, 3D models such as organ and spheroid culture have been used to study the behaviour of novel drug compounds. Multi-Cellular Tumour Spheroids (MCTS) are spherical cell aggregates which, by being grown in a 3-dimensional structure, are able to better simulate the physiological features of solid tumours compared to cell monolayers. This includes the development of an ECM, contact between cells and cell-cell communication\(^{56}\), zones of differing proliferative activity as well as oxygen\(^{57}\) and nutrient gradients. These regions, and the spatial distribution of various proteins across them, have been investigated using MALDI-MS-based imaging\(^{58}\).

MCTS have also been proven to resemble minor metastases during the initial phases of avascular growth\(^{59}\). Spheroids can be cultured using various techniques, such as liquid overlay culture on non-adherent surfaces or as suspension cultures inside spinner flasks\(^{57}\). MCTS are good mimics of the tumour microenvironment but are essentially an inverted tumour, as shown in Figure 1.5.
The periphery of the spheroid receives the greatest concentration of oxygen and nutrients from the surrounding media and is composed of actively proliferating cells. The diffusion limitations in solid tumours are also present in spheroids, where the maximum diffusion distance of most molecules, including oxygen, is 150-200 µm\textsuperscript{60}, resulting in a quiescent hypoxic zone which contains cells capable of differentiation into more aggressive phenotypes\textsuperscript{61}. This diffusion limit also leads to the inefficient clearance of metabolic waste and the establishment of a necrotic core, and means that tumours with a diameter >500 µm will typically exhibit all three zones of interest (section 1.2).

MCTS have been used to study the responses to a range of tumour treatments including radiotherapy, chemotherapy, immunotherapy and combinations thereof. For chemotherapy, they can be used to study the mechanisms of drug metabolism and distribution by incubation in media dosed with the compound of interest. Various methods for assessing treatment effectiveness also exist, such as measuring spheroid diameter to determine growth, sectioning to assess antigen expression by immunoenzymology and spheroid dissociation to analyse single cells\textsuperscript{54}. For compounds which are inherently fluorescent the distribution of the compound can be observed over time using confocal microscopy, and the uptake and retention of metal-containing compounds can be determined by accumulation studies.

Overall, MCTS are considered excellent in vitro models which closely resemble many of the microenvironmental features of solid tumours and are important tools in collecting critical data required for progressing in the drug development process. However, they are not a...
substitute for animal models which can provide vital information regarding the pharmacokinetics, pharmacodynamics and bioavailability of novel drugs, properties which are often found to be undesirable for novel inorganic drug candidates. Therefore, spheroids are an accessible and replicable laboratory tool for investigating both the biological traits of solid tumours and drug behaviour at a moderately complex level and are therefore employed in this work.

1.4 Metal complexes in chemotherapy

1.4.1 Platinum anticancer complexes

Platinum(II) drugs are amongst some of the most common and lucrative chemotherapeutic agents available worldwide today. Since the serendipitous discovery of the first generation platinum drug cisplatin in the 1960’s, over 3000 new platinum compounds have been investigated, but with only a small proportion of them progressing to clinical evaluation. Following the success of cisplatin, the second generation analogue carboplatin was developed, which despite low in vitro cytotoxicity showed a remarkable therapeutic effect in vivo, perhaps attributable to its improved water solubility over cisplatin. Both cisplatin and carboplatin possess a high therapeutic efficacy against a wide range of cancers, however due to slower aquation kinetics carboplatin causes fewer systemic side effects such as the extreme nephrotoxicity observed with cisplatin. Shortly after the regulatory approval of carboplatin, the drive behind discovering new platinum therapeutics shifted towards circumventing mechanisms of intrinsic and acquired drug resistance, and oxaliplatin gained momentum due to its success in patients with colorectal cancer. In oxaliplatin the two bidentate ligands, cyclohexane-1,2-diamine (chxn) and oxalato (ox), replace the four monodentate ligands in cisplatin resulting in the formation of alternative DNA-platinum adducts, which escape repair by the DNA-mismatch repair system and thereby overcome cisplatin resistance.

Figure 1.6 Clinically employed platinum(II) complexes: (l-r) Cisplatin, carboplatin, oxaliplatin.
The cellular transformation of cisplatin and similar Pt(II) complexes has been researched extensively, largely in an effort to overcome the problem of acquired resistance by developing compounds which exert their effect via alternate pathways. Following administration, cisplatin is believed to exist largely in its neutral, intact form in the bloodstream due to the high chloride ion concentration (~ 100 mM)\textsuperscript{66,67} which inhibits aquation. Studies have shown that at this extracellular concentration, 68% of the cisplatin is still intact and 24% exists in the monoaquated form\textsuperscript{67}. In the case of cisplatin, both these forms experience passive diffusion across the cell membrane despite the acquired singly cationic charge of the monoaquated form. Along with the well researched and accepted passive diffusion of these complexes\textsuperscript{68-73}, active and facilitated transport mechanisms are also believed to play a role in the cellular uptake of platinum drugs. Cisplatin and other Pt(II) drugs are vulnerable to attack by blood plasma proteins, particularly those containing thiol groups on cysteine residues such as glutathione and human serum albumin\textsuperscript{74,75} and this can result in deactivation of the platinum complex.

Following influx into the cell, the decrease in chloride ion concentration (3-20 mM) results in further aquation at the equatorial leaving group positions, and the platinum centre is then able to bind covalently to the N7 of the guanine bases in the major groove of nuclear DNA to form DNA-platinum adducts\textsuperscript{76}. The nature of these adducts can either be mono- or bifunctional, depending on the number of displaced leaving groups on the Pt(II) complex\textsuperscript{77}, and can either occur between guanine bases on the same strand or by crosslinking adjacent DNA strands\textsuperscript{78}. The nature of the non-leaving groups influences the type of DNA adducts which are formed. For example, carboplatin contains the bidentate 1,1-cyclobutanedicarboxylato ligand in place of the two chlorido ligands on cisplatin, which slows the rate of ligand substitution as it is a more stable leaving group. After aquation, the complex is identical to cisplatin and as such has a similar DNA binding profile and overall efficacy, but with fewer toxic side effects. Oxaliplatin, despite being at least as cytotoxic as cisplatin and in some cell lines more so, forms a significantly lower number of DNA adducts than cisplatin\textsuperscript{79,80}. Ultimately, the DNA-adducts formed with platinum complexes cause distortion of the DNA strand, which is recognised by DNA binding proteins. These proteins can either initiate DNA damage repair, or induce cell death by apoptosis\textsuperscript{81-83}. 
1.4.1.1 Platinum(IV) complexes

The impetus towards designing Pt(IV) complexes has been based on the Pt(IV) centre’s inherent kinetic inertness compared to their labile Pt(II) counterparts. This inertness confers a slower rate of ligand exchange with other molecules \textit{in vivo} so that the complex may reach and penetrate the tumour without undergoing aquation or ligand exchange with various biological molecules \textit{en route}. Ideally, activation via a reductive mechanism should occur only once the Pt(IV) complex is inside the target tumour cells\textsuperscript{84}. The mechanism by which this occurs is shown in Figure 1.7.

![Figure 1.7 Example of reduction in vivo by endogenous single electron reductases. The Pt(IV) complex is reduced to Pt(II) and loses the two axial ligands (X).](image)

Pt(IV) complexes possess two additional ligands in the axial position due to their six coordinate octahedral geometry, and there are various ways in which the axial ligands can be used to achieve desirable pharmacological properties for the Pt(IV) prodrug. The first of these is altering the rate of reduction of the metal complex. In principle, if the Pt(IV) compound remains intact before reduction to the active Pt(II) complex, systemic toxicity should be minimised, and the concentration of the Pt(IV) drug within the tumour site should be maximised. This is because many Pt(II) drugs undergo protein binding in the bloodstream and subsequent excretion before reaching the tumour site\textsuperscript{85}. Hambley and co-workers have demonstrated that in the case of cisplatin-like Pt(IV) prodrugs with an [N\textsubscript{2}Cl\textsubscript{2}] equatorial coordination sphere, complexes with halide ions in the axial positions are most readily reduced, followed by those with carboxylato and hydroxido ligands respectively\textsuperscript{86,87}. This is consistent with the reduction potentials of the complexes, with the most readily reduced complexes having the least negative $E_p$ values. From this, they concluded that complexes which are very difficult to reduce can achieve toxicities similar to that of the parent Pt(II) compound. Investigations into the relationship between the axial ligands and rate of reduction for Pt(IV) congeners of oxaliplatin, with an [N\textsubscript{2}O\textsubscript{2}] bidentate equatorial coordination sphere,
have shown that a different trend is observed to that of cisplatin-based complexes. The compounds investigated did not show the expected correlation between reduction potential and reduction rate by ascorbate, which was attributed to the lessened ability of the amine and carboxylato ligands to form bridging species with the reducing agents to facilitate electron transport \(^{88}\).

The axial ligands can also be used to alter the lipophilicity of the complex to control cell membrane permeability and uptake via passive diffusion. This mechanism of drug uptake has the potential to circumvent drug resistance as it removes reliance on energy dependent drug uptake which is depleted in drug resistant cell lines. Despite being sound in theory, this hypothesis has not been supported by in vitro studies, with poor correlation between lipophilicity and cellular accumulation observed across platinum complexes \(^{89-91}\). This is with the exception of Kelland and co-workers who achieved high in vitro cytotoxicity with the butyrate analogues of the Pt(IV) prodrug JM221 which was attributed to the highly lipophilic nature of the compounds \(^{92}\) and therefore improved transport across the cell membrane into the cell.

### 1.4.2 Ruthenium anticancer complexes

After early pioneering work into the anticancer activity of ruthenium complexes by Dwyer and co-workers in the early 1950's \(^{93}\), the usefulness of ruthenium complexes as anti-cancer agents was somewhat overshadowed by the discovery of cisplatin by Rosenberg et al in the 1960's \(^{94}\). Ruthenium, however, is an attractive alternative to platinum for several salient reasons, the first of which revolves around the diverse synthetic chemistry available for tuning of the reactivity and structure of ruthenium systems. Although typically found in vivo as Ru\(^{III}\), the range of oxidation states (Ru\(^{II}\), Ru\(^{III}\), Ru\(^{IV}\)) which may exist at physiological conditions, due to the presence of biological oxidants and reductants, also contributes to its synthetic versatility. These oxidation states allow for the exploitation of in vivo reduction for compound activation as well as presenting the opportunity for various structural conformations in the design of inhibitor Ru complexes, such as the staurosporine structural mimics developed by Meggers and co-workers \(^{95}\). Additionally, ruthenium complexes are generally regarded as less toxic than their platinum counterparts \(^{96}\), and this has been attributed, at least in part, to their ability to mimic endogenous iron atoms. As such, they are able to bind to proteins such as transferrin and albumin, and the overexpression of transferrin receptors on the surface of cancer cells results in the selective delivery of ruthenium-based drugs to cancer cells instead of normal, healthy cells \(^{97,98}\).
Some of the earliest ruthenium complexes screened for antitumour activity were ruthenium-dimethylsulfoxide (DMSO) compounds, including \textit{cis}- and \textit{trans}-Ru(DMSO)$_4$Cl$_2$. Following screening in C75B1 and BD2F1 female mice with lung carcinomas, it was found that the \textit{trans}-isomer was 20-fold more active compared to its \textit{cis}-congener, due to much more rapid binding with DNA and greater disruption of DNA by crosslinking\textsuperscript{99,100}. Analogues of these compounds containing derivatised chelating DMSO ligands have been found, despite high levels of accumulation within DNA, to be non-cytotoxic. A non-classical variation of the typically synthesised Ru-DMSO complexes, imidazolium \textit{trans}-[tetrachloro(DMSO)(imidazole)ruthenate(III)] (NAMI-A), was the first ruthenium compound to enter clinical trials as a drug candidate against non-small cell lung cancer (NSCLC)\textsuperscript{101}. Despite its lack of activity \textit{in vitro} (up to a concentration of 0.1 mM), NAMI-A inhibited the formation of lung metastases and also caused a reduction of metastases weight, with no concomitant effect on the primary tumour\textsuperscript{102}, however it was shown to remove metastatic tumour cells from heterogenous primary tumour tissue\textsuperscript{103,104}. To date, there is no data published which supports DNA being the primary target for NAMI-A, but strong serum protein binding has been observed which suggests that receptor-mediated delivery by transferrin may be occurring\textsuperscript{105}. Its effect on metastatic tissue comes from the blockage of cell cycle progression at the G$_2$M pre-mitotic phase as well as the regulation of the actin-dependant processes of adhesion and cytoskeleton remodelling\textsuperscript{102}. When given \textit{in vivo} to mice and rats an increase in spleen volume, circulating lymphocytes and lymphocytic spleen depletion was measured, consistent with the proposed stimulated interaction between tumour cells and tumour-infiltrating lymphocytes\textsuperscript{106,107}. A recent Phase II clinical trial study into NAMI-A in combination with the nucleoside analogue Gemcitabine, as a second-line therapy against NSCLC, at University Trieste has been suspended\textsuperscript{102}.
The other similarly successful Ru$^{119}$ compound shown in Figure 1.8 is indazolium trans-[tetrachlorobis(1H-indazole)ruthenate(III)] (originally KP1019, now FFC14a), which shows remarkably different in vitro cytotoxicity to NAMI-A, as evidence by its induction of apoptosis in the colorectal cancer cell lines HT29 and SW480$^{108}$. In vivo evaluation against MAC15A chemoresistant colorectal tumours in rats showed that KP1019 was highly effective, even more so than the clinically used 5-fluorouracil, at reducing tumour growth and significantly more so than cisplatin which was found to be inactive. As with NAMI-A, this was in part attributed to its strong binding to the iron-binding pockets of transferrin. However, unlike NAMI-A it does not exhibit any antimetastatic activity and is limited to behaving as a cytotoxin which induces apoptosis via the mitochondrial pathway, generating reactive oxygen species (ROS) in the process. KP1019 also displays a similar DNA binding profile to cisplatin but with less potency, as it is highly efficient in forming monofunctional compared to bifunctional adducts and forms 15-fold less intrastrand crosslinks than cisplatin$^{109}$. Having already completed Phase I clinical trials, KP1019 has shown a very similar pharmacokinetic profile to NAMI-A with respect to protein binding and elimination from the body, and from a total of eight patients, six of whom were evaluable, five showed disease stabilisation$^{109}$. Despite not making it to clinical trials, there are several other classes of both classical and non-classical ruthenium complexes which have shown potential as anticancer agents. Ruthenium polyaminocarboxylate complexes consisting of ligands such as 1,2-
propylenediaminetetraacetate (pdta)\textsuperscript{110,111}, ethylenediaminetetraacetate (edta)\textsuperscript{112}, and ethylenediamine-\textit{N,\textit{N}-di-3-propionate (eddp)}\textsuperscript{113} have also possessed chlorido ligands which rapidly undergo aquation, allowing them to induce either conformational changes or cleavage of DNA. Even more extensively studied are organometallic ruthenium-arene complexes, although none are yet to enter clinical trials. Early work by Clarke and co-workers\textsuperscript{114} was the impetus behind the development of ruthenium(II)-arene compounds, as they proposed that the cytotoxic activity of many ruthenium(III) compounds was the result of reduction to the more labile Ru(II). It was found that the presence of an arene ligand has a stabilising effect on the Ru(II) centre, and in particular the “half-sandwich” mono-arene complexes are relatively inert and resist ligand exchange in physiological conditions\textsuperscript{115}. “RAPTA” complexes (RAPTA = Ruthenium-Arene-PTA (PTA = 1,3,5-triaza-7-phosphatricyclo[3.3.1.1]decane)), first described by Dyson and co-workers\textsuperscript{116}, built upon the traditional structure of half-sandwich compounds: a monodentate ligand, bidentate ligand and arene moiety, all of which can be modified to tune the properties of the complex. It was found that RAPTA-C was capable of inducing pH-dependent DNA unwinding which presented an opportunity for selective targeting of cancer cells. However, further experiments showed that proteins and RNA, demonstrated by the coordinative N-donor binding to the 14-mer oligomer (5'-ATACATGGTACATA-3'), are in fact the primary intracellular targets of these compounds\textsuperscript{117}. In general, anticancer activity for all organometallic Ru(II)-arene complexes has been shown to increase with the size of the coordinated arene\textsuperscript{115}, with toxicity towards A2780 ovarian cancer cells comparable to cisplatin and carboplatin for ruthenium complexes containing tetrahydroanthracene (THA) and biphenyl (bip) arene ligands respectively\textsuperscript{118}.

1.4.2.1 Ruthenium polpyridyl complexes

The anti-cancer properties of ruthenium polypyrrolidyl complexes were discovered by Dwyer \textit{et al} over 40 years ago, and was considered unusual given their inherently unreactive properties. The first complex of this type was tris(3,4,7,8-tetramethyl-1,10-phenanthroline)ruthenium(II) dichloride which was found, despite its inert and hydrophobic nature, to inhibit the growth of Landschütz ascites tumour cells in mice\textsuperscript{119}. Polypyrrolidyl-Ru systems were traditionally used as molecular DNA probes due to their fluorescent properties and ability to intercalate DNA, an attribute which contributes to their cytotoxic potential.

The polypyrrolidyl ligands are both large and rigid and their multidentate coordination to Ru centres also confers both shape and chirality, which can be exploited to fine tune DNA-binding
properties. Typical ligands of this type include bipyridine (bpy), 1,10-phenanthroline (phen), 2,2':6,2''-terpyridine (terpy) and dipyrido[3,2-\textit{f}:2',3'-\textit{h}]-quinoxaline (dpq), shown in Figure 1.9.

![Figure 1.9 Structures of commonly employed polypyridyl-type ligands: (l-r) bpy, phen, terpy and dpq.](image)

Amongst the earlier compounds which contained these ligands were cis-[Ru(bpy)$_2$Cl$_2$] and mer-[Ru(terpy)Cl$_3$]. In terms of cytotoxicity, the IC$_{50}$ of mer-[Ru(terpy)Cl$_3$] was determined to be 7 and 8 µM in L1210 and HeLa cells respectively, remarkably lower than for cis-[Ru(bpy)$_2$Cl$_2$] which was hindered by solubility issues.$^{120}$ The cytostatic behaviour of the terpyridine complex was attributed to its ability to bind covalently to guanine residues on DNA in a trans configuration. This results in the formation of ~2% interstrand crosslinks, however they differ from the cis crosslinks formed by cisplatin and carboplatin.$^{121}$ In order to improve the DNA-intercalating abilities the two chlorido ligands were substituted with large, aromatic, polycyclic ligands such as benzo[i]dipyrido[3,2-a:2',3'-c]phenazine (dppn). The lack of any leaving group ligands in this case points towards a non-covalent mode of action, and this was confirmed by a decrease in the impedance of the cell following administration, indicative of an interaction with the cell membrane and its components.$^{122}$ This was confirmed by more recent work by Dyson et al, who investigated a series of ruthenium tris(bipyridyl) complexes in which the bipyridyl ligands were derivatised to confer varying degrees of lipophilicity to the complexes. It was found that the most lipophilic compound, [Ru(DEA-bpy)$_3$]PF$_6$ (where DEA-bpy = 4,4'-Diethylamino-2,2'-bipyridine), was the most cytotoxic, with an IC$_{50}$ < 1µM in A2780 and A2780cisR cell lines. This was attributed to accumulation at the plasma membranes, rather than the result of its diethylamine groups becoming protonated in the surrounding medium, as determine by pKa measurement.$^{123}$
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Figure 1.10 Structures of $[\text{Ru(DEA-bpy)}_3]_{2}\text{PF}_6$ and $[\text{Ru(tBu}_2\text{bpy)}_2\text{(phox)}]_{\text{PF}_6}$.

However, the most comprehensive study of Ru(II) polypyridyl complexes to date is that of Meggers and co-workers, who used a combinatorial approach to screen a library of 560 Ru(II) complexes of general formula $[\text{Ru(X-X)(Y-Y)(Z-Z)}]_{\text{PF}_6}$ against HeLa cells\textsuperscript{124}. Structure-activity relationship studies led to the discovery of $[\text{Ru(tBu}_2\text{bpy)}_2\text{(phox)}]_{\text{PF}_6}$ (where tBu\textsubscript{2}bpy = 4,4'-di-tert-buty-2,2'-bipyridine and Hphox = 2-(2'-hydroxyphenyl)oxazoline), the structures shown in Figure 1.10, which displayed cytotoxicity similar to the aforementioned tris(bipyridyl) complex of Dyson and co-workers. This complex also displayed antiproliferative and apoptotic effects in vincristine- and doxorubicin- resistant BJAB cells, however no further investigations into this compound have been reported, perhaps due to the success of NAMI-A and KP1019.

Ru(II)-polypyridyl complexes have also been investigated as agents for photodynamic therapy (PDT) due to their well-documented photosensitivity. Providing that the complex is stable to aquation, non-toxic prior to irradiation, prone to accumulation in cancer cells and absorbs light in the range of 640-850 nm\textsuperscript{125} it may be suitable for PDT. One of the first compounds of this type, cis-(Cl,Cl)-$[\text{Ru}^{\text{II}}\text{(terpy)(NO)}\text{Cl}_2]\text{Cl}$, was reported by Reedijk and co-workers. It was found that the nitrosyl group was readily released following irradiation with a mercury lamp, and significant cytotoxicity was observed in A2780 cells ($IC_{50} = 0.49 \mu M$).
1.5 Enzyme-activated prodrugs

While traditional drug development has focused on generating highly toxic compounds which are indiscriminate in their cytotoxicity, enzyme-activated drug development strategies focus on structure-based design, where the cytotoxin is tethered via a linking moiety to a bioorganic molecule capable of being cleaved by an enzyme of interest, subsequently yielding the active drug. The localised activation of prodrugs can be achieved by targeting pathophysiological molecules indigenous to specific sites or disease phenotypes. The creation of organic prodrugs which are selectively activated at therapeutically significant locations has been widely considered in drug development in the past. However, the application of this more sophisticated design archetype has scarcely been considered in inorganic therapeutic design, and has only become more prominent in the past 5-10 years. The bioconjugation of metals and metal complexes to peptides and peptide motifs has the potential to address the problems discussed in section 1.7. The potential of this class of drugs is dependent on the level of knowledge of enzyme-substrate interactions and as such will likely be augmented by advances in the fields of genomics, proteomics and metallomics.

Various enzymes have been employed as activators for chemotherapeutics to date, including oxidoreductases, transferases, hydrolases and lysases. To be effective, either the enzyme required for activation must be selectively present in the target (tumour or organ), or the target should selectively take up the prodrug\(^\text{126}\).

Kageyama et al, using this approach, investigated the activation of cisplatin-like complexes following stereoselective ester hydrolysis by intracellular hydrolase enzymes as a means of selective targeting. Alkyl chains were attached via ester, ether and amide linkages to various cisplatin derivatives to improve the lipophilicity of the complexes for cell entry via passive diffusion. The greatest cytotoxicity was observed for the complexes with the longest hydrophobic alkyl chains tethered by an ester linkage. Comparison of the growth inhibition observed in rat liver cancer cells (Anr4) and rat hepatoma cells (H4-II-E), which exhibit high and low stereoselectivity for acyl group hydrolysis respectively, suggested that enzyme specificity contributed to stereoselective activation of the Pt(II) complexes\(^\text{127}\).

Site-specific activation of metal based prodrugs is a concept which will likely gather further interest and momentum in the future as the molecular pathologies of disease states are revealed through advances in other fields.
1.6 Matrix metalloproteinases

In order to achieve tumour-localised enzyme-activation of a prodrug, the enzyme of interest must be found at increased levels in tumour regions. One important group of enzymes which are implicated in tumour progression are matrix metalloproteinases. These zinc dependent endopeptidases are responsible for the degradation of several components of the extracellular matrix such as fibrillar and nonfibrillar collagens, fibronecton, laminin and basement membrane proteoglycans\textsuperscript{128}. There are now more than 20 enzymes which have been identified as MMPs and these are classified according to their preferred substrate – collagenases, gelatinases, stromelysins and membrane-type (MT-) MMPs. The regulation of cell behaviour by extracellular signalling pathways is typically performed at, or near, the cell surface – hence the covalent tethering of MT-MMPs to the cell membrane. The other secreted MMPs however are still capable of localising at the cell surface via various cell-surface-associated biomolecules such as integrins, CD44, heparin sulphate proteoglycans and collagen type IV\textsuperscript{129}.

Following their synthesis, MMPs exist as inactive zymogens, and they are kept as this precursor by the interaction that occurs between a cysteine-thiol moiety present in the propeptide domain and the Zn\textsuperscript{2+} ion which is bound in the catalytic domain\textsuperscript{129}. Activation typically occurs by other activated MMPs or serine proteases on the cell surface exterior so that MMP activity is confined to the peritumour environment\textsuperscript{130}. The regulation of their action is achieved through endogenous inhibitors, the salient one being the plasma protein α2-macroglobulin which binds to MMPs and upon binding to scavenger receptors is cleared by endocytosis and subsequent degradation. Other molecules capable of inhibiting MMP action include those which contain a subdomain structurally similar to that of tissue inhibitors of metalloproteinases (TIMPs)\textsuperscript{131}.

Structurally, almost all MMPs consist of four discrete domains: the N-terminal pro-domain, the catalytic domain, the hinge region, and the C-terminal hemopexin-like domain\textsuperscript{132}. Membrane-type MMPs (MT-MMPs) also contain a transmembrane domain that allows them to anchor to the cell surface. Early work into structure elucidation by Lovejoy and coworkers revealed that the active site of the catalytic domain is a shallow cleft with a flat non-prime side and a narrow prime side centered around the S1’ pocket. This work has been supplemented by more studies solving the structure of other MMP-inhibitor complexes, revealing that the active sites of most MMPs share a significant similarity in sequence residues\textsuperscript{133}. This similarity leads to broad substrate specificities as confirmed by various digestion studies\textsuperscript{132,134}. However the variation between the amino acid residues of the substrate binding groove within the catalytic region,
with the substrate binding sites labelled S#, determine the substrate selectivity in the enzymes. In a similar fashion, the positions, P#, on substrates will be assigned the same numbering as the sites that they occupy, with P1’ and P1 adjacent to the scissile bond as shown in Figure 1.11\textsuperscript{135}. The substrate docking is determined by the geometry of this binding site, in particular the depth of the S1’ pocket and the length as well as composition of the loop which constitutes the outside wall of the S1’ pocket\textsuperscript{136}. This is perhaps why such a significant interaction occurs between the S1’ subsite and the P1’ residue, with longer-chain aliphatic residues in the P1’ position increasing potency for MMP-3 and decreasing potency for MMP-1, while aromatic substituents seem to have a broad spectrum effect (see Figure 1.12)\textsuperscript{137}.

Figure 1.11 Two possible hexapeptide substrate (P#)-MMP substrate binding site (S#) arrangements. Taken from Schechter et al.\textsuperscript{135}

The determination of MMP cleavage site motifs by a combinatorial library approach has been performed by Turk and co-workers\textsuperscript{138}. Their results were in accordance with previously published substrate specificity studies as well as crystallographic and mutagenesis studies\textsuperscript{139,140}, which all found that the deep S1’ pocket has the greatest affinity for hydrophobic amino acids such as leucine in the P1’ position.
1.6.1 MMPs and cancer

The strong correlation between the altered expression of MMPs and poor patient prognosis in cancer therapy has been published extensively\textsuperscript{142}. There is significant evidence that the increased expression of MMP-1, -2, -3, -7, -9, -13, -14 in both primary tumours and metastases is positively associated with tumour progression as measured by poor tumour differentiation, the invasive stage of cancer, metastasis of secondary organs and shorter survival time\textsuperscript{130}. In particular, high MMP-2 expression has been shown to correlate with poor patient prognosis for gastric cancer and non-small cell lung carcinoma (NSCLC) in studies comprising 203 and 193 patients respectively\textsuperscript{143,144}. In a study of 45 patients with ovarian cancer, a similarly positive correlation between high expression of MMP-9 and poor patient prognosis was also found\textsuperscript{145}.

There are five significant instances in which MMPs play a role in tumour progression: these are the processes of growth, invasion, metastasis, angiogenesis and apoptosis.

MMPs are involved in the regulation of tumour growth via two primary mechanisms. Firstly, the induction of cell growth signals from neighbouring cells is facilitated by MMPs through
type of proteolysis known as ectodomain shedding, whereby the extracellular domains of proteins are released from the cell surface\textsuperscript{146}. This, along with release from matrix binding makes growth regulating molecules, such as TGF-α, TNF-α, Delta, FGF-R, SCF and ephrin\textsuperscript{147} bioavailable to other cells which are not in direct physical contact. MMPs can also act on non-matrix substrates, such as cell-membrane bound growth factors, as in the case of TGF-β liberation which causes growth inhibition\textsuperscript{148}.

The homeostatic functions involved in physiological invasion are similar to those mechanisms employed in tumorigenic invasion. However, the first is tightly regulated and the latter appears to be a perpetual process. The first step in invasion is for the cancer cells to cross the epithelial basement membrane in order to invade the surrounding stroma. In order to do this, cancer cells must undergo migration, whereby they must detach from both neighbouring cells and the surrounding matrix\textsuperscript{129}. In human cancers, MMP-14 is colocalised with 5-laminin, the cleavage of which triggers cell motility\textsuperscript{149,150}. The CD44 receptor is also cleaved by MMP-14, releasing the extracellular domain and triggering mobility. When the receptor cleavage site is mutated, it has been shown that cell migration is inhibited\textsuperscript{151}. Interestingly, CD44 also binds MMP-9 to promote tumour invasion and angiogenesis, so when the CD44-MMP-9 complexes are disrupted by MMP-14, tumour invasiveness is inhibited \textit{in vivo}\textsuperscript{152}. MMP localisation to specialised cellular protrusions known as invadopodia is also required for invasion. Via different mechanisms, MMP-2, -9 and -14 are known to localise to these surface protrusions by binding to αvβ3 integrins, recruitment by CD44 and recruitment via transmembrane and cytoplasmic domains respectively\textsuperscript{153}.

Following the preliminary course of invasion, MMPs are also implicated in the later stages of metastases, where cancer cells must pass into the blood vessels and lymphatic system to relocalse then exit, having survived. Although it was originally believed that MMPs facilitated metastasis by destroying the basement membrane and other structural components of the ECM, it is now more widely accepted that the overexpression of MMPs in relation to ECM assembly correlates with progression to a metastatic phenotype\textsuperscript{154}. This is supported by work which TIMP-1 overexpressing cancer cells are equally successful at extravasation as control cells, but yield fewer and smaller metastases due to diminished cell growth following exit from the blood stream. This suggested that MMPs support proliferation in the establishment of secondary tumours at a new site\textsuperscript{155}.

All the stages of tumour progression described thus far share a common feature – they are limited by the process of angiogenesis. New vascular structure is required for gas and nutrient
exchange by the rapidly proliferating cells to sustain growth, and without this the tumour mass is limited in size to within a tissue-diffusion distance of 200 µm. MMPs are known to play both a pro- and antiangiogenic role, where in healthy tissue the balance is tightly controlled and in tumour tissue, perturbed\textsuperscript{156}. The primary MMPs involved in angiogenesis, MMP-2, MMP-9 and MMP-14\textsuperscript{157} originate from both epithelial and stromal cells. In the epithelial cells, MMPs can be found in membrane vesicles, with localised angiogenic stimulation of cells by bFGF or VEGF causing shedding of the vesicles and MMP release which induces the angiogenic phenotype\textsuperscript{158}. Their pro-angiogenic role involves the deposition and breakdown of vascular basement membranes to allow for the proliferation and migration of endothelial cells\textsuperscript{146}. They are also implicated in the release and activation of VEGF, bFGF and TGF-β which induce angiogenesis by signalling through their respective receptors on endothelial cells\textsuperscript{156}. MMP-2 exhibits pro-angiogenic behaviour through its cell surface localisation to the invading edge of migrating endothelial cells via αvβ3 binding, and also plays a role in anti-angiogenesis through its own degradation, where its soluble PEX domain binds to the αvβ3 integrin thereby inhibiting MMP-2-αvβ3 binding and angiogenesis. The generation of endogenous angiogenesis inhibitors such as angiostatin from the hydrolysis of plasminogen through the proteolysis of ECM components is also a result of MMP activity, in particular MMP-2, -7, -9 and -10\textsuperscript{159-161}.

As in the case of angiogenesis, MMPs have both apoptotic and anti-apoptotic actions, in particular MMP-3, -7, -9 and -11. The degradation of laminin by overexpressed MMP-3 has been shown to induce apoptosis by modification of the ECM which in turn changes integrin signalling\textsuperscript{162-164}. The release of membrane-bound FASL byt MMP-7 is capable of inducing apoptosis in neighbouring cells, or decreasing apoptosis by facilitating cleavage of FASL, which is a ligand for the death receptor FAS\textsuperscript{165,166}. MMP-11 has been hypothesised as having a similar mechanism of action for inhibiting apoptosis through the release of IGFs\textsuperscript{167}. In general, MMPs are also believed to contribute to the characteristic rounding up of apoptotic cancer cells by cleaving the VE-cadherin, PECAM-1 and E-cadherin glycoproteins\textsuperscript{168-170}.

1.6.2 Exploiting MMPs in cancer therapy

1.6.2.1 Activation by MMPs

To date, various enzymes have been targeted in an effort to improve the selective delivery of chemotherapeutics. These include prostate-specific antigen, legumain, plasmin and other tumour-associated proteases\textsuperscript{171-174} which are overexpressed in the tumour environment. Fundamental work into elucidating the peptide cleavage motifs of various MMPs\textsuperscript{138} has made
these enzymes a viable target, and targeting MMPs for selective activation has become a more prominent design strategy.

A novel approach to the selective delivery of a galactosyalated liposomal carrier was investigated by Terada et al who employed an MMP-2 specific cleavage sequence for hepatocellular carcinoma-selective targeting. The compound, Gal-PEG-PD-liposome (Gal = galactose, PEG-PD = MMP-2-cleavable PEG-Peptide-dioleoylphosphatidylethanolamine) was not expected to be taken up by normal hepatocytes due to steric hindrance, but would be activated by the increased MMP-2 levels around HepG2 hepatocarcinoma cells, enabling the receptor-mediated uptake of the liposomes. In experiments where HepG2 cells were pretreated with human MMP-2 (hMMP-2) it was found that increasing liposomal delivery was positively correlated with increasing hMMP-2 concentration, suggesting PEG-PD cleavage by MMP-2.

Another member of the MMP family which is overexpressed in tumour regions is MMP-9, and work by Van Valckenborgh et al has focused on exploiting this enzyme for tumour-specific activation of the prodrug EV1-FITC (where FITC = fluorescein isothiocyanate) in 5T33 multiple myeloma (MM) mouse model cell homogenates. The drug EV1 contains the L-proline adduct of 1-(3-aminopropylamine)anthraquinone, a dual inhibitor of DNA topoisomerase I and II, as the active agent, and is attached to the C-terminus of an MMP-9 peptide substrate. The presence of the anthraquinone chromophore caused internal quenching of the FITC fluorophore, and cleavage of the MMP-9 peptide substrate in bone marrow and spleen cells resulted in a return of fluorescence which was measured spectroscopically, confirming cleavage. Treatment with the MMP-2/-9 inhibitor CTT saw a dose-dependent inhibition of fluorescence which confirmed cleavage by MMPs.

The widely recognised anticancer drug doxorubicin has also been conjugated to an MMP selective sequence in an attempt to improve its efficacy and therapeutic index. Hu et al synthesised a range of peptide-doxorubicin prodrugs and tested their cleavage by MMP-2, MMP-9 and MMP-14, as well as the serum protease neprilysin to investigate non-specific cleavage. To determine selectivity for tumour tissue, the concentration of doxorubicin found in HT1080 xenografts in mice was compared to that found in the heart, with ratios ranging from 6.5 to 17, showing tumour selectivity.

Tauro and co-workers have used the selective activation approach in the design of anti-tumour therapeutics delivered by hydrogel matrices. The cisplatin-like molecules were tethered to the
peptide (through the oxygen atoms of aspartic acid) which was attached to the hydrogel via poly(ethyleneglycol) acrylate spacers. Upon cleavage by the extracellular MMPs which are overexpressed in tumour tissue, cisplatin and cisplatin-peptide conjugates were released and able to induce cell death via the formation of classical Pt-DNA adducts. Figure 1.13 depicts the mechanism by which this was believed to occur. Progressive fine tuning resulted in a hexapeptide substrate for tumour specific MMP-2 and MMP-9 which resulted in improved selectivity for tumour tissue and decreased systemic release. Prior to this study, literature on the selective activation of metal-based drugs is scarce.

Figure 1.13 Structure of the cisplatin-peptide-hydrogel matrix prepared by Tauro and co workers. Figure adapted from [178].

1.6.3 Visualising MMP activity

Imaging MMP activity is a field still relatively in its infancy, with the gradual elucidation of the roles played by MMPs in the growth and metastasis of tumours giving rise to the development of methods for imaging tumours based on their high levels of proteolytic activity. For the purpose of drug development, visualising MMPs provides a means of observing compound activation relative to the localisation of MMPs which can assist in determining the modes of action of compounds which target MMPs specifically.

One of the earliest developed methods for measuring proteinase activity in vitro, zymography, is still extensively used in vitro but is not suitable for in vivo studies. Also, whilst it provides excellent and accurate quantitative information, it provides no spatial resolution for visualisation of MMP activity. In the last decade, a NIR (NIR) fluorescence sensor was developed by Bremer et al, which could not only give a measurement of MMP-2 activity but also provided an image of MMP-2 localisation. The sensor was synthesised by covalently attaching a NIR fluorochrome to a methoxy polyethylene glycol (MPEG)-modified poly-lysine
backbone via an MMP-2 specific peptide substrate. *In vitro* activation by MMP-2 showed an 850% increase in NIR fluorescence signal in HT1080 breast cancer cells which are known to overexpress MMP-2. A control probe, synthesised in the same fashion but incorporating a scrambled MMP-2 substrate sequence, remained optically silent. The probe was then applied to *in vivo* studies, with NIR fluorescence imaging of mice implanted with an HT1080 fibrosarcoma or BT20 adenocarcinoma (MMP-2 negative) revealing a quantitatively significant difference between the fluorescent signal intensity between the two tumours.

Fluorescence imaging has since been applied more frequently to the visualisation of MMP activity *in vitro*, with Yang and co workers exploiting the Förster Resonance Energy Transfer (FRET) phenomenon to identify sites of MMP activity using a genetically coded fluorescence indicator. The two fluorescent proteins, yellow and cyan (YFP and CFP respectively) were linked by a 12 amino acid MMP substrate and this construct was anchored to the cell surface to detect extracellular MMPs. When intact, the fluorescence emission by CFP overlaps the excitation spectrum of YFP, such that only fluorescence emitted by the YFP will be observed. Upon cleavage, this transfer of energy between the two fluorescent proteins is terminated, and a return of CFP fluorescence is observed. In MCF-7 cells which express low levels of MMPs, real time FRET was observed as the fluorescent probe remained intact and following incubation with MMP-2, FRET ceased. MDA-MB 435s cells secrete high levels of MMP-2 and as such no FRET effect was observed, with the respective emission profiles for both YFP and CFP being visible.

MMPs are implicated in various other biological processes aside from tumour growth and metastasis. In particular, there is now ample evidence that the gelatinases MMP-2 and -9 play a role in the development, plasticity, diseases and repair of the central nervous system. To study the trafficking and secretion of MMP-2, -9 and their inhibitor TIMP-1 in neuronal cells, Sbai and co-workers adopted a molecular biology approach, synthesising plasmid constructs which encoded mouse MMPs and their inhibitor fused to green or red fluorescent proteins (GFP and RFP respectively). These proteins were then expressed in mouse neuroblastoma cells and visualised using fluorescence microscopy, as shown in Figure 1.14.
Figure 1.14 Secretion of vesicles containing MMP-9-GFP by transfected N2a neuronal cells (A). Inset of A (a’). Image taken from [184].

This method proved to be highly sensitive, provide excellent spatial resolution, and offer the advantage of requiring no prior treatment by extrinsic chemicals, which could potentially alter or disrupt some cellular functions. In addition, the absence of a chemical probe means that this technique could be used to simultaneously monitor the behaviour of fluorescent MMP-targeted compounds in the vicinity of active MMPs, without potential competition from chemical probes.

1.7 Peptide bioconjugation to metal complexes

The attachment of peptides, including enzyme-specific sequences, to metal complexes is a rapidly increasing area of interest in medicinal inorganic chemistry, in line with the ever-increasing elucidation of the biological hallmarks of disease. Given that examples of peptide-metal conjugates for enzyme-selective activation have already been discussed in this chapter, this section will focus on the use of peptides as uptake modulators which can direct the localisation of the attached metal complex.

Given that most chemotherapeutic metal complexes exert their action on DNA, a large proportion of the research on the directing effects of peptides has focused on improving delivery of cytotoxic agents to the cell nucleus using a nuclear localisation sequence (NLS). NLS peptides are short positively charged basic peptides that actively transport large proteins across the nuclear membrane from the cytosol to the cell nucleus\textsuperscript{185,186}. Kirin and co-workers
achieved conjugation of the chelating amino acid modified bis(picolyl)amine (bpa) ligand with a heptapeptide derivative of the SV 40 virus antigen NLS with the sequence (H-PKKKRKF-OH), which was then complexed with Cu$^{2+}$, Zn$^{2+}$, and more recently Co$^{2+}$ metal centres$^{187}$. Increased uptake was reported for the metal-NLS conjugates, likely the result of the cationic charge conferred to the conjugate by the multiple lysine residues in the NLS rather than any directing effects by the NLS, as it has previously been determined that NLS is not a good vector for improving cellular uptake$^{188}$.

![Diagram of metal complexes containing peptide sequences](image)

**Figure 1.15** Examples of metal complexes containing peptide sequences a) The Co-bpa-PNA-NLS complex synthesised by Kirin et al. b) The Pt(IV) complex containing RGD peptide motifs and c) NGR peptide motifs synthesised by Mukhopadhyay et al.

Most recently, the bpa ligand was also conjugated to a peptide nucleic acid oligomer (PNA) sequence prior to the NLS and showed increased cellular accumulation as a result$^{189}$. HT-29 human colon adenocarcinoma cells treated with Co-bpa-NLS, Co-bpa-PNA and Co-bpa-PNA-NLS (see Figure 1.15a) favoured uptake of the respective complexes compared to unconjugated Co-bpa, as determined by atomic absorption spectroscopy (AAS), with the similar concentrations of Co-PNA and Co-PNA-NLS after 24 hrs suggesting that the PNA sequence is predominantly responsible for the enhanced uptake.

In similar work, Noor et al.$^{190}$ synthesised cobaltocenium-peptide bioconjugates containing the same SV 40 antigen NLS. A Co metalloocene was chosen over ferrocene due to its higher kinetic
stability and redox potential. Nuclear localisation was visualised by attaching a fluorescent label (fluorescein isothiocyanate (FITC)) to the various cobaltocenium compounds and free NLS. It was observed that free NLS was unable to traverse the cell membrane, whereas the cobaltocenium-NLS species showed intracellular accumulation and co-localisation with the Hoechst 3342 nuclear stain in HepG2 cells. This study indicated the importance of the organometallic moiety for endocytosis into the cell, and as such the potential for metal-peptide conjugation to overcome the limitations of the separate components and exhibit different and unique biological properties.

Another example where the metal-peptide conjugate possesses very different biological properties to its building blocks was reported by Neundorf et al\textsuperscript{191}, where the cell-penetrating peptide hCT(18-32)k7 was used to enhance the cellular penetration of cymantrene [CpMn(CO)\textsubscript{3}] MCF-7 breast cancer cells. Prior to conjugation, the respective peptide and cymantrene have no cytotoxic properties, and confocal microscopy of a fluorescently tagged hCT(18-32)k7 peptide showed primarily vesicular localisation, with none present in the cell nucleus. Contrastingly, the fluorescently tagged hCT(18-32)k7-CpMn(CO)\textsubscript{3} conjugate was distributed throughout the cytosol, with a large amount of fluorescence also observed inside the nucleus and a measured IC\textsubscript{50} of 36 µM.

Conversely, metal complexes can also be exploited as chaperones for peptides in order to improve either their cell or tissue-penetrating properties. In this vain, metal-peptide bioconjugation of the neuropeptide leucine-enkephalin ([Leu\textsuperscript{5}]-Enk,Tyr-Gly-Gly-Phe-Leu) to ferrocene or the cobalocenium cation was intended to improve its limited penetration across the blood-brain-barrier (BBB) by increasing its lipophilicity. Indeed, conjugation to the ferrocenoyl moiety resulted in a great logP value seven-fold greater than the free [Leu\textsuperscript{5}]-Enk, and subsequent addition of the FITC fluorophore led to a further increase in lipophilicity again and suitable for transport across the BBB. Visualisation in cells showed increased uptake of the Ferrocene-[Leu\textsuperscript{5}]-Enk in HeLa and HepG2 cells after 24 hrs, confirming the enhanced penetration across cell membranes, however these compounds did not display any cytotoxic activity.

An example of peptide-directed tumour targeting comes from Mukhopadhyay et al, who tethered peptide motifs to the axially coordinated succinato ligands of a Pt(IV) cisplatin analogue via amide linkages, shown in Figure 1.15b and 1.15c. The peptide motifs RGD (Arg-Gly-Asp) and NGR (Asn-Gly-Arg) were employed as “tumour homing devices” intended to recognise the upregulated αvβ3 and αvβ5 integrins on the membranes of metastatic tumour
cells. In addition, two cyclic pentapeptides containing the RGD sequence were also investigated as their enhanced recognition by endothelial cells over linear peptides is well established\textsuperscript{192}. It was found that the cyclic RGD-Pt(IV) complexes had a potent inhibitory effect on cell proliferation, and upregulated integrins were likely mediators in the internalisation of the Pt(IV)-peptide complex into the tumour cells\textsuperscript{193}. Similarly, Ndinguri and co workers synthesized two cyclic mPEG-CNGRC-Pt(II) (PEG = polyethyleneglycol) complexes and investigated their uptake and cytotoxicity\textsuperscript{194}. The NGR motif is known to bind selectively to the CD13 receptors present on the surface of some tumour cells. The peptide moiety was conjugated via the amine-functionalised malonato ligand, with the remaining equatorial positions occupied by two ammine ligands or a bidentate en (ethylenediammine) ligand, see Figure 1.16. In cellular accumulation studies, the ammine compound was found to have 12-fold greater uptake than its analogue carboplatin, and 4-fold greater uptake than the en conjugate in CD13 positive PC-3 cells. This data showed no direct correlation with the cytotoxicity observed for the two conjugates in comparison to carboplatin, as the IC\textsubscript{50} values for the two compounds were 149 mM and 115 \(\mu\)M. This confirms that the form of the Pt complex is still highly influential in the effectiveness of the drug, and it is not simply a case of increasing Pt uptake.

![Figure 1.16](image.png)

**Figure 1.16** Generic structure of cyclic mPEG-CNGRC-Pt(II) prepared by Ndinguri et al.

These studies show how the conjugation of biologically relevant molecules, including peptides, can be an effective technique for improving the selective delivery of metal complexes to their site of action. This can be useful for overcoming fundamental cellular uptake issues, resulting in previously inactive complexes being able to exert their cytotoxic effect following optimised
localisation. As more knowledge is inevitably garnered about disease states, it is likely that this mode of targeted design will be increasingly relevant and more widely employed.

1.8 Imaging of peptide-metal bioconjugates

1.8.1 Fluorescence imaging

Many of the studies mentioned thus far have employed fluorescence imaging as a diagnostic tool for the in situ monitoring of compound localisation and in some cases, drug activation. Fluorescence occurs when a photon of light is absorbed by a molecule and subsequently emitted after a very short period (approximately $10^{-8}$ s) as the molecule transitions from a higher to lower electronic state\textsuperscript{195,196}. For fluorescent emissions, these emitted photons are, apart for a few exceptions, the result of relaxation from the $S_1 \rightarrow S_0$ singlet states as shown in Figure 1.17. Due to the small dissipation of energy incurred during the excited state, the emitted radiation is always produced at a slightly longer wavelength than that absorbed, known as the Stokes shift.

![Perrin-Jablonski diagram showing the relative energy transitions of absorption, fluorescence and phosphorecence spectra.](image)

**Figure 1.17** Perrin-Jablonski diagram showing the relative energy transitions of absorption, fluorescence and phosphorecence spectra. Taken from [196].

Imaging fluorescence using microscopic techniques provides invaluable insights into the behaviour of new compounds. However, conventional fluorescence microscopy is inherently problematic due to the emission and scattering of light by surrounding tissue not within the focal plane. Confocal microscopy ensures that both the illumination and detection systems are focused on the same region, such that any fluorescent signals arise only from the area being
visualised, known as spatial filtering\textsuperscript{197}. For these reasons, confocal microscopy is ideal for visualising live cells which are adherent on the bottom of plates, as fluorescence from floating debris, autofluorescent particles or any other fluorescent molecules will not be detected to any significant degree. It can also achieve a higher optical resolution than standard microscopy, which provides more information on the spatial localisation of the analyte\textsuperscript{197}, as cellular compartments and components including organelles are able to be visualised.

1.8.1.1 Fluorescence for detection and distribution

The detection of particular chemical and biological entities such as ions, molecules, organelles or cell types is an important feature of fluorescent imaging. Achieving this also generates the ability to follow particular cellular processes and changes in the physicochemical environment through the use of fluorescent probes or tracers, whether they are tethered to cellular entities or molecules intended to interact with the cell.

Fluorescent probes for detection of metal ions include Calcium green-1 and Oregon green for the imaging of calcium ions in tissues\textsuperscript{198}, ZP4 for zinc\textsuperscript{199}, and coumarins for iron\textsuperscript{200}. The detection of proteins and peptides makes use of their variety of chemical functional groups for which specific fluorochromes exist, the most typical being modification of the amine moieties and the most widely employed example is fluorescamine. The original spiro compound is intrinsically nonfluorescent, but upon reaction with primary amines generates a blue-green fluorescent isoindole. Other compounds such as o-phthaldialdehyde and naphthalene-2,3-dialdehyde are also essentially nonfluorescent prior to reaction with a primary amine\textsuperscript{198}. This phenomenon, whereby fluorescence is either enhanced or quenched upon metal coordination or reaction with the target functional group has been appropriated by medicinal chemists for monitoring compound activation. Yamamoto and co-workers investigated the behaviour of two Co(III) complexes containing the fluorescent coumarin-343 and coumarin-343 hydroxamic acid ligands, where complexion to the metal centre resulted in complete fluorophore quenching. Upon hypoxia-induced reduction from Co(III) to Co(II), ligand release and a return of fluorescence was used to visualise the site of reduction and observe localisation of the free coumarin-343 in the cell nucleus\textsuperscript{201}.

Apart from detecting particular moieties or sites of activity, fluorophores are also valuable in simply observing the distribution of molecules within cells and tissues. For new chemotherapeutics, the visualisation of compound penetration in solid tumour models is particularly pertinent to the process of rational drug design. This is well demonstrated by Noor
et al whose original work on the cellular uptake of a cobaltocenium-NLS-PNA conjugate and subsequent investigations into ferrocene- and cobaltocene-NLS derivatives used FITC as a fluorescence marker to visualise compound distribution\textsuperscript{202}. HepG2 cells which were concurrently stained with endosomal and nuclear fluorescent markers were treated with both mono- and di-substituted metalloocene-NLS-FITC, as well as a metalloocene-scrambled NLS-FITC compound. By observing the different cellular uptake and intracellular localisation profiles, they were able to conclude that the metal moiety is predominantly responsible for assisting the NLS peptide in entering cells, rather than any increase in lipophilicity such as for the disubstituted compound, with the charge on the metalloocene having little effect on uptake. They were also able to conclude that the NLS was solely responsible for shuttling the compound into the nuclei.

Fluorescence images for spheroid tumour models treated with Pt(II)-anthraquinone complexes have also provided invaluable insights into the effect of complex charge on the penetration and distribution of these compounds\textsuperscript{41}. The results, displayed in Figure 1.18, were compared to the distribution of the free anthraquinone ligand (1C3), with the doubly cationic [Pt(1C3)(dien)]\textsuperscript{2+} showing a much more uniform delivery throughout the spheroid when compared to the neutral cis-[PtCl\textsubscript{2}(1C3)(NH\textsubscript{3})] complex which remained primarily in the peripheral regions of the spheroid.

![Image](image.png)

**Figure 1.18** Fluorescence images of DLD-1 spheroids treated with (L-R) the free ligand (1C3), cis-[PtCl\textsubscript{2}(1C3)(NH\textsubscript{3})] and Pt(1C3)(dien)\textsuperscript{2+}.

Information such as this is likely to shape future design of chemotherapeutic agents, and as such reconfirms fluorescence imaging as a powerful tool in anticancer drug development.
1.8.2 Chemical mapping using X-ray fluorescence (XRF)-based techniques

In the absence of fluorescently-tagged proteins or molecules, mapping the elemental distribution of metals derived from both endogenous and exogenous compounds requires powerful techniques with excellent resolution, in order to detect and measure metal ions at low concentrations and with high accuracy. In order to obtain elemental maps which are representative of metal distribution in the cellular environment, sample preparation techniques should not involve extensive pretreatment such as chemical fixing, which can cause redistribution, contamination and loss of metal ions.

1.8.2.1 Synchtron radiation-induced X-ray emission

Synchtron radiation-induced X-ray emission (SRIXE) is the only technique that is capable of achieving trace element sensitivity (in the parts per million range)\textsuperscript{203} and sub-micron resolution while simultaneously being compatible with fully hydrated biological samples such as whole cells and tissue sections\textsuperscript{204}.

The physical principle behind SRIXE analysis is best described using the Bohr atom model, as shown in Figure 1.19. Following irradiation by a highly-focussed, high flux of X-rays (> 1keV), a core-shell electron is ejected, generating a vacancy which is filled by the demotion of a higher-shell electron. This process involves the emission of a photon of energy, the energy of which is equal to the difference in binding energies between the two energy shells involved in the transition. This photon is of a discreet energy, as the binding energy of each energy level for a particular element is proportional to the square of the nuclear charge. The energy of the emitted photon is therefore characteristic for a particular element, and can be used to determine their internal spatial distribution due to the high penetrative depth of hard X-rays.
The applications of SRIXE mapping have been extensive, including elemental imaging of bacteria\textsuperscript{205}, Alzheimer’s and Parkinson’s disease-affected brain tissue\textsuperscript{206-209}, toxicological studies of Chromium in lung tissue\textsuperscript{207,210}, and the mapping of anticancer drugs in cells and tumour models. In particular, micro-SRIXE has been used by Hall and coworkers to map the cellular distribution of a library of platinum-based chemotherapeutics in A2780 human ovarian cancer cells\textsuperscript{211}. In the majority of platinum-based therapeutics, the platinum centre is responsible for the biological activity of the compound. Therefore, as SRIXE mapping directly confers the distribution of platinum in a sample it is a more accurate method compared to the inferences drawn from fluorescently-labelled platinum compounds.

1.8.2.2 X-ray absorption near-edge structure spectroscopy

X-ray absorption near-edge structure (XANES) Spectroscopy is a complementary Synchrotron-based technique which can be used to extract more information from SRIXE mapping regarding the oxidation state and coordination sphere of a mapped element.

In XANES spectroscopy, the absorbance of X-rays by a particular sample is represented by the absorption coefficient, which is a measure of the decay in intensity of the X-ray beam with distance. The absorption coefficient is then plotted as a function of the incident X-ray energy.
As depicted in 1.20, peaks caused by intense rises in absorption are referred to as edges, and the XANES region is located within approximately 30 eV above an energy edge\textsuperscript{212}. The energy position of the edges is characteristic for a particular element, as described in section 1.2.8.1, and the oscillatory features of the XANES region contain detailed information about interatomic distances and oxidation numbers.

XANES spectroscopy has been employed to investigate the oxidation, reduction and speciation of both endogenous and exogenous elements in a range of systems, including complex biological matrixes\textsuperscript{211,213-218}. Recent work by Hall and co-workers on the reduction of platinum(IV) drugs \textit{in vitro} has confirmed that the relative ratios of Pt(IV) to Pt(II) in biological samples can be determined by measuring the peak height ratio a/b, Figure 1.21, at the absorption edge of the XANES spectrum and comparing it to a calibration curve constructed from the peak heights of various mixtures of Pt(II) and Pt(IV) complexes of known ratios\textsuperscript{214}.

\textbf{Figure 1.20} Schematic view of the x-ray absorption coefficient as a function of incident photon energy. Energy edges are labelled L3, L2, L1 and K. Image taken from [210].
Figure 1.21 Examples of XANES spectra of Pt(II) (red) and Pt(IV) (black) complexes. The difference in peak height is evident, with the Pt(IV) peak height being greater than that of the Pt(II) complex. The parameters $a$ (edge height maxima) and $b$ (post-edge minima) were used to determine the peak height ratio $a/b$. For normalised spectra, $b = 1$. 
1.9 Objectives

The objective of this study is to exploit the unique features of the local tumour microenvironment to develop anti-cancer agents which are more selective for solid tumours over healthy tissue. As described in section 1.2.1, solid tumours exhibit distinctive physiological, chemical and biomolecular characteristics which can selectively activate chemotherapeutics which have been administered in an inert prodrug form, reducing systemic toxicity to the patient.

In particular, this project focuses on the overexpression of MMPs in a range of solid tumours, and will exploit the cleavage of an enzyme-specific peptide sequence as a means of prodrug activation. The prodrug will be inactivated through the presence of a cell-uptake blocking moiety, which following enzymatic cleavage will allow for the delivery of a cell-permeable payload. In the presence of the enzyme, such as within solid tumours, the sequence should be cleaved, detaching the uptake-blocker and delivering the payload to tumour cells. In healthy tissue, which expresses minimal levels of the enzyme, the sequence should remain intact and not taken up by cells, thus sparing healthy cells from the active drug component.

A series of model peptide prodrugs will first be investigated in order to understand the behaviour of these enzyme-specific sequences. These model peptides will contain fluorophores as model cytotoxins, such that their physicochemical properties and behaviour in biological systems can be monitored.

A series of cytotoxin-containing peptide sequences will then be investigated as potential prodrug compounds. Methods for the attachment of the platinum(IV) congener of oxaliplatin to peptides will be explored. In the absence of fluorophores, Synchrotron radiation-based techniques will be used to observe the distribution and speciation of the platinum species in three-dimension tumour models.

A ruthenium(II) cytotoxic complex-peptide conjugate will also be investigated, based on the identical MMP-2 specific peptide sequence. The difference between cellular uptake of the free ruthenium(II) complex and its peptide analogue will be monitored using confocal fluorescence microscopy due to the inherent fluorescence of this class of compounds.

In order to better understand the behaviour of these MMP-2 selective compounds in two- and three-dimensional cell models, molecular biology techniques will be used to visualise the secretion of MMP-2 and MMP-9 enzymes through the generation of plasmid constructs that
encode human MMP-2 and MMP-9 fused to a cyan fluorescent protein. Additionally, commercially available fluorogenic peptide substrates will be used to observe colocalisation between fluorophore-peptide model prodrugs and active MMPs in vitro.

Through this body of work, it will be possible to understand the behaviour of this class of enzyme-activated prodrugs and ascertain whether this rational design concept is a viable means of exploiting the tumour microenvironment for prodrug activation. The outcomes of this study will build upon the understanding of ways in which prodrugs can be activated, and therefore contribute to the development of better tumour targeted therapies.
Chapter 2

MATERIALS AND INSTRUMENTATION
2.1 Materials

2.1.1 Chemical synthesis

All solvents and chemicals were of laboratory grade and used without further purification unless otherwise stated.

K$_2$[PtCl$_4$] was purchased from Precious Metals Online.

(±)-trans-cyclohexane-1,2-diamine and methyl bromoacetate were obtained from Sigma-Aldrich.

(+)-Tartaric acid, sodium azide, H$_2$O$_2$ (30% w/v) and acetic acid were all obtained from Ajax FineChem.

Oxalic acid dihydrate and succinic anhydride were obtained from Merck.

cis, cis, trans-2-azidoaceto[(1R,2R)-cyclohexane-1,2-diamine-N,N']methoxidoxalato-platinum(IV) was kindly synthesised and provided by Dr Jenny Zhang.

Preloaded fmoc-amino acid-Wang resins and fmoc-protected amino acids were purchased from GLBiochem.

Fluorescein-5-isothiocyanate was obtained from Toronto Research Chemicals.

Rhodamine B, triisopropylsilane (TIS) and hydroxybenzotriazole (HOBt) were obtained from Sigma-Aldrich.

DMF (peptide synthesis grade) was obtained from Labscan.

Piperidine and acetonitrile (HPLC grade) were obtained from Merck.

Trifluoroacetic acid (peptide synthesis grade) was obtained from Ajax FineChem.

N,N-Diisopropylethylamine (DIPEA) and O-Benzotriazole-N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU) were obtained from Alfa Aesar.

RuCl$_3$.3H$_2$O and DMF were obtained from Sigma-Aldrich.

4,4'-Di-tert-butyl-2,2'-bipyridine and 4-methyl-4'-carboxy-2,2'-bipyridine were purchased from CMS Chemicals Ltd.
Aluminium oxide, activated, neutral, Brockmann I, \(\sim 150\) mesh was obtained from Sigma-Aldrich.

### 2.1.2 Molecular biology

Forward and reverse oligonucleotides were purchased from Integrated DNA Technologies and diluted to a concentration of \(1 \text{ mg/mL}\) with sterile Milli-Q water before use.

Matrix metalloproteinase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase) plasmid was purchased from the DNASU Plasmid Repository (Biodesign Institute, Arizona State University).

LongAmpTaq, 2x Master Mix, T4 DNA ligase and restriction enzymes (SalI-HF™, HindIII-HF™, KpnI-HF™, EcoRI-HF™, XbaI) were purchased from New England Biolabs® Inc.

Ultrapure™ Agarose for gel electrophoresis was obtained from Invitrogen. 100 bp and 1 kbp DNA ladders were purchased from New England Biolabs® Inc. Ethidium bromide was obtained from Sigma Aldrich.

LongRange 2Step RT-PCR Kit, QIAprep Spin Miniprep Kit, Plasmid Maxi Kit, QIAquick Gel Extraction Kit, RNeasy Extraction Kit were all purchased from Qiagen.

pAmCyan was purchased from Clontech Laboratories, Inc.

pcDNA™3.3-TOPO® TA Cloning Kit was purchased from Invitrogen™.

### 2.1.3 Biological studies

All cell lines were obtained from ATCC and used within 6 months of resuscitation.

Advanced Dulbecco’s Modified Eagle Medium (DMEM), L-glutamine, fetal calf serum (FCS), trypsin and Antibiotic + Antimyotic (AA) for cellular studies were obtained from Gibco. Agarose was obtained from Sigma-Aldrich. All cellular stains were obtained from Molecular Probes.

MMP-2 enzyme was obtained from Anaspec. \(p\)-aminophenylmercuric acetate was obtained from Sigma Aldrich.
2.2 Instrumentation

2.2.1 Nuclear Magnetic Resonance Spectrometry

Proton and Carbon ($^1$H and $^{13}$C) nuclear magnetic resonance (NMR) spectra were collected on a Bruker 300 MHz NMR spectrometer at 300 K. All samples were dissolved in commercially available deuterated solvents. TSP (3-(trimethylsilyl)propionic acid) was used as an internal reference in D$_2$O. In all other solvents, isotopic impurities were used as internal reference signals.

Platinum ($^{195}$Pt) NMR spectra were collected on a Bruker 400 MHz NMR instrument. All samples were referenced to sodium tetrachloroplatinate(II). $\delta$ (Na$_2$[PtCl$_4$]) = -1620 ppm.

2.2.2 Mass Spectrometry

Mass spectrometry was performed on a Finnigan LCQ mass spectrometer using electro-spray ionization (ESI) and monitoring in both positive and negative ion modes.

2.2.3 Infrared Spectrometry

Diffuse reflectance infrared Fourier transform spectra (DRIFTS) were collected on a Varian FT-IR 400 Scimitar Series over the range 4000 – 400 cm$^{-1}$. All spectra were collected with potassium bromide as the supporting matrix and background.

2.2.4 Microanalysis

Microanalysis (C, H, N) was performed by the Campbell Microanalytical Laboratory at the University of Otago.

2.2.5 X-ray crystallography

Crystal structures were performed by Dr Jack Clegg of the School of Chemistry at the University of Sydney.
2.2.6 Inductively Coupled Plasma-Mass Spectrometry (ICP-MS)

All ICP-MS measurements were made with an Agilent 7500cs ICP-MS equipped with a MicroMist glass concentric nebulizer, a Quartz-Scott spray chamber (Peltier cooled, 2 °C) and Nickel cones. Baseline® HNO₃ (67-70%) was purchased from SEASTAR™, and all metal ion standards were obtained from Choice Analytical.
Chapter 3: Visualising Matrix Metalloproteinases In Vitro
3.1 Background and strategy

The visualisation of MMP expression is a fundamental step towards understanding the biological behaviour of the MMP-2 specific peptide sequences presented in Chapters 4 and 5 of this thesis. In particular, any colocalisation of the fluorophore-peptide conjugates or their fragments with MMP-2 would provide invaluable insights into the cleavage and activation properties of potential prodrugs based on the MMP-2 specific sequence.

MMPs, in particular MMP-2 and MMP-9, are secreted enzymes which mediate the cleavage of pericellular substrates. The exocytotic release of active MMP-2 and -9 as well as their proenzyme forms has previously been monitored by immunofluorescent labelling in highly malignant A7 human melanoma cells\textsuperscript{219}. The cytoplasmic vesicles, containing discreet populations of the two enzymes, were found to be aligned along the microtubular network, and administration of paclitaxel to impair the function of the microtubular network impaired the secretion of the MMPs. Despite the success of indirect immunofluorescence, it was necessary for the cells to be fixed in methanol prior to treatment with the MMP-specific antibodies and the fluorophore-conjugated secondary antibodies. This strategy, despite being highly specific, is not appropriate for the imaging of cells which have been treated with methanol-soluble compounds (see Chapters 4 and 5), as fixation would lead to the redistribution of the administered compounds and give an inaccurate distribution profile.

Alternatively, fluorescent protein fusions have been used successfully for studying the secretion and trafficking of MMPs, and represent a highly selective method for visualising specific MMPs. Sbai and co-workers generated plasmid constructs which encoded the MMP-2, MMP-9 and their endogenous inhibitor TIMP-1 genes fused to green fluorescent protein (GFP)\textsuperscript{184}. Similarly, Suzuki and co-workers were able to observe the real-time secretion of MMP-2 from migrating cells through the construction and transfection of a DNA plasmid encoding MMP-2 DNA fused to the Gaussia luciferase fluorescent protein.

Given the success of these approaches for visualising MMP-2 and -9 in vitro, a similar approach was adopted in this study using recombinant DNA technology to generate a stable cell line expressing MMP-2 or MMP-9 fused to a derivative of cyan fluorescent protein (AmCyan) for visualisation using confocal fluorescence microscopy. AmCyan is a fluorescent protein isolated from the \textit{Anemonia majano} sea anemone and belongs to the same family as GFP, which is isolated from the jellyfish \textit{Aequorea victoria}. It shares approximately 29% identity and 40% of its amino acid sequence with GFP, and differs by one amino acid at the chromophore precursor...
site: S-Y-G in GFP and M-Y-G in CFP. Fluorescence activation occurs post-translationally via the O₂-dependent spontaneous internal cyclisation of the precursor site and dehydration of the methionine residue. AmCyan was chosen over the yellow, green and red fluorescent proteins (YFP, GFP, RFP) in order to prevent spectral overlap between the fluorescent protein and the peptide-fluorophore conjugates investigated in Chapter 4. With an excitation maximum at 458 nm and emission maximum at 489, AmCyan can be excited and the emission collected independently to fluorescein isothiocyanate (FITC) and rhodamine B (RhB) which are excited at 488 nm and 559 nm respectively. The simultaneous imaging of MMP-2 distribution and peptide-fluorophore distribution facilitates the understanding of how these potential prodrug models are activated by MMP-2 in cell monolayers and 3D tumour models.

The DNA that encodes for MMP-2 and MMP-9 was reverse transcribed from mRNA, and following purification, ligated with AmCyan to generate a construct that encodes a fluorescently tagged MMP protein. Following this, the construct insertion into a plasmid vector which is suitable for introduction and expression of the DNA construct in mammalian cells was performed.

Figure 3.1 A simplified representation of the salient steps to be undertaken in this study. The MMP-2/9-AmCyan vectors was constructed following standard cloning protocols, and introduced into cells using a mammalian cell cloning vector, where expression of the vector should occur.
In order for this strategy to be successful, the final plasmid vector must contain several important features. Firstly, an appropriate promoter sequence positioned upstream of the gene of interest is required to define where transcription of the gene by RNA polymerase should begin. In the case of this work the full-length human cytomegalovirus (CMV) immediate-early promoter was used, which demonstrates a high level of effectiveness across a wide range of cell lines\textsuperscript{221}. Secondly, and particularly for MMPs and other secreted proteins, an endogenous signal sequence is necessary to ensure that following transcription the protein undergoes secretion out of the cell. For MMP-2, the signal sequence allows for its entry into the endoplasmic reticulum ready for secretion, and the presence and quality of this signal sequence has been shown to be critical for its movement out of the cell\textsuperscript{222}. Finally, the inclusion of an antibiotic resistance gene is required for the establishment of stable cell lines through antibiotic selection. This is based on the fact that cells which have undergone successful transfection and are expressing the recombinant DNA will also exhibit resistance to the particular antibiotic encoded in the plasmid vector. Cells which are not expressing the desired DNA will also not exhibit resistance, and as such passaging the cells into antibiotic-containing selective medium will result in only the survival of the successfully transfected cells.

### 3.2 Materials and methods

#### 3.2.1 General methods

##### 3.2.1.1 Polymerase Chain Reaction (PCR)

DNA amplification by Polymerase chain reaction (PCR) was performed using LongAmp\textsuperscript{®} Taq 2x Master Mix, which was used followed a 1:2 dilution to contain 60 mM Tris-SO\textsubscript{4}, 20 mM (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, 2 mM MgSO\textsubscript{4}, 125 units/mL LongAmp\textsuperscript{®} Taq DNA Polymerase, 0.3 mM dNTPs, 3% glycerol, 0.06% IGEPAL\textsuperscript{®} CA-630, 0.05% Tween-20 at pH 9.0. MMP-2 and -9 specific forward and reverse primers were used.

To the diluted LongAmp\textsuperscript{®} Taq buffer, 1 µg of template DNA was added as well as the forward and reverse primers to a final concentration of 0.4 µM. Nuclease-free water was used to increase the volume of the reaction mixture to 50 µL. Standard thermocycling conditions for the PCR are given in Table 3.1.
Chapter 3: Visualising Matrix Metalloproteinases In Vitro

Table 3.1 PCR thermocycling conditions.

<table>
<thead>
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<th>Stage</th>
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<th>Time</th>
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</thead>
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<td>Initial denaturation</td>
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</tr>
<tr>
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<td>10 minutes</td>
</tr>
<tr>
<td>Hold:</td>
<td>4 °C</td>
<td></td>
</tr>
</tbody>
</table>

3.2.1.2 Restriction enzyme digestion

Approximately 2 µg of plasmid DNA was digested with 20 U of restriction enzyme in the manufacturer’s recommended buffer (50 mM Potassium Acetate, 20 mM Tris-acetate, 10 mM Magnesium Acetate, 100 µg/ml BSA, pH 7.9 @ 25°C). The digestion mixture was incubated for a minimum of 1 hr at 37 °C.

3.2.1.3 Agarose gel electrophoresis

Restriction digested DNA samples were separated on 1% (w/v) agarose, containing 0.5 µg/mL ethidium bromide, in 1x TAE buffer (4.84 g/L Tris-base, 11.42% (v/v) glacial acetic acid, 1 mM EDTA, pH 8). The DNA samples, 100 bp or 1 kb DNA ladders were mixed with 6x Gel Loading Dye (Blue) in the ratio 5:1 respectively before being loaded into wells. Electrophoresis was performed at 7 V cm⁻¹ for 90 min. DNA bands were visualised using the DNR BIS 303 PC Bioimaging system and the gel images exported for analysis. DNA fragments were excised while the gel was illuminated by low-UV light by a UVP transilluminator.

3.2.1.4 DNA ligations

DNA ligation reactions were set up in a 5:1 ratio of insert (ng) : vector (ng), composed of 1x T4 Ligase reaction buffer (50 mM Tris-HCl, pH 7.6, 10 mM MgCl₂, 1mM ATP, 1mM DTT, 5% (w/v) polyethylene glycol-8000), 100 U T4 DNA ligase and made up to 20 µL with autoclaved Milli-Q water. Ligations were incubated at room temperature for a minimum of 1 hr.
3.2.1.5 Transformations

5 µg of plasmid DNA was added to pre-chilled chemically competent One Shot® TOP10 *E. Coli* cells and incubated on ice for 10 minutes. The cells were heat shocked at 42 °C for 30 seconds to facilitate entry of the plasmid into the cells, then returned to ice. The TOP10 cells were then transferred to a transformation vial containing 500 µL of Luria-Bertani (LB) broth at room temperature and incubated for 1 hr at 37 °C with vigorous shaking at 200 rpm.

A 200 µL aliquot of the transformation mixture was then plated onto an LB-carbenicillin (100 µg/mL) agar plate and incubated at 37 °C for 16 hrs.

3.2.1.6 Plasmid DNA extraction

Isolated single bacteria colonies were cultured in 2 mL or 500 mL LB-carbenicillin (100 mg/mL) broth and grown for 16 hrs at 37 °C with vigorous shaking at 200 rpm. Plasmid DNA from the 2 mL cultures was extracted using the QIAprep Spin Miniprep Kit or QIAGEN Plasmid Maxi Kit for 500 mL cultures as per the manufacturer’s instructions. In principle, both kits isolate plasmid DNA by a modified alkaline lysis method. In brief, bacteria were pelleted by centrifugation and the LB broth decanted. The bacteria were then resuspended with buffer and lysed by treatment with NaOH and SDS. Cell debris and genomic DNA were pelleted by centrifugation while plasmid DNA in the supernatant was applied to the QIAGEN anion-exchange resin. Impurities (RNA, proteins, and low molecular weight impurities) were removed by medium-salt wash and plasmid DNA eluted with high-salt buffer or Milli-Q water, pH 7.0. The DNA was then concentrated and desalted by isopropanol precipitation.

3.2.1.7 DNA purity and quantification

The concentrations of DNA samples were measured photometrically by reading the absorbances at 260 nm and 280 nm. The samples were diluted 1:25 in Milli-Q water and Milli-Q water was used as a blank reference. DNA quality was assessed using the \( A_{260}:A_{280} \) ratio, with a value of 1.8 indicative of pure DNA, and the yield calculated assuming 50 µg/mL solution of DNA gives an absorbance of 1.0 at 260 nm.

3.2.1.8 Cell lines

DLD-1 human colon carcinoma cells, A549 human lung carcinoma cells and HT1080 human fibrosarcoma cells were obtained from ATCC and used within 6 months of resuscitation. Cells were maintained as monolayers in complete media: Advanced DMEM (Invitrogen) supplemented with 2% foetal calf serum (FCS), 1% L-glutamine and 1% antibiotic antimycotic

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Cells were incubated under standard culturing conditions (37 °C with 5% (v/v) CO₂ under humidified conditions).

3.2.1.9 Fluorescence spectroscopy

Spectra of media collected after 24 hrs incubation were run at room temperature using a Varian Cary Eclipse fluorescence spectrophotometer, with a 1 cm x 1 cm quartz cuvette (Starna). Scans were run without dilution, at 120 nm/min with excitation and emission slit widths of 5 nm.

3.2.1.10 Confocal fluorescence microscopy of spheroids

Spheroids were formed by coating the wells of a 96-well plate with agarose (sterile, 0.75% w/v in PBF) and plating with 100 µL of cell suspension (1.5 x 10⁵ and 2.5 x 10⁵ cells/mL for medium and large spheroids respectively) in complete media. The cells were left to aggregate for 72 hrs, then transferred into a 35 mm glass-bottom culture dish (MatTek) containing 2 mL of complete medium or PBS.

Images were collected on an Olympus FV1000 inverted microscope using an Olympus UPLAPO 10x/NA:0.40 objective lens in a humidified live-cell chamber maintained at 37 °C (Solent Scientific). A scan rate of 4.0 µs/pixel and sequential collection of the red and green channels were applied for all images. Cyan fluorescence was collected by excitation with a 458 nm argon laser and collected in the 475-525 nm range.

3.2.2 Specific procedures

3.2.2.1 Extraction and purification of total RNA

RNA was extracted from DLD-1 human colorectal adenocarcinoma cells and A549 human alveolar adenocarcinoma cells grown in cell monolayer and DLD-1 three-dimensional tumour model spheroids. Monolayer cells were grown in 70 cm² Corning™ flasks and harvested at 50% confluency (approximately 1 x 10⁷ cells). Spheroids were grown in 96-well plates and all cells per plate were harvested (approximately 2 x 10⁶ cells).

RNA extraction was performed following the Qiagen RNeasy protocol for animal cells. Cells were disrupted in the appropriate buffer and homogenised. Ethanol was then added to the lysate to facilitate selective binding of RNA to the RNeasy membrane. As the total RNA remained membrane-bound contaminants were washed away and the purified RNA eluted in RNase-free H₂O.
3.2.2.2 Isolation and purification of MMP-2 and MMP-9 encoding DNA

Reverse transcription of extracted mRNA to cDNA was performed following the LongRange 2Step RT-PCR Protocol with some modification. In addition to the Oligo-dT primer, gene-specific reverse primers (IDTDNA, USA) for MMP-2 and -9 were employed.

MMP-2 Reverse Primer: 5’-GCT ATT GGT CGA CGC GCA GCC TAG CCA GTC GGA TTT GAT GCT TCC-3’

MMP-9 Reverse Primer: 5’-GGC GAC CGG TAC CGC GTC CTC AGG GCA CTG CAG GAT GTCATA G-3’

In brief, the RNA was thawed on ice, during which a Master Mix was prepared containing a 1:5 dilution of 5x LongRange RT Buffer, dNTP mix, the Oligo-dT and MMP-specific primers to a final concentration of 1 µM, Long Range Reverse Transcriptase enzyme. Following the addition of 1 µg of the template RNA, the volume was made up to 20 µL with RNase-free water and incubated for 90 mins at 42 °C. Heating at 85 °C for 5 mins was then performed to inactivate any enzyme. cDNA mastermixes were stored at -20 °C until required.

Amplification of cDNA to give MMP-2 and MMP-9 genes was achieved by PCR using LongAmp® Taq 2x Master Mix with the following primers (IDTDNA, USA). PCR was performed using standard thermocycling conditions.

MMP-2 Long Forward: 5’-CTA GAA GCT TAT GGA GGC GCT AAT GGC CCG GGG CGC GCT-3’

MMP-2 Long Reverse: 5’-GCT ATT GGT CGA CGC GCA GCC TAG CCA GTC GGA TTT GAT GCT TCC-3’

MMP-9 Long Forward: 5’-GTC GAC AGT CGA CATGAG CCT CTG GCA GCC CCT GGT CCT GGT GCT-3’

MMP-9 Long Reverse: 5’-GGC GAC CGG TAC CGC GTC CTC AGG GCA CTG CAG GAT GTCATA G-3’

Due to low yield, MMP-9 was also isolated by PCR of a premade MMP-9-containing plasmid using the same forward and reverse primers.

Purification of the desired MMP-2 and MMP-9 fragments was performed by gel extraction using QIAquick Gel Extraction Kit following agarose gel electrophoresis of the entire PCR reaction mixture.
3.2.2.3 Formation of constructs

Construction of the MMP-2/9-AmCyan vector was achieved by performing restriction digests of the MMP-2 and MMP-9 genes and pAmCyan vector with the endonucleases shown in Table 3.2, followed by ligation.

Ligation of the sticky ends of MMP-2/9 cleaved fragments and the pAmCyan vector fragment was performed using DNA ligase enzyme in T4 DNA ligase buffer.

<table>
<thead>
<tr>
<th>Table 3.2</th>
<th>Restriction endonucleases used to prepare MMP-2 and -9 fragments for ligation into pAmCyan vector.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP2-AmCyan</td>
<td>MMP9-AmCyan</td>
</tr>
<tr>
<td>MMP-2</td>
<td>Sall-HF</td>
</tr>
<tr>
<td>HindIII-HF</td>
<td></td>
</tr>
<tr>
<td>pAmCyan</td>
<td>Sall-HF</td>
</tr>
<tr>
<td>HindIII-HF</td>
<td></td>
</tr>
</tbody>
</table>

3.2.2.4 Transformation and purification of plasmid constructs

Transformation of the MMP-2/9-AmCyan plasmid vectors was performed according to the protocol outlined in section 3.2.1.5.

4-6 bacterial colonies were selected for lysis and plasmid DNA isolation, which was performed by following the QIAprep Spin Miniprep Protocol. Isolated plasmids were then subjected to restriction digests with the enzymes shown in Table 3.3 to confirm insertion of the respective MMP-2/9 DNA.

<table>
<thead>
<tr>
<th>Table 3.3</th>
<th>Restriction endonuclease used to confirm MMP-2 and -9 fragments ligation into pAmCyan vector.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP2-AmCyan</td>
<td>MMP9-AmCyan</td>
</tr>
<tr>
<td>Sall-HF</td>
<td>Sall-HF</td>
</tr>
<tr>
<td>HindIII-HF</td>
<td>KpnI-HF</td>
</tr>
</tbody>
</table>
3.2.2.5 Isolation of DNA constructs from pAmCyan vector

PCR was performed using LongAmp® Taq 2x Master Mix and the primers (IDTDNA, USA) shown below.

AmCyan Reverse Primer: 3’-GTT GGA ATT CTC GCG TCG C-5’

MMP-2 Forward Primer: 5’-CTA GAA GCT TAT GGA GGC G-3’

MMP-9 Forward Primer: 5’-GTC GAC AGT CGA CAT GAG C-3’

The annealing temperature for the PCR reaction was changed to 53 °C and 57 °C for MMP-2 and MMP-9 constructs respectively, due to the composition of the new primers.

Purification of the desired MMP2-AmCyan and MMP9-AmCyan constructs was performed by gel extraction using QIAquick Gel Extraction Kit following agarose gel electrophoresis of the entire PCR reaction mixture.

3.2.2.6 TOPO® cloning and transformation for expression in mammalian cells

The purified PCR product and TOPO® Vector were combined in the ratio 2:1, to which salt solution and autoclaved Milli-Q water were added as per the manufacturer’s instructions to reach a final volume of 6 µL. The reaction was gently mixed and incubated for 5 mins at room temperature.

Transformation of the TOPO® Cloning reaction was then performed by adding 2 µL of the reaction mixture to pre-chilled chemically competent One Shot® TOP10 E. Coli cells. The transformation was then performed according to the protocol described in section 3.2.1.5. Plasmid DNA extraction was then performed on a large scale, using the QIAGEN Plasmid Maxi Kit. DNA was quantified and tested for purity using the method described in section 3.2.1.7.

3.2.3 Transfection into mammalian cells

3.2.3.1 Transfection using Attractene transfection reagent

Attractene is a nonliposomal lipid formulation for transfection in adherent cells with low cytotoxicity. Transfection was performed as per the manufacturer’s instructions for Fast-Forward Transfection in a single well of a 6-well plate. In brief, 1.2 µg of the DNA plasmid was diluted to 100 µL with medium without serum or antibiotics. Attractene (4.5 µL) was added, mixed by vortexing and then incubated for 15 mins at room temperature to allow formation of the transfection complex. While incubation was occurring, cells were harvested by
trypsinisation and suspended in culture medium containing serum and antibiotics, and adjusted to a cell density of $1 \times 10^5$ cells/mL. 2000 µL of the cell suspension was added to the well of a 6-well plate, followed by the transfection complexes. These were mixed well by pipetting then left to incubate overnight at 37 °C and 5% CO$_2$.

### 3.2.3.2 Transfection using Superfect transfection reagent

Superfect is an activated-dendrimer transfection reagent, with a defined spherical architecture formed by radiating branches which terminate at charged amino groups. This adopted structure packages DNA into compact structures within positively charged complexes, optimising entry into the cell and facilitating transport across the cell membrane by binding to negatively-charged receptors. Transfection was performed as per the manufacturer’s instructions in a single well of a 6-well plate. The day before transfection, $2 \times 10^5$ cells were seeded and left to adhere overnight at 37 °C and 5% CO$_2$. On the day of transfection, 2 µg of the DNA plasmid was diluted to 100 µL with medium without serum or antibiotics. Superfect (10 µL) was added, mixed by vortexing and then incubated for 10 mins at room temperature to allow formation of the transfection complex. While incubating, the growth medium was gently aspirated from the 6-well plate, and cells were washed once with PBS. 600 µL of culture medium containing serum and antibiotics was added to the transfection complex and mixed by pipetting. The entire transformation-complex solution was added to the well of a 6-well plate, then left to incubate for 2-3 hrs at 37 °C and 5% CO$_2$. After this, the medium was removed and cells were washed once with PBS. Fresh growth medium containing serum and antibiotics was then added, and the cells incubated for 24 hrs.
3.3 Discussion of plasmid construction

In order to visualise the secretion of endogenous MMPs in cancer cells, genetic modification of the genes coding for MMP-2 and -9 was performed so that secreted MMPs would fluoresce in the range of AmCyan, a modified cyan fluorescent protein.

Initially, PCR experiments to isolate the encoding DNA of MMP-2 and MMP-9 were performed using RNA extracted from two- and three-dimensional (spheroid) cell cultures of DLD-1 cells. The lack of initial success, confirmed by the absence of DNA bands at approximately 2000 bp after gel electrophoresis, was attributed to one of two reasons: either the template RNA was of poor quality due to degradation, or the DLD-1 cultures were not expressing sufficient levels of MMPs to isolate the encoding DNA regions. The integrity of the RNA was verified by gel electrophoresis, as shown in Figure 3.2. The presence of two sharp bands, the larger of which is approximately twice as intense matched the 2:1 ratio of 28S and 18S RNA bands was indicative of intact RNA. Therefore, as the quality of RNA was not the issue, RNA was instead extracted from A549 cells, which have been shown to contain more MMP activity than DLD-1 cells.

A 2-step RT-PCR protocol, where the conversion of RNA to cDNA is performed in a separate reaction tube prior to PCR, was chosen as PCR reactions to this point had produced only dimerised primers. By performing the reverse transcription stage separately, a different set of primers to those used in the PCR step could be employed. The primers used for the synthesis...
of cDNA were Oligo-dT and a short reverse primer for either MMP-2 or MMP-9. The Oligo-dT primer is a string of deoxythymidylic acid residues that hybridise to the poly(A) tail of all mRNA, and is therefore not specific for any particular gene. This was desirable, as the lack of DNA in all previous PCR attempts was perhaps caused by the high specificity of the long MMP-2/9 forward and reverse primers used in previous 1-step RT-PCR reactions. Moreover, the long primers may have undergone bending into a hairpin shape, thereby losing their ability to recognise and bind to the intended sections of RNA. To stop this from happening, shorter MMP-2/9 specific reverse primers were used. PCR was then performed using the longer MMP-2/-9 primers and LongAmp® Taq, and gel electrophoresis confirmed that PCR had successfully generated DNA fragments of the expected length for MMP-2 and MMP-9, as shown in Figure 3.4a.

The brightness of the DNA band at ~ 2000 bp in lane 2 compared to the similarly sized band in lane 3 is indicative of the quantity of DNA present, and given that MMP-2 is expressed at higher levels in A549 cells than MMP-9, this difference in quantity was expected. These bands were excised and purified in preparation for ligation to the AmCyan fluorescent protein.

AmCyan was obtained as the plasmid vector pAmCyan, with the AmCyan gene flanked by two multiple cloning sites (MCS) at the 5’ and 3’ ends. Two restriction enzyme sites were chosen upstream (5’) of the AmCyan gene to allow insertion of the MMP-2 and MMP-9 DNA into the pAmCyan vector. Figure 3.3 illustrates the steps which were undertaken, and the composition of the resulting plasmids.
Figure 3.3 Schematic of the steps undertaken for the construction of the MMP-2-AmCyan and MMP-9-AmCyan constructs. i) MMP-2 and MMP-9 DNA was cleaved at restriction sites which flanked the gene. The pAmCyan ligand was cleaved at identical restriction sites as the MMP which was to be inserted; ii) MMP-2 and MMP-9 DNA were ligated into their respective cleaved pAmCyan vectors.
In order to confirm the insertion of the MMP-2 and -9 genes into the pAmCyan vector, restriction digests using the enzymes described in Table 3.3 were performed after DNA amplification by transformation. However, transformation by TOP10 cells repeatedly yielded no bacterial colonies for the pMMP-9AmCyan vector, despite generating a moderate number of colonies for pMMP-2AmCyan. As the only observable difference between the MMP-2 and MMP-9 DNA was the quantity, it was decided that sourcing the MMP-9 gene from a commercially purchased plasmid may generate a more lucrative DNA yield. PCR was performed using the MMP-9 primers described in section 3.2.2.4 and showed a significantly higher quantity of MMP-9 DNA. The MMP-9 was again isolated by gel extraction, and cleaved by KpnI and SalI restriction enzymes ready for insertion into the pAmCyan vector. Transformation of the new pMMP-9AmCyan vector resulted in a significant increase in the number of bacterial colonies.

As these enzymes were identical to those used for insertion of the MMP genes, their cleavage sites bordered the MMP DNA and thus it was simple to observe successful ligation based on the presence of a fragment at ~ 2000 bp, corresponding to the MMP genes. The results of the restriction digest, shown in Figure 3.4c-d, showed that three bacterial colonies had amplified the correct MMP-2/9AmCyan plasmid (Figure 3.4c lane 2 and lane 7, Figure 3.4d lane 6), so these were retained and employed in the next step.
In order to obtain the free MMP-2/9-AmCyan construct, PCR of the pMMP-2AmCyan and pMMP-9AmCyan plasmids was performed using the primers described in section 3.2.2.5. The PCR products can be seen in Figure 3.4e, where the faint band at ~ 2700 bp corresponds to the MMP-2/9AmCyan constructs, and the brighter band at ~ 5500 bp corresponds to the original plasmids. This showed that, despite being low-yielding, the PCR reaction was successful.
Therefore, 5 replicates of each PCR were performed, and the desired DNA fragments were isolated, combined and concentrated by gel electrophoresis and extraction, as seen in Figure 3.4f.

**Figure 3.5** Restriction enzyme digests confirming successful insertion of MMP-2-AmCyan and MMP-9-AmCyan construct into pcDNA™3.3-TOPO® vector. i) Expected fragment size of TOPO vector with no construct inserted when cut with EcoRI and HindIII. ii) Expected fragment sizes of TOPO-MMP-9-AmCyan when cut with EcoRI and XbaI. a) Gel of restriction digests of TOPO-MMP-2-AmCyan: lane 1 – 1 kB ladder, lane 2-7 – restriction digests of plasmid DNA extracted from different colonies, lane 9 – undigested TOPO-MMP-2-AmCyan, lane 10 – 1 kB ladder. b) Gel of restriction digests of TOPO-MMP-9-AmCyan: lane 1 – 1 kB ladder, lane 2-9 – restriction digests of plasmid DNA extracted from different colonies, lane 10 – 1 kB ladder.

The final step before the construct could be expressed in human cancer cell lines was insertion into a mammalian expression vector. The pcDNA™3.3-TOPO® vector contains the human cytomegalovirus (CMV) immediate-early enhancer/promoter gene, which allows for high-level gene expression, as well as a neomycin resistance gene, which is required for the Geneticin®-selective establishment of stable cell lines. Supplied in its linearised form, the TOPO® vector has single overhanging 3’ deoxythymidine (dT) residues, making it suited for ligation with the MMP-2/-9AmCyan PCR products that were generated using the LongAmp Taq system which have overhanging deoxyadenine (dA) 3’ ends. To confirm the insertion of the vectors into the
plasmid, restriction digests were performed on the DNA extracted from the transformation bacterial colonies, and the results are shown in Figure 3.5a-b.

For comparison purposes, Figure 3.5i-iv shows the expected restriction digest fragments without (i-ii) and with (iii-iv) the correct vector inserted. Moreover, the pcDNA™3.3-TOPO® is designed, by way of the d(T) residue overhangs, not to self-close without the insertion of any vector. As can be seen in Figure 3.5a-b, the fragment sizes observed following gel electrophoresis correspond to the expected fragment sizes, 2731 and 5421 bp for TOPO-MMP-2AmCyan and 2869 and 5399 bp for TOPO-MMP-9AmCyan.

The results indicated that the MMP-2/-9AmCyan constructs had been successfully inserted into the mammalian expression vector, and transfection into the appropriate cell lines was now possible.
3.4 Discussion of cell transfection

3.4.1 In cell monolayers

Transfection of the MMP2-AmCyan and MMP9-AmCyan plasmids was first attempted in DLD-1 cells, as they are readily grown into spheroids which would allow for the visualisation of MMP-2 and MMP-9 secretion in 3D tumour models as well as cell monolayers. Transfection was first performed in accordance with the Attractene transfection reagent Fast-Forward Transfection protocol. After 24 hrs, the cells were washed with fresh medium and then transfection medium was replaced with fresh complete medium and following another 24 hrs incubation, the success of the transfection was assessed using fluorescence microscopy without replacing the media.

No intra- or extracellular fluorescence was observed in the cyan channel which was attributed to the low expression of MMP-2 and 9 in DLD-1 monolayer cell cultures. For this reason, transfection was then attempted in A549 and HT1080 cell lines. While A549 cells exhibit moderate expression of MMP-2, the HT1080 human fibrosarcoma cell line secretes high levels of MMP-2 and moderate levels of MMP-9. It was believed that by transfecting cell lines which expressed higher levels of MMP-2 and -9, a corresponding increase in the expression of the MMP-2/9-AmCyan constructs would be observed.

After following the same transfection procedure, again no cell-localised fluorescence was immediately visible. Being secreted proteins, MMPs generally undergo vesicular release into the extracellular surrounds, which in the case of cultured cell monolayers is the surrounding media. One possibility was that the secreted MMP-2/9-AmCyan was too dilute in the surrounding media to generate a fluorescent signal which could be visualised. For this reason, the cell media was collected and analysed by fluorescence spectrophotometry, with the resulting emission spectra shown in Figure 3.6.
For both cell lines, there was a decrease in fluorescence observed in the transfected cell media compared to that of the control cells. This was unexpected, but indicated that the inherent fluorescence characteristics of the media had been in some way modified by an additional substance in the media, potentially the AmCyan-modified MMPs. The relative degree of fluorescence quenching for both cell lines was similar, with MMP-2-AmCyan transfected cells showing a marginally higher amount of quenching than MMP-9-AmCyan transfected cells. This correlated directly with the levels of MMP-2 and -9 expression expected for both cells lines, supporting the hypothesis that the cells are undergoing some expression of the plasmid DNA, but visualisation of AmCyan is being prevented.
The inherent fluorescence in cell culture medium is due to the presence of phenol red indicator (excitation 488 nm; emission >500 nm (wide)) and riboflavin (excitation 450-490 nm; emission 500-560 nm), both of which are known quenching agents for fluorescent proteins. Bogdanov and co-workers have investigated the effect of cell culture medium on the fluorescent photostability of green and cyan fluorescent proteins, and they found that the presence of cell-permeant electron acceptors such as vitamins (including riboflavin), FAD and NAD$^+$ facilitated the oxidative reddening of members of the GFP family, causing photobleaching. Given the decrease in fluorescence observed between 500-550 nm in Figure 3.6, the fluorescence behaviour of riboflavin was considered further. Riboflavin, which belongs to the flavin family, and other flavin compounds can exist in three redox states which are the flavoquinones, Fl$^{ox}$H, the flavosemiquinones, Fl.H$_2$, and the flavohydroquinones, Fl$^{red}$H$_3$. The structures of anionic, neutral and cationic riboflavin species in the oxidised and reduced states are shown in Figure 3.7.

Figure 3.7 The structures of cationic, neutral, and anionic riboflavin species in their oxidised and reduced forms (taken from [227]). $R = -\text{CH}_2(\text{CHOH})_2\text{CH}_2\text{OH}$.

While in its oxidised form (Fl$^{ox}$_H), riboflavin is weakly fluorescent, upon reduction to Fl$^{red}$H$_3$ its fluorescence is quenched entirely. If oxidative reddening of the AmCyan fluorescent protein is
occurring in the transfected cell media, the concomitant reduction of riboflavin would explain the decrease of fluorescence observed in Figure 3.6.

### 3.4.2 In 3D tumour models

Following the lack of fluorescence in the cyan channel for DLD-1 monolayers transfected with the MMP-AmCyan plasmids, it was decided to grow the transfected cells into spheroids, as previous work has shown that the level of active MMPs in DLD-1 cells is significantly higher in 3D models\(^\text{128}\). The spheroids were then imaged by fluorescence confocal microscopy and the results are shown in Figure 3.8.

![Confocal microscopy images of DLD-1 medium spheroids made from DLD-1 cells transfected with A: TOPO-MMP-2-AmCyan; B: TOPO-MMP-9-AmCyan and C: control cells. Images were obtained in the cyan channel. Scale bar represents 100 µm.](image)

**Figure 3.8** Confocal microscopy images of DLD-1 medium spheroids made from DLD-1 cells transfected with A: TOPO-MMP-2-AmCyan; B: TOPO-MMP-9-AmCyan and C: control cells. Images were obtained in the cyan channel. Scale bar represents 100 µm.
A similar trend as was observed for the fluorescence emission spectra of collected media (see Figure 3.6) was seen in the spheroids, with the control spheroids exhibiting the greatest fluorescence emission followed by those transfected with MMP-9-AmCyan then MMP-2-AmCyan. Again, this difference in fluorescence suggests that the transfection has been successful, but the oxidative reddening of AmCyan is causing the expected cyan fluorescence to be significantly red-shifted, so that emission observed in the 500 – 550 nm range is due to the concomitant quenching of riboflavin fluorescence upon reduction.

Given the time frame of this project, an alternative technique was investigated for visualising MMPs in vitro. Tumour spheroids were treated with the commercially available MMP substrate MMPSense 680 (PerkinElmer™), which also has a discrete excitation and emission profile from the fluorophores FITC and RhB used in Chapter 4. The advantage of MMPSense 680 is that it allows for the visualisation of MMP activity, as opposed secretion of both active and inactive MMP-2 and -9 as afforded by the AmCyan plasmid constructs.
3.5 Summary

The results presented in this Chapter show that recombinant DNA techniques can be successfully used to generate DNA constructs that can modify MMPs that are expressed in vitro. It was found that in order to generate DNA that encodes for MMP-2 and MMP-9 by reverse transcription, the precursor RNA must be isolated from a cell system which expresses significant levels of these enzymes. In this case, A549 cell monolayers yielded high amounts of MMP-2, but low expression of MMP-9 resulted in a low DNA yield which proved problematic in further experiments and was rectified by substituting the MMP-9 DNA obtained by reverse transcription with DNA purchased commercially.

Moreover, this study achieved the fusion of MMP-2 and MMP-9 DNA to the AmCyan fluorescent protein gene to create a DNA construct which can be inserted into a mammalian transfection vector. Transfection into three cell lines that exhibit different levels of MMP-2 and MMP-9 expression was evaluated by measuring the fluorescence emission in the 480 – 550 nm range.

Transfection into cell monolayers was assessed by collecting the media and measuring its fluorescence. The media collected from A549 cells showed a decrease in fluorescence for cells transfected with the MMP-2/9-AmCyan vectors, and an even greater decrease of fluorescence was observed for HT1080 cells which are known to express higher levels of MMPs than A549 cells. This phenomenon was attributed to the unique interaction between green and cyan fluorescent proteins and riboflavin, an endogenous agent in cell culture media. This interaction is catalysed by the photooxidative reddening of the AmCyan protein which in turn causes reduction of the riboflavin compound. Simultaneously, this causes a significant red-shift of the fluorescence emission of AmCyan and quenches the fluorescence for riboflavin in the 480 – 550 nm range. A similar effect was observed in DLD-1 spheroids, which were grown from transfected cells as whereas MMP expression in DLD-1 cell monolayers is low, it increases in 3D cell culture.

Overall, these results demonstrate the potential for monitoring MMP expression and secretion in monolayer and 3D cell culture by transfection with MMP-fluorescent protein plasmid vectors. However, employing AmCyan as the fluorescent protein has resulted in an unexpected interaction with the surrounding media which should be investigated further. The use of other fluorescent proteins should also be pursued, as well as the use of cell-culture media which is riboflavin-free.
Chapter 4

**Fluorophore-Peptide Substrates for Investigating MMP-2 Activity**
4.1 Background and strategy

Matrix metalloproteinases (MMPs) are a family of zinc-containing endopeptidases which are minimally expressed in healthy physiological conditions. However due to their role in tissue growth and remodelling, have increased levels of expression and activation in almost all human cancers. The involvement of MMPs in cancer-cell growth, migration, invasion, metastasis and angiogenesis is predominantly through their cleavage of the structural components of the extracellular matrix, as well as growth factor-binding proteins, growth-factor precursors, receptor tyrosine kinases, cell-adhesion molecules and other proteinases\textsuperscript{129}. There is a high degree of correlation between MMP levels and cancer invasion, cancer cell migration and cancer cell-mediated tissue remodelling\textsuperscript{130}, thus MMP levels are considered an important prognostic factor in determining cancer progression and patient survival.

MMPs present a unique means of targeting drug molecules to solid tumours by exploiting the cleavage of an MMP-specific peptide substrate for selective drug activation. In particular, MMP-2 is an attractive enzymatic target as it is overexpressed in a range of tumour-derived human cell lines including mammary adenocarcinoma, prostate carcinoma, osteosarcoma, fibrosarcoma, brain carcinomas and melanoma\textsuperscript{223}. The cleavage of peptide substrates by MMPs to deliver drug molecules to tumour cells has been reported previously\textsuperscript{175-177,180}. By exploiting the properties of the drug molecule to render it either inactive or indisposed to cellular uptake prior to MMP cleavage, the site-specific overexpression of MMPs within tumours can result in localised drug activation.

In this work, the target compound has been designed to incorporate an uptake-blocking moiety on the unprimed end of the peptide substrate, and two fluorophores attached to amino acids on opposite sides of the MMP cleavage site, as shown in Figure 4.1. Prior to enzymatic cleavage, the uptake-blocking group should prevent influx of the prodrug into cells and promote paracellular transport which may enhance tumour penetration. Following cleavage, the unprimed side, tagged by the fluorophore will, providing that it is intrinsically cell-penetrable, be able to enter cells while the primed side remains outside of the cell.
Selectivity for MMP-2 can be achieved through the use of a peptide substrate which is preferentially cleaved by MMP-2 compared to other members of the MMP family. An oriented peptide library approach has been used by Turk and co-workers to determine the cleavage motif for MMP-2 to be \( \text{NH}_2\text{-Asp-Ile-Pro-Val-Ser-Leu-Arg-Ser-COOH} \). This motif is incorporated into an extended peptide, which also contains a polyanionic tetraaspartate domain on the N-terminus for uptake inhibition as well as strategically positioned lysine residues for the conjugation of fluorophores. The uptake-limiting properties of polyanionic groups have been demonstrated by Aquilera et al\(^\text{229}\) who successfully employed a polyglutamate group to moderate the uptake of a polyarginine cell-penetrating peptide (CPP) prior to its detachment following cleavage by MMP-2.

The orthogonally conjugated fluorophores are important in order to track the distribution of the intact peptide and its post-cleavage fragments in 2D and 3D cellular studies. The positioning of two discrete fluorophores on alternate sides of the cleavage site will allow for the simultaneous monitoring of localisation and cleavage of the compound. When imaged in combination with the techniques used to observe MMP activity discussed in Chapter 3, important insights into the behaviour of these compounds was obtained.

The primary aim of this part of the project was to synthesise a library of fluorophore-tagged peptides in order to investigate the effectiveness of the peptide at improving selectivity for MMP-2.

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**Figure 4.1** Diagram showing enzymatic cleavage of potential prodrug containing an MMP-2 specific peptide cleavage motif. Top: the intact peptide contains two fluorophores on either side of the cleavage site, plus an uptake blocking group to prevent influx into cells. Bottom: after cleavage by MMP-2, the primed side (RHS) is free to enter cells, while the unprimed side retains the uptake blocking group, preventing cell penetration.
solid tumours. The effect of the polyanionic domain in blocking cellular uptake was also studied. The behaviour of these compounds in biological systems was also studied, including their selectivity for MMP-2, enzymatic cleavage profiles, and distribution of both the intact peptide and the peptide fragments.

4.2 Methods

4.2.1 LC-MS

Liquid chromatography-mass spectrometry (LC-MS) was performed on a Shimadzu LC-MS 2020 system using a LC-20AD Pump and a SPD-20A detector. Separation was achieved using a Waters SunFire™ series C18 column (150 x 2.1 mm, 5 µm particle size), at a flow rate of 0.2 mL/min over a linear gradient from 0% to 100% solvent B over 30 minutes (solvent A: 100:0.1 v/v Milli-Q water/formic acid, solvent B: 100:0.1 v/v acetonitrile/formic acid).

4.2.2 Preparative HPLC

Preparative HPLC was performed on a Waters 600 HPLC system fitted with a Waters 2998 Photodiode Array Detector. Separation was achieved using a Waters Sunfire™ C18 (5 µm, 19 x 150 mm) preparative column at a flow rate of 7 mL/min. A linear gradient of 5 to 60% solvent B minutes (solvent A: 100:0.1 v/v Milli-Q water/TFA, solvent B: 100:0.1 v/v acetonitrile/TFA) over 70 mins was used. Signals were detected at 254 and 400 nm depending on the presence of the rhodamine B fluorophore. Relevant fractions were collected manually.

4.2.3 Fluorescence Spectra

Spectra of model peptides and blocking peptides were collected at room temperature using a Varian Cary Eclipse fluorescence spectrophotometer, with a 1 cm x 1 cm quartz cuvette (Starna). Scans were run as 70 µM solutions in H_2O adjusted to pH 7.4, at 120 nm/min with excitation and emission slit widths of 5 nm. All compounds were prepared as 1 mM stock solutions and stored at -20 °C, and diluted immediately prior to analysis.

4.2.4 Cleavage assays

4.2.4.1 Fluorescence

Preliminary measurements were carried out on a Shimadzu RF-5301PC fluorimeter. Measurements were taken in a 2 mm x 2 mm quartz cuvette held in an FCA2 adaptor (Starna).
Solutions were prepared in TCN buffer (50 mM Tris, 5 mM CaCl2, 0.3 M NaCl, 0.05% Brij-35, pH adjusted to 7.4) and Dulbecco’s Modified Eagle’s Medium (without phenol red) immediately prior to taking measurements. Scans were run at 120 nm/min with excitation and emission slit widths of 1.5 nm unless specified otherwise. Excitation was performed at 488 or 559 nm depending on the experiment and emission data was collected in the range of 500-650 nm.

4.2.4.2 LC-MS

100 units of MMP-2 (AnaSpec) were activated by incubation with 1 mM 4-aminophenylmercuric acetate (APMA) in TCN buffer at 37 °C for 1 hr. Peptides were added (from 1 mM stock solution) to give a final concentration of 2 x 10^{-5} M and incubated at 37 °C for the appropriate length of time (4 or 24 hrs). The reaction was stopped by denaturation of the enzyme with 4 volumes of absolute EtOH and incubation on ice for 20 mins. The sample was then centrifuged at 14 000g for 20 mins, the supernatant removed and dried under N₂. Before analysis by LC-MS, the sample was redissolved in 50 µL of EtOH.

Liquid chromatography-mass spectrometry (LC-MS) was performed on a Shimadzu LC-MS 2020 system using a LC-20AD Pump and a SPD-20A detector. Separation was achieved using a Waters SunFire™ series C18 column (150 x 2.1 mm, 5 µm particle size), at a flow rate of 0.2 mL/min over a linear gradient from 0% to 100% solvent B over 30 minutes (solvent A: 100:0.1 v/v Milli-Q water/formic acid, solvent B: 100:0.1 v/v acetonitrile/formic acid).

4.2.5 Cell lines

DLD-1 human colon carcinoma cells and A549 human lung carcinoma cells were obtained from ATCC and used within 6 months of resuscitation. Cells were maintained as monolayers in complete media: Advanced DMEM (Invitrogen) supplemented with 2% foetal calf serum (FCS), 1% L-glutamine and 1% antibiotic antimycotic (AA). Cells were incubated under standard culturing conditions (37 °C with 5% (v/v) CO₂ under humidified conditions).

4.2.6 Confocal microscopy

4.2.6.1 Monolayer cell culture

Approximately 2.5 x 10⁵ cells were plated onto 35 mm glass-bottom culture dishes (MatTek) and allowed to adhere overnight. Depending on the experiment, cells were either plated in complete media, or serum-free medium. Cells were treated with the compounds for 4 or 24
hrs under standard incubation conditions. The cells were subsequently imaged with either the compound still present in the media, or with the medium replaced with fresh medium of an equal volume.

Images were collected on an Olympus FV1000 inverted microscope using an Olympus UPLSAPO 60x/NA:1.2 water objective lens in a humidified live-cell chamber maintained at 37 °C (Solent Scientific). A scan rate of 4.0 µs/pixel and sequential collection of the red and green channels were applied for all images. Green fluorescence was measured by excitation with a 488 nm multi-argon laser and collected in the 500-540 nm emission range. Red fluorescence was measured by excitation with a 559 nm multi-argon laser and collected in the 580-660 nm range. Near Infra-red fluorescence was measured by excitation with a 633 nm argon laser and collected in the 680-700 nm range.

4.2.6.2 Spheroids

Spheroids were formed by coating the wells of a 96-well plate with agarose (sterile, 0.75% w/v in PBF) and plating with 100 µL of cell suspension (1.5 x 10^5 and 2.5 x 10^5 cells/mL for medium and large spheroids respectively) in complete media. The cells were left to aggregate for 72 hrs without motion, after which a single spheroid had formed per well. 12 hrs prior to treating with the compound, the media was replaced with serum-free media. The spheroids were treated with 20 uM of compound for 4 or 24 hrs, then transferred into a 35 mm glass-bottom culture dish (MatTek) containing 2 mL of serum-free medium.

Images were collected using the same instrumental parameters as in section 4.2.6.1, but using an Olympus UPLAPO 10x/NA:0.40 objective lens.

4.2.6.3 Live microscopy of spheroids

Spheroids were grown in the manner described in section 4.2.6.2. The spheroid was transferred on to a black, µ-clear 96-well imaging plate (Greiner Bio-One) which had been pre-coated with agarose to prevent spheroid outgrowth. The spheroid was treated with the compounds immediately prior to image collection, which was performed using the same parameters described in section 4.2.6.2. Images were collected every 15 minutes over the course of 12 hrs.
4.3 Synthesis

4.3.1 General method for peptide synthesis

All peptides described in this work were synthesised using standard solid phase peptide synthesis (SPPS) techniques following the conventional Fmoc strategy. Wang resins which were preloaded with the first amino acid of the sequence: fmoc-Pra-Wang resin (0.78 mmol/gram), fmoc-Lys(mtt)-Wang resin (0.66 mmol/gram), fmoc-Asp(OtBu)-Wang resin (0.34 mmol/gram), fmoc-Ser(tBu)-Wang resin (0.80 mmol/gram) were used. The resin was swollen in DMF for 1 hr in a fritted syringe (Brand). Fmoc protecting groups were removed by shaking in 20% piperidine in DMF for 20 minutes, followed by rinsing with 5 x 2-5 mL of DMF (depending on reaction scale). The coupling reactions were performed using 4 equivalents of the respective amino acids, which were added to HTBU (4 equivalents) and HOBt (4 equivalents) and dissolved in 2-5 mL of DMF. To activate the carboxylic acid towards forming the peptide bond, DIPEA was added (12 equivalents) and allowed to activate for 1 minute before addition to the syringe. Coupling reactions for most amino acids were allowed to continue for 40 minutes, the exceptions being fmoc-Arg(Pbf)-OH and fmoc-Lys(mtt)-OH which were coupled for 1 hr due to their bulky protecting groups. To monitor coupling reactions, the Kaiser test was used to test for the presence of free 1° amine groups (see section 4.2.2.1). The coupling process was repeated until the Kaiser test indicated that no free amines were present. Once coupling was complete, this deprotection-coupling procedure was repeated for subsequent amino acids.

The coupling of fluorophores to the peptides was achieved by the orthogonal removal of the mtt (methyltrityl) and mmt (monomethoxytrityl) protecting groups on lysine residues. Prior to deprotection, the resin was washed with 5 x 2-5 mL DCM followed by 5 x 2-5 mL of MeOH to shrink the resin. Mtt was removed by shaking in 2-5 mL aliquots of a 1.5% TFA, 5% TIS and 93.5% DCM solution for 2 mins, until the yellow solution turned colourless and the Kaiser test indicated the presence of free amines. Mmt was deprotected by shaking in 2-5 mL aliquots of a solution of acetic acid : trifluoroethanol : DCM (1 : 2 : 7) for 20 mins until the red/orange solution turned colourless. After removal of mtt or mmt, the resin was washed with DCM, and swollen in DMF for 30 mins. For coupling with rhodamine B, the resin was shaken with a solution of rhodamine B, HBTU and HOBT (all 4 equivalents) dissolved in DMF and DIPEA (12 equivalents) and shaken for 6 hrs. For coupling with FITC, the resin was shaken with FITC (4 equivalents) dissolved in DMF and DIPEA (4 equivalents) overnight. For the peptides which contain both fluorophores, mmt deprotection of the N-terminal lysine and subsequent
coupling of the first fluorophore was performed before deprotection of the Lys(mtt) residue at the C-terminus, then coupling the second fluorophore.

At the end of the synthesis, the terminal fmoc group was deprotected with 20% piperidine in DMF, and the resin washed as described above with DMF, DCM and finally shrunk with MeOH. The peptide was cleaved from the resin with a solution of 95% TFA, 2.5% TIS and 2.5% H₂O for 2 hrs. The solution containing the cleaved peptides was expelled through the fritted syringe and precipitated by the addition of ice-cold diethyl ether. The precipitate was collected by centrifugation, drying and lyophilisation. Purification was achieved by preparative HPLC (Linear gradient: 0 to 100% MeCN in 50 mins, eluents: H₂O and MeCN, both containing 0.1% TFA).

4.3.1.1 The Kaiser test

The following three solutions were prepared:

1. 5% ninhydrin in EtOH: 0.5 g ninhydrin in 12 mL EtOH
2. 80% phenol, 20% ethanol: 3.1 g phenol in 750 µL EtOH
3. 0.001 M KCN in pyridine: 1.3 mg KCN in 0.4 mL H₂O in 20 mL pyridine.

After fmoc deprotection and coupling reactions, the resin was washed thoroughly with DMF. A few beads of the resin were placed into a 1 mL centrifuge tube and 1-2 drops of each of the Kaiser test solutions (1-3) were added. The mixture was heated to 100° C for 2 mins. A blue colouration of the beads indicated the presence of free amines. The Kaiser test could not be used to determine the fmoc deprotection of secondary amines such as proline, or the success of coupling amino acids to proline. In these instances, double coupling was performed to ensure no free amines were present.
4.3.2 Conditions and characterisation of peptides

All peptides were synthesised following a similar protocol, purified by preparative HPLC and their purity analysed by LC-MS. Retention times using LC-MS (Liner gradient: 0% to 100% solvent B over 30 minutes (solvent A: 100:0.1 v/v Milli-Q water/formic acid, solvent B: 100:0.1 v/v acetonitrile/formic acid).) All peptide-fluorophore conjugates are > 95 % purity. All LC-MS traces can be found in Appendix A3.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Resin</th>
<th>Amino acids (C -&gt; N)</th>
<th>MS</th>
<th>LC-MS t, (min)</th>
<th>Yield</th>
<th>Molecular mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>LRSK(RhB) (4.7)</td>
<td>fmoc-Lys(mtt)-Wang resin</td>
<td>fmoc-Ser(tBu)-OH/fmoc-Arg(pbf)-OH/fmoc-Leu-OH/rhodamine B</td>
<td>927.6 ([M]⁺), 464.5 ([M]²⁺)</td>
<td>15.79</td>
<td>73 mg (31%)</td>
<td>928.15</td>
</tr>
<tr>
<td>K(FITC)DIPVS (4.6)</td>
<td>fmoc-Ser(tBu)-Wang resin</td>
<td>fmoc-Val-OH/fmoc-Pro-OH/fmoc-Ile-OH/fmoc-Asp(OtBu)-OH/fmoc-Lys(mtt)-OH/FITC</td>
<td>1047.5 ([M]⁺), 524.5 ([M]²⁺)</td>
<td>14.17</td>
<td>20 mg (19%)</td>
<td>1047.14</td>
</tr>
<tr>
<td>K(FITC)DIPVSLRSK(RhB) (4.5)</td>
<td>fmoc-Lys(mtt)-Wang resin</td>
<td>fmoc-Ser(tBu)-OH/fmoc-Arg(pbf)-OH/fmoc-Leu-OH/fmoc-Ser(tBu)-OH/fmoc-Val-OH/fmoc-Pro-OH/fmoc-Ile-OH/fmoc-Asp(OtBu)-OH/fmoc-Lys(mmt)-OH/FITC/rhodamine B</td>
<td>978.60 ([M]²⁺)</td>
<td>17.98</td>
<td>43 mg (22%)</td>
<td>1957.27</td>
</tr>
</tbody>
</table>
### Chapter 4: Fluorophore-Peptide Substrates for Investigating MMP-2 Activity

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Peptide Sequence</th>
<th>Mascotte Masses</th>
<th>Purity</th>
<th>Yield</th>
<th>MW</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDDDK(FITC)DIPVS (4.9)</td>
<td>fmoc-Ser(tBu)-Wang resin</td>
<td>fmoc-Val-OH, fmoc-Pro-OH, fmoc-ile-OH, fmoc-Asp(OtBu)-OH, fmoc-Lys(mtt)-OH, fmoc-Asp(OtBu)-OH, fmoc-Asp(OtBu)-OH, fmoc-Asp(OtBu)-OH, fmoc-Asp(OtBu)-OH, FITC</td>
<td>1508.6 ([M]+), 754.7 ([M]+²)</td>
<td>14.57</td>
<td>61 mg (16%)</td>
</tr>
<tr>
<td>DDDDK(FITC)DIPVSLRSK(RhB) (4.8)</td>
<td>fmoc-Lys(mtt)-Wang resin</td>
<td>fmoc-Ser(tBu)-OH, fmoc-Arg(pbf)-OH, fmoc-Leu-OH, fmoc-Ser(tBu)-OH, fmoc-Val-OH, fmoc-Pro-OH, fmoc-ile-OH, fmoc-Asp(OtBu)-OH, fmoc-Lys(mmt)-OH, fmoc-Asp(OtBu)-OH, fmoc-Asp(OtBu)-OH, fmoc-Asp(OtBu)-OH, fmoc-Asp(OtBu)-OH, FITC, rhodamine B</td>
<td>1209.2 ([M]+²), 806.5 ([M]+³), 605.2 ([M]+⁴)</td>
<td>18.02</td>
<td>24 mg (20%)</td>
</tr>
<tr>
<td>DDDDK(RhB)D (4.4)</td>
<td>fmoc-Asp(OtBu)-Wang resin</td>
<td>fmoc-Lys(mtt)-OH, fmoc-Asp(OtBu)-OH, fmoc-Asp(OtBu)-OH, fmoc-Asp(OtBu)-OH, rhodamine B</td>
<td>574.1 ([M]+²)</td>
<td>16.29</td>
<td>30 mg (11%)</td>
</tr>
<tr>
<td>Fluorophore-Peptide Substrates</td>
<td>Reaction Conditions</td>
<td>Mass Spectrum</td>
<td>Yield</td>
<td>Mass (mg) (%)</td>
<td>Molecular Mass</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>--------------------</td>
<td>---------------</td>
<td>-------</td>
<td>---------------</td>
<td>----------------</td>
</tr>
<tr>
<td>K(RhB)D (4.2)</td>
<td>fmoc-Asp(OtBu)-Wang resin</td>
<td>fmoc-Lys(mtt)-OH rhodamine B</td>
<td>686.5([M]^+) , 343.9 ([M]^2+)</td>
<td>16.82</td>
<td>14 mg (8%)</td>
</tr>
<tr>
<td>DDDDK(FITC)D (4.3)</td>
<td>fmoc-Asp(OtBu)-Wang resin</td>
<td>fmoc-Lys(mtt)-OH fmoc-Asp(OtBu)-OH fmoc-Asp(OtBu)-OH fmoc-Asp(OtBu)-OH fmoc-Asp(OtBu)-OH FITC</td>
<td>556.0 ([M]^+)</td>
<td>14.53</td>
<td>27 mg (10%)</td>
</tr>
<tr>
<td>K(FITC)D (4.1)</td>
<td>fmoc-Asp(OtBu)-Wang resin</td>
<td>fmoc-Lys(mtt)-OH FITC</td>
<td>651.3([M]^+) , 326.3 ([M]^2+)</td>
<td>13.03</td>
<td>15 mg (9%)</td>
</tr>
</tbody>
</table>
4.4 Discussion of synthesis

All peptides described in this chapter were synthesised by solid phase peptide synthesis (SPPS) protocols, which involve the sequential addition of amino acids to a solid support while allowing for the orthogonal conjugation of non-amino acid molecules with ease. SPPS was originally described by Merrifield as a means of overcoming the difficulties in solubility and purification typically associated with the assembly of longer, complicated peptide chains by solution-phase chemistry. The N-terminal carboxybenzoxyl (cbz) protecting group employed by Merrifield was removed by 10% hydrogen bromide in glacial acetic acid; treatment which also led to hydrolysis of the ester bond tethering the peptide to the polystyrene resin. This resulted in a decrease in peptide yield, and was superseded by the tert-Butyloxycarbonyl (BOC) group, which is also acid labile and requires neutralisation of the N-α-amino group before the next peptide coupling step can occur. Still, the presence of acid-sensitive peptide bonds as well as the potential for acid-catalysed side reactions has led to the development of the base-labile N-α-amino protecting group, 9-fluorenylmethoxycarbonyl (fmoc), which is employed in this work.

The use of the base-labile fmoc strategy required the choice of a solid resin that was base-stable and, due to the necessary fluorophore conjugation, resistant to the orthogonal side-chain deprotection methods which would be required. For this reason, the widely employed C-terminal Wang resin was chosen. Cleavage of intact peptides from Wang resins was achieved with a mixture of TFA:TIS:H₂O (95:2.5:2.5). Therefore it was also necessary to ensure that all amino acids which contained reactive side chains (Asp, Ser, Arg), with the exception of those intended for fluorophore conjugation (Lys), incorporated protecting groups which were only labile under strongly acidic conditions. This allowed for the complete deprotection of the peptide in unison with cleavage from the resin.

The synthetic pathway for building K(FITC)DIPVSLRSK(RhB) (4.5) is shown in Scheme 4.1, and the general method applies to all peptides described in this chapter. Depending on the C-terminal amino acid of the peptide being synthesised, the appropriate amino acid-loaded Wang resin was employed, and peptides were synthesised on 0.05, 0.1, 0.2 or 0.25 mmol scales depending on the availability of reactants and the purpose of the peptide.
Scheme 4.1 Synthetic pathway for K(FITC)DIPVSLRSK(RhB) \((4.5)\). The structures of the protecting groups have been shown once, then represented by their respective abbreviations. (i) 20% piperidine in DMF (ii) 4 eq. fmoc-Ser(tBu)-OH, 4 eq. HBTU, 4 eq. HOBT, 12 eq. DIPEA, in DMF (iii) repeat (i) and (ii) with sequential fmoc-protected amino acids (iv) acetic acid : trifluoroethanol : DCM \((1 : 2 : 7)\); 4 eq. FITC, 4 eq. DIPEA in DMF (v) TFA : TIS : DCM \((1.5 : 5 : 93.5)\); 4 eq. rhodamine B, 4 eq. HBTU, 4 eq. HOBT, 12 eq. DIPEA, in DMF (vi) 20% piperidine in DMF (vii) TFA : TIS : H\(_2\)O \((95 : 2.5 : 2.5)\).
The Kaiser test was used to test for any cases of incomplete deprotection of fmoc protecting groups or partial coupling of amino acids, both of which result in the formation of incomplete peptide chains, reducing the purity and yield of the final peptide. As the Kaiser test is only able to test for the presence of free primary amines, the proline residue could not be monitored using this method. For this reason, fmoc deprotection was repeated and the isoleucine residue was double coupled to proline, to ensure complete attachment. The addition of fmoc-Arg(pbf)-OH was also repeated on most occasions, with the inefficient coupling attributable to the bulky pbf protecting group.

Also, in the case of peptides built on the fmoc-Lys(mtt)-Wang resin, heating to the conventional 110 °C yielded a positive (blue) Kaiser test result even when no fmoc deprotection had been performed due to the susceptibility of the mtt group to high temperatures. For this reason, the temperature was reduced to 100 °C as it was observed that this did not give the same immediate positive result seen at 110 °C.

4.4.1 Orthogonal coupling of fluorophores

Preliminary attempts to couple the FITC and rhodamine B fluorophores followed the procedure described by Yamamoto et al\textsuperscript{232}, where the first fluorophore was attached to the C-terminal lysine prior to the addition of the N-terminal lysine and attachment of the second fluorophore. It was found that this method resulted in some degradation of the rhodamine B fluorophore during fmoc deprotection and coupling of the second lysine residue, reducing purity and yield. An improved method has been described in section 4.3.2, where the commercially available fmoc-Lys(mmt)-OH amino acid was incorporated into the sequence in place of a second Lys(mtt) residue. This allowed for the entire peptide sequence, including the tetra-aspartate blocking group as in DDDDK(FITC)DIPVSLRSK(RhB) (4.8), to be built prior to any fluorophore conjugation. The mmt group could then be selectively removed leaving the mtt group in place and the first fluorophore, FITC, could be attached prior to deprotection of the mtt protecting group and peptide coupling of rhodamine B. This meant that the only synthetic step required in the presence of both fluorophores was deprotection of the N-terminal fmoc group. The purity of the crude peptide was significantly improved by this procedure, and the yield increased to > 20%, from 4.2% for the equivalent peptide in the literature\textsuperscript{232}.
4.5 Model peptides

In order to assess the viability of the tetraanionic moiety as a cellular uptake-blocker, a series of preliminary experiments were performed. These experiments also served to establish the proof of principle that fluorophores conjugated to the side chains of amino acid residues can be used to monitor distribution and localisation of the peptides.

The first set of dipeptides, containing lysine and aspartic acid residues, were synthesised in order to determine whether the fluorescence properties of FITC and rhodamine B were altered upon coordination to an amino acid. The second set of peptides included four additional aspartic acid residues, in order to investigate the effectiveness of the polyanionic chain at hindering uptake of the peptides by cells.

4.5.1 Dipeptide-fluorophore conjugates

Two dipeptide-fluorophore conjugates, $\text{H}_2\text{N}-\text{K(FITC)}\text{D-COOH (K(FITC)}\text{D, (4.1))}$ and $\text{H}_2\text{N}-\text{K(RhB)}\text{D-COOH (K(RhB)}\text{D, (4.2))}$, were synthesised by following the procedure outlined in section 4.3.2. The Lys-Asp dipeptide was chosen as it mimicked the N-terminus of the target peptide, $\text{H}_2\text{N-K(FITC)}\text{DIPVSLRSK-COOH}$ and was believed to represent a polypeptide more accurately than a single lysine residue. In order to determine whether these fluorophores were suitable for labelling the target peptides, the fluorescence spectra of the dipeptide-fluorophore conjugates were compared to those of the free fluorophores, FITC and rhodamine B.

![Figure 4.2](image_url) Structures of fluorophore-dipeptide conjugates.
A comparison of the fluorescence spectra can be seen in Figure 4.3. When comparing the fluorescence profile of pure FITC to that of the FITC-conjugated dipeptide K(FITC)D (4.1), a reduction in fluorescence intensity of approximately 5-fold is apparent. However, no significant shift in the excitation and emission spectra was caused by conjugation to the lysine residue. In the case of rhodamine B and its dipeptide derivative, the level of fluorescence is reduced by a factor of 2.5 and the excitation and emission maxima are blue-shifted by approximately 10 nm.

![Fluorescence Spectra](image)

**Figure 4.3** The fluorescence excitation and emission spectra of the free fluorophores FITC and rhodamine B, and their dipeptide congeners K(FITC)D (4.1) and K(RhB)D (4.2).

The reduction in fluorescence may possibly be explained by considering the chemical structures of the FITC and rhodamine B molecules, derivatives of the classical fluorescein and rhodamine dyes. In solution, both structures exist in an equilibrium between their “open”/fluorescent and “closed”/non-fluorescent spirolactam form, as shown in Figure 4.4.
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Figure 4.4 Diagram showing the pH-dependent equilibrium which exists between the “open” and closed forms of (a) FITC and (b) rhodamine B.

While FITC and rhodamine B were obtained commercially and used without further purification, the dipeptide-fluorophore conjugates had undergone preparative HPLC which involves a significant amount of time (approximately 1 hr) spent in a predominantly aqueous solution. It is possible that this time allowed for a greater proportion of the spirolactam form of each fluorophore to form via loss of the delocalised proton. This process has been shown to be at least partially reversible, as it is a pH dependent cyclisation. It has been demonstrated that by adjusting the pH to acidic (< 3) from the physiological pH of 7.4, a significant return in fluorescence can be observed. As performing biological experiments at pH 3 to increase the fluorescence was not feasible, it was decided that dosing would be performed at a concentration of 25 µM instead of the more conventional 10 µM to ensure fluorescence was detectable. For clarity, all further discussion of the rhodamine B fluorophore and its derivatives will refer to the “open”/fluorescent form, as it is this form which will be observed in confocal microscopy experiments.

The behaviour of these fluorescently labelled peptides was then investigated in vitro, and compared to that of the corresponding free fluorophore. DLD-1 cells were dosed with 10 µM of the compound and incubated for 4 hrs in order to observe the cellular localisation of the unmodified fluorophore. As shown in Figure 4.5, free FITC does not undergo cellular uptake and remains in the media, causing the cells to appear as dark shadows against a background of bright green fluorescence. Conjugation to the lysine residue in the K(FITC)D (4.1) dipeptide did alter the fluorescence distribution pattern, with a small punctate delineation of the cells being
visible once the media had been changed to remove excess compound. This suggests that perhaps the peptide is causing the FITC to associate with the cell membrane, but is still unable to traverse the membrane and enter cells.

**Figure 4.5** Confocal microscopy images of DLD-1 cells treated with A: rhodamine B + Mitotracker®, B: FITC, C: K(FITC)D (4.1), D: K(FITC)D (4.1) with media changed, E: K(RhB)D (4.2), F: K(RhB)D (4.2) with media changed. A1: shows rhodamine B fluorescence collected in the red channel; A2: shows Mitotracker® fluorescence collected in the green channel; A3: shows the red and green channel overlaid. Their respective bright field images are also shown. Incubation time was 4 hrs. Images of FITC and derivative were obtained in the green channel. Images of rhodamine B and its derivative were obtained in the red channel. Images of Mitotracker® were obtained in the green channel. Scale bar represents 20 µm.
This property of FITC therefore made it ideal for labelling the target peptide in a position where its uptake-blocking properties would be beneficial. Contrastingly, the free rhodamine B fluorophore can be seen to undergo cellular uptake and subcellular localisation in a pattern resembling mitochondrial distribution. This was confirmed by also staining with green Mitotracker®, as shown in Figure 4.5A, where superimposition of the red (rhodamine B) and green (Mitotracker®) channels reveals significant co-localisation (shown in yellow). This was expected, as xanthene based dyes such as rhodamine B are widely used as mitochondrial probes. This result indicated that the rhodamine B fluorophore would be best attached to the target peptide in a position which was intended, following cleavage by MMP-2, for cellular uptake. The dipeptide K(RhB)D (4.2), despite having a different distribution pattern to the free fluorophore, also underwent cellular uptake. The compound appears to be dispersed in the cytoplasm, and again removal of excess compound by changing the original media led to the visualisation of small, punctate fluorescence within the cell, reminiscent of endosomal localisation.

The overall charge of the two fluorophore-dipeptide conjugates may also explain the differences in cellular uptake. At the physiological pH of 7.4, the α-amino group of lysine is protonated and the α-carboxylic acid group of aspartic acid is deprotonated, with pKa values of 8.95 and 2.09 respectively, such that the peptide exists in a zwitterionic form. The aspartic acid side chain, with a pKa of 3.86 is also deprotonated at physiological pH, lending a -1 charge to the dipeptide. In the case of K(RhB)D (4.2), this charge is countered by the positive charge on the rhodamine B molecule, when in its “open”, fluorescent form making the overall compound neutral. This does not happen in the case of the neutral FITC, where the FITC-dipeptide conjugate remains with an overall charge of -1. It has been well-established that cells undergo decreased uptake of negatively charged species, due to reduced adhesion to the negatively charged phospholipid bilayer of the cell membrane and reduced uptake by endocytotic mechanisms. However, the effect of charge on cell uptake is likely to be minor compared to the inherent properties of the free fluorophores, as evidenced by the cellular uptake patterns of the free fluorophores themselves. Importantly, this work showed that the fluorophore-dipeptide conjugates had relatively similar biological properties to the fluorophores with which they were labelled, which is beneficial for their incorporation into the target compound.
4.5.2 Tetraanion-fluorophore conjugates

In order to assess the ability of a polyanionic moiety to block cellular penetration, a pair of blocking group-fluorophore derivatives, H$_2$N-(Asp)$_4$-Lys(FITC)-Asp-COOH (DDDDK(FITC)D, (4.3)) and H$_2$N-(Asp)$_4$-Lys(RhB)-Asp-COOH (DDDDK(RhB)D, (4.4)) were synthesised.

![Structures of dipeptide-fluorophore conjugates with the inclusion of the tetra-aspartate uptake-blocking group.]

As FITC’s cell uptake blocking properties had already been established, the rhodamine B analogue was also synthesised. It was believed that by conjugating a highly cell-penetrable moiety such as rhodamine B, the true effect of the tetra-aspartate group could be observed.

As mentioned previously, the pKa for the side chain carboxylic acid residues is 3.86, meaning that it is deprotonated at physiological pH. This imparts a charge of -5 and -4 on DDDDK(FITC)D (4.3) and DDDDK(RhB)D (4.4) respectively. Figure 4.7C depicts the cellular penetration of DDDDK(FITC)D (4.3) which, given the uptake profile of K(FITC)D (4.1) described in section 4.5.1, was not expected to exhibit any intracellular fluorescence. The same absence of cellular penetration was observed, but interestingly the punctate membrane-associated fluorescence observed for K(FITC)D (4.1) was not present when cells were treated with its tetra-aspartate congener DDDDK(FITC)D (4.3).
This may be due to the increased anionic charge, causing sufficient electrostatic repulsion between either the cell membrane or adjacent fluorophore-peptide molecules to prevent them adhering to the cell surface or forming aggregates with each other. In either case, it was still necessary to assess the suitability of the anionic blocking group for preventing cell uptake by examining the fluorescence patterns observed when the cells were treated with DDDDK(RhB)D (4.4). As shown in Figure 4.7D, the cytoplasmic hotspots of fluorescence in the red channel were no longer visible in the presence of the polyanionic group, and although a very minor amount of intracellular fluorescence can be observed, it was considered to be negligible. Moreover, as all settings were kept consistent between images, the fluorescence of rhodamine B in the media is greater for DDDDK(RhB)D (4.4) than its dipeptide equivalent, indicative of more compound remaining in solution.

Even when conjugated to a dipeptide, rhodamine B is capable of penetrating the cell membrane and localising in the cytoplasm. However, the presence of the tetra-aspartate moiety significantly hindered cellular uptake and therefore could be employed in the target peptide for this purpose. It was also proven that conjugation of FITC to the tetra-aspartate residues did not change the uptake-blocking ability of the anionic group, making it a suitable fluorophore for monitoring the peptide fragment intended to remain outside cells.
4.6 Target peptides

The structure of the target compound designed to be selectively activated by MMP-2 is shown in Figure 4.8. Through the presence of the two fluorophores on opposite sides of the cleavage site, the biological behaviour of the intact peptide as well as its respective fragments is able to be monitored, and provides the basis for the design of the cytotoxin-peptide compounds described in Chapter 5.

![Figure 4.8](image)

As discussed in section 4.1, the peptide backbone of this target compound is the MMP-2 specific sequence H2N-Asp-Ile-Pro-Val-Ser-Leu-Arg-Ser-COOH, with some modifications. Attached to the N-terminus is a tetra-aspartate moiety which prevents cellular uptake of the entire compound through its polyanionic charge. With the inclusion of this group, the overall charge of the compound is -3, which was believed to be sufficient to prevent cell uptake based on the results described in section 4.5.2. A lysine residue was inserted between the uptake-blocking group and the MMP-2 selective sequence in order to attach one of the two fluorophores, FITC. In previous work, the Lys(FITC) moiety was tethered to the N-terminus of the MMP-2 cleavage sequence, and this appeared to inhibit cleavage of the peptide by MMP-2 such that after 18 hrs, the majority of the peptide remained intact. It was thought that by incorporating an amino acid to act as a spacer between the MMP-2 sequence and the blocking group, some degree of cleavage could be restored. As such, two MMP-2 cleavable peptides...
were synthesised – one containing the blocking group and one without, to act as a control. As it has already been shown that peptide conjugation did not greatly affect the fluorescence properties of the fluorophores, FITC was chosen to label the side of the peptide which was not required to enter cells, based on its intrinsic uptake-blocking properties discussed in section 4.5.1. Based on the same set of results, rhodamine B was chosen to label the side of the peptide intended for cell uptake following MMP-2 cleavage. This led to the synthesis of two target peptides, H$_2$N-Lys(FITC)-Asp-Ile-Pro-Val-Ser-Leu-Arg-Ser-Lys(RhB)-COOH (K(FITC)DIPVSLRSK(RhB), (4.5)) and H$_2$N-(Asp)$_4$-Lys(FITC)-Asp-Ile-Pro-Val-Ser-Leu-Arg-Ser-Lys(RhB)-COOH (DDDDK(FITC)DIPVSLRSK(RhB), (4.8)) containing the tetra-aspartate blocking group. As the charge on the intact peptide is being exploited as a meaning of impeding cellular uptake, consideration of the charges of the entire peptide family is fundamental to understanding their behaviour in vitro.

Table 4.2 The charge present on intact peptides and their fragments at physiological pH, 7.4.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Charge</th>
<th>Peptide</th>
<th>Charge</th>
</tr>
</thead>
<tbody>
<tr>
<td>K(FITC)DIPVSLRSK(RhB)</td>
<td>1+</td>
<td>DDDDK(FITC)DIPVSLRSK(RhB)</td>
<td>3-</td>
</tr>
<tr>
<td>K(FITC)DIPVS</td>
<td>1-</td>
<td>DDDDK(FITC)DIPVS</td>
<td>5-</td>
</tr>
<tr>
<td>LRSK(RhB)</td>
<td>2+</td>
<td>LRSK(RhB)</td>
<td>2+</td>
</tr>
</tbody>
</table>

The small family of target peptides and their respective fragments is shown in Figure 4.9. It should be noted that the rhodamine B-containing fragment LRSK(RhB) (4.7) was common to both peptides.
Figure 4.9 Structures of target compounds and their cleaved fragments.
4.7 Cleavage assays

4.7.1 Fluorescence

Given the position of the two fluorophores attached to opposite sides of the cleavage site in target peptides K(FITC)DIPVSLRSK(RhB) (4.5) and DDDDK(FITC)DIPVSLRSK(RhB) (4.8), it was anticipated that cleavage of the peptide by MMP-2 could be monitored using fluorescence spectrophotometry. Moreover, the fluorescence spectra of FITC and rhodamine B and their peptide derivatives showed a degree of overlap between the FITC emission spectrum and rhodamine B excitation spectrum, indicating the possibility of Förster (or Fluorescence) Resonance Energy Transfer (FRET) behaviour between the pair of fluorophores. If a true FRET interaction existed between this pair of fluorophores, it would mean that excitation of FITC at 488 nm would result in emission by the rhodamine B fluorophore only, with no emission seen for FITC. This is a result of the nonradiative transfer of energy from the excited fluorophore (FITC) to the acceptor fluorophore (rhodamine B) when they are held in close proximity to each other, such as in the intact peptide K(FITC)DIPVSLRSK(RhB) (4.5). Cleavage of the intact peptide by MMP-2 would generate two peptide fragments containing the respective fluorophores, but they would no longer be restricted in their distance from each other, preventing the transfer of energy from donor to acceptor fluorophore from occurring. As a result, a return in fluorescence for FITC would be observed. Figure 4.10 shows the fluorescence spectra, measured in H₂O adjusted to pH 7.4, for K(FITC)DIPVSLRSK(RhB) (4.5) and its respective fragments. The excitation wavelengths were 488 nm (FITC) and 559 nm (rhodamine B) in order to achieve consistency with the available argon laser wavelengths used in subsequent confocal microscopy experiments.
Firstly, fluorescence spectra were measured for the two peptide fragments, K(FITC)DIPVS (4.6) and LRSK(RhB) (4.7) together in solution in order to establish the characteristic emission spectra and intensity for the free fragments. When excited at 488 nm, the emission spectrum has a predominantly FITC character, with some broadening observable above 500 nm. However, no apparent shoulder peak was visible for rhodamine B emission. When the same solution was excited at 559 nm, the excitation wavelength for rhodamine B, a distinct emission spectrum (red) was observed at almost double the intensity of the emission for K(FITC)DIPVS (4.6) (green).

When excited at 488 nm, the intact peptide K(FITC)DIPVSLRSLK(RhB) (4.5) showed a predominantly rhodamine B emission profile, but this was difficult to observe due to the low fluorescence intensity. By increasing both the excitation and emission slit widths to 5 nm (purple dashed line), the FRET interaction between the two fluorophores becomes evident. The diminished fluorescence in the FITC emission range (500-550 nm) suggests that a transfer of energy from FITC to rhodamine B is occurring, as for the fragment LRSK(RhB) (4.7) no fluorescence is observed when excited at 488 nm. The salient emission peak in the spectrum

**Figure 4.10** Fluorescence emission spectra for K(FITC)DIPVSLRSLK(RhB) (4.5) and a mixture of its fragments. The spectra were collected in H₂O adjusted to pH 7.4.
of the intact peptide K(FITC)DIPVSLRSK(RhB) (4.5) was confirmed to be a result of rhodamine B emission by comparing it to the emission spectrum (pink) of the same compound following excitation at 559 nm. Despite some degree of FRET occurring, a significant loss of fluorescence was observed for the acceptor fluorophore, rhodamine B, the result of inefficient donor-acceptor energy transfer. The efficiency of the energy transfer between donor and acceptor fluorophore is dependent on a number of factors including the amount of spectral overlap between the acceptor and donor fluorophore respectively, as depicted by the grey area in Figure 4.11, as well as the physical distance between the two fluorophores and the relative orientations of the donor-acceptor transition dipole moments.

![Figure 4.11](image)

*Figure 4.11* Spectral overlap of the emission spectrum of the donor fluorophore (Dem) with the excitation spectrum of the acceptor fluorophore (A-abs). Image taken from [231].

With the purpose of using FRET as a method for monitoring the cleavage of K(FITC)DIPVSLRSK(RhB) (4.5) *in situ*, the fluorescence properties of the intact peptide were investigated in the recommended TCN buffer for MMP cleavage assays. The emission spectra which were collected are shown in Figure 4.12, and are markedly different to those collected in H₂O.

The emission spectra of both K(FITC)DIPVSLRSK(RhB) (4.5) and a mixture of its fragments K(FITC)DIPVS (4.6) and LRSK(RhB) (4.7) show a significant lack of fluorescence when excited at 559 nm (red and pink-dashed lines respectively). Also, dissolution in the TCN buffer appears to have enhanced the fluorescence of FITC in the intact peptide by approximately two-fold when compared to the equivalent emission spectrum in Figure 4.12. The non-radiative energy transfer in FRET occurs over much greater distances than short-range solvent effects, and is largely independent of the dielectric nature of the surrounding molecules, ie solvent and host.
molecules. As these changes in fluorescence are consistent across all peptides and not caused by any FRET-related phenomena, it is believed that the TCN buffer is modulating the fluorescence characteristics of the FITC and rhodamine B fluorophores, rather than directly affecting the FRET interactions between the two fluorophores.

Figure 4.12 Fluorescence emission spectra for K(FITC)DIPVSLRSK(RhB) (4.5) and a mixture of its fragments. The spectra were collected in TCN buffer adjusted to pH 7.4.

The reduction in the brightness of rhodamine B has been widely investigated, where brightness is defined as the product of the extinction coefficient and the quantum yield \((\varepsilon \times \phi)\). The photophysical properties of rhodamine B, including fluorescence lifetime and quantum yield, have been shown to vary greatly with solvent composition and temperature, as well as the molecules to which it is tethered. Rhodamine B is known to exhibit a strongly viscosity-dependent quantum yield\(^238\), and is known to form aggregates in aqueous solutions which also cause a decrease in the quantum yield of the dye\(^239,240\). The formation of these aggregates, likely to be enhanced by the high salt concentration in the TCN buffer, is known to occur when the concentration of a Rhodamine in solution is in the range of \(10^{-6}\) to \(10^{-5}\) M. Not only does the formation of these aggregates change the shape of the fluorescence excitation and emission spectra, dimer and higher order aggregate formation by the dye leads to poorer solvation and a reduction in fluorescence through self-quenching\(^239\). This quenching has been
attributed to long-range dipole-dipole energy transfer from the excited state of the rhodamine B monomer to the aggregates. This behaviour of rhodamine B in aqueous-type solutions explains the almost complete loss of fluorescence observed when excited at 559 nm in TCN buffer, a phenomenon which was also observed in Advanced DMEM medium (without the addition of phenol red). This meant that, despite the observation of FRET-like activity as shown in Figure 4.10, fluorescence spectrophotometry would not be a suitable method for monitoring the cleavage of K(FITC)DIPVSLRSK(RhB) (4.5) and DDDDK(FITC)DIPVSLRSK(RhB) (4.8) by MMP-2.

### 4.7.2 LC-MS

In order to investigate the cleavage properties of K(FITC)DIPVSLRSK(RhB) (4.5) and DDDDK(FITC)DIPVSLRSK(RhB) (4.8) by MMP-2, cleavage assays were performed as described in section 4.2.4.2, and the resulting solutions analysed by LC-MS. The chromatograms, measured at 254 nm in order to best detect both the FITC and rhodamine B fluorophores, are shown in Figure 4.13a-d. Despite being unable to provide full quantitative information, differences in the relative peaks heights of the intact peptides and fragments do indicate that the cleavage characteristics of K(FITC)DIPVSLRSK(RhB) (4.5) and DDDDK(FITC)DIPVSLRSK(RhB) (4.8) are not identical.

Figure 4.13a and 4.13b show the extent of cleavage of K(FITC)DIPVSLRSK(RhB) (4.5) by MMP-2 at 4 and 24 hrs respectively. The peak at 13.9 mins corresponds to the K(FITC)DIPVS (4.6) fragment as confirmed by mass spectrometry, with the LRSK(RhB) (4.7) fragment eluting with a retention time of 15.5 mins. The results clearly demonstrate that the peptide is cleaved into its respective fragments after 4 hrs, with a small proportion of the intact peptide visible at 17.7 mins. After 24 hrs (Figure 4.13b), the peak heights of the two fragments have increased relative to the intact peptide, suggesting that cleavage has progressed but is not complete after 24 hrs.
Similarly, Figure 4.13c shows the LC-MS spectrum of DDDDK(FITC)DIPVSLRSK(RhB) (4.8) after treatment with MMP-2 for 4 hrs. Again, peaks for the two fragments DDDDK(FITC)DIPVS (4.9) and LRSK(RhB) (4.7) are visible at 14.1 and 15.5 mins respectively. However, the peak height of both fragments is significantly lower than the fragments seen in the LC-MS of K(FITC)DIPVSLRSK(RhB) treated with MMP-2 for 4 hrs. Following the same trend, the chromatogram collected after treatment for 24 hrs shows that the peak at 17.8 mins corresponding to uncleaved DDDDK(FITC)DIPVSLRSK(RhB) (4.8) is still significant, suggesting that cleavage of this peptide by MMP-2 is not occurring readily.

As the major difference between these two peptides is the presence of the tetra-aspartate uptake-blocking group, the attenuated cleavage observed is most likely due to a reduction in affinity for the peptide substrate by the MMP-2 enzyme. The anionic charge present on the
tetra-aspartate group may interfere with binding site interactions between the enzyme and peptide by causing intra-strand folding within the DDDDK(FITC)DIPVSLRSK(RhB) due to attractive forces between the positive arginine and rhodamine B moieties and the aspartate group. This could cause steric hindrance which limits access of the enzyme to the cleavage site, or alter the orientation of the peptide such that binding site recognition and fit is compromised. This could also be caused by electrostatic repulsion between the tetra-anionic charges and the binding site, and could be probed further by measuring the actually peptide-enzyme affinities and using computer modelling techniques to determine structure-activity relationships to improve upon the rational design of these peptides.

Overall, investigations into the cleavage properties of K(FITC)DIPVSLRSK(RhB) (4.5) and DDDDK(FITC)DIPVSLRSK(RhB) (4.8) have shown that the tetra-aspartate uptake-blocking group significantly interferes with cleavage by MMP-2. This occurs even despite the insertion of a lysine residue to act as a spacer between the cleavage sequence and the uptake-blocking moiety. However, as some level of cleavage is still detectable by LC-MS analysis, both K(FITC)DIPVSLRSK(RhB) (4.5) and DDDDK(FITC)DIPVSLRSK(RhB) (4.8) were investigated further.

4.8 Confocal microscopy

4.8.1 Cell lines

In order to assess the behaviour of these MMP-2 specific peptide sequences, it was necessary to use cell lines which were known to express MMP-2, as well as cell lines which did not. Previous work\(^{150,223,228}\) has shown that A549 human alveolar basal epithelial adenocarcinoma cells have high levels of MMP-2 activity, while DLD-1 human colorectal adenocarcinoma cells have minimal MMP-2 activity. While this does not necessarily mean that DLD-1 cells do not express MMP-2 at all, it does mean that they are not being activated and therefore are unable to cleave peptide substrates. Since DLD-1 cells can be grown into spheroids, where they have been shown to have low but significant levels of MMP-2 activity\(^{232}\), they are suitable models for investigating the \textit{in vitro} behaviour of MMP-2 cleavable peptides. In order to ensure that no cleavage was occurring due to exogenous MMPs or non-specific proteases, all cells and spheroids were maintained in serum-free media for a minimum of 12 hrs before dosing with the desired compounds, as foetal calf serum contains endogenous MMPs.
4.9 Confocal microscopy of 2D-dimensional cell models

Fluorescence confocal microscopy was used to investigate the in vitro behaviour of these selectively-activatable peptides. A549 and DLD-1 cells were treated with the intact peptides and their fragments and images collected in the red and green channels.

4.9.1 Cellular accumulation of peptide fragments

The cellular localisation of the three peptide fragments, shown in Figure 4.14, was investigated in both A549 and DLD-1 cells, in order to expose any cell-line specific localisation patterns and more so to exemplify what would be expected if the intact MMP-2 peptide sequences were cleaved completely. The fragment intended for cell penetration, LRSK(RhB) shows a high degree of cell uptake in A549 cells and a moderate level of uptake in DLD-1s, as settings on the microscope (laser power, imaging settings) were kept consistent for all experiments. In both cell lines, the compound is localised in the cytoplasm, and in the A549s cells, which show a more punctate staining effect, a small amount of nuclear staining is also present. While this was expected, given the mitochondrial-staining applications of rhodamine B, it also suggests that the RHS fragment may tend to adopt the cellular accumulation properties of its payload, such as the cytotoxic moieties described in Chapter 6, and that this should be considered in future applications of this sequence. The possibility that the similarity between the observed fluorescence distribution of the LRSK(RhB) (4.7) and free rhodamine B (see Figure 4.5A) is a result of the peptide losing the fluorophore upon incubation was considered, but the results presented in section 4.7.2 showed that no free rhodamine B was detected after incubation for 24 hrs, suggesting that it does not readily undergo detachment form the peptide. The left hand side (LHS) fragments K(FITC)DIPVS (4.6) and DDDDK(FITC)DIPVS (4.9) are also shown in Figure 4.14, with no cell penetration achieved by the compounds as evidenced by a lack of intracellular fluorescence in the green channel. This also was expected given the uptake-blocking properties of FITC discussed in section 4.5.1.
These results suggested that cleavage of the intact peptide sequences $\text{K(FITC)}\text{DIPVSLRSK(RhB)}$ (4.5) and $\text{DDDDK(FITC)}\text{DIPVSLRSK(RhB)}$ (4.8) should give rise to two different distribution profiles for the fragments, making cleavage observable by confocal microscopy. This was investigated by dosing cells with equimolar mixtures of the two fragments simultaneously, to ensure that no fluorescence bleed-through was occurring.
Figure 4.15 shows that there is no fluorescence bleed through from the green into the red, and that the presence of the two peptide fragments in solution does not affect the uptake or blocking properties of either peptide.
4.9.2 Cellular accumulation of target peptides

4.9.2.1 In the absence of MMP-2

The intact peptides were tested in DLD-1 cell monolayers due to their minimal expression of MMP-2. Figure 4.16A shows cellular penetration in both the green and red channels for K(FITC)DIPVSLRSK(RhB) (4.5), which when superimposed shows a significant degree of colocalisation within the cell. As made evident by the results in section 4.9.1, the free fragment K(FITC)DIPVS (4.6) does not undergo cellular uptake by itself, meaning that a large proportion of the intact peptide must be entering the cells before it is cleaved. Extracellular fluorescence is also observed in the green channel, but not for the red, indicative of a small degree of peptide cleavage occurring extracellularly, perhaps by non-specific proteases.

![Confocal microscopy images](image)

**Figure 4.16** Confocal microscopy images of DLD-1 cells treated with A: K(FITC)DIPVSLRSK(RhB), B: DDDDK(FITC)DIPVSLRSK(RhB) (4.8). Their respective bright field images are also shown. Incubation time was 4 hrs and the media was replaced for all images. Images of FITC derivatives were obtained in the green channel. Images of rhodamine B derivatives were obtained in the red channel. Scale bar represents 20 µm.

When compared to the fluorescence distribution of DDDDK(FITC)DIPVSLRSK(RhB) (4.8), containing the uptake-blocking group, a marked difference in uptake can be seen. Apart from minimal intracellular fluorescence in the red channel, no colocalisation with fluorescence in the green channel is observed. This indicates that the red and green fluorescence patterns are the result of two different species, most likely the cleaved peptide fragments LRSK(RhB) (4.7)
Chapter 4: Fluorophore-Peptide Substrates for Investigating MMP-2 Activity

and DDDDK(FITC)DIPVS (4.9). Furthermore, the absence of any FITC-related fluorescence with the cells suggests that the intact peptide is remaining outside cells until it is cleaved, allowing the free LRSK(RhB) (4.7) to enter cells. As this is not observed in the absence of the tetra-aspartate blocking group, it suggests that the cell-penetrating ability of rhodamine B is greater than the uptake-blocking nature of FITC, reinforcing the need for an additional uptake-blocking moiety.

4.9.2.2 In the presence of MMP-2

The behaviour of intact peptides was also studied in A549 cells, which have been shown to possess MMP-2 activity as discussed in section 4.8.1. A similar but diminished effect is observed for K(FITC)DIPVSLRSK(RhB) (4.5) (Figure 4.17A) in A549 as in DLD-1 cells (Figure 4.16A), whereby a small amount of colocalised red and green fluorescence is visible within the cells, indicative of a slight amount of uptake of the intact peptide. However, in contrast to the DLD-1 cells, a much greater amount of fluorescence in the red channel which shows no overlap with the green is observed.

![Figure 4.17](image_url)

**Figure 4.17** Confocal microscopy images of A549 cells treated with A: K(FITC)DIPVSLRSK(RhB) (4.5), B: DDDDK(FITC)DIPVSLRSK(RhB) (4.8). Their respective bright field images are also shown. Incubation time was 4 hrs and the media was replaced for all images. Images of FITC derivatives were obtained in the green channel. Images of rhodamine B derivatives were obtained in the red channel. Scale bar represents 20 µm.
This again is indicative of a separate species to the intact peptide giving rise to the red fluorescence, which could be attributable to the cleaved fragment LRSK(RhB).

$\text{DDDDK(FITC)} \text{DIPVSLRSK(RhB)}$ (4.8) shows a similar distribution pattern in the red channel as for $\text{K(FITC)} \text{DIPVSLRSK(RhB)}$ (4.5) suggesting that the same species is present in both cases, however the fluorescence intensity is diminished. Additionally, fluorescence within the cell is not visible in the green channel, which correlates with the results reported in section 4.9.2.1. This implies that cleavage of the peptide fragment is taking place outside of the cell, and that the tetraaspartate group is effective at hindering the uptake of compounds containing the highly cell-penetrating rhodamine B molecule. Moreover, the lower level of intracellular fluorescence could be attributable to the slower rate of cleavage observed for $\text{DDDDK(FITC)} \text{DIPVSLRSK(RhB)}$ (4.8) compared to $\text{K(FITC)} \text{DIPVSLRSK(RhB)}$ (4.5), as discussed in section 4.7.2.

To avoid the addition of exogenous MMPs, all DLD-1 and A549 cell monolayers were plated in serum-free media. However, the cellular distribution patterns of the target peptides were also investigated in DLD-1 monolayers in which the medium had been supplemented with FCS.

![Figure 4.18](image-url) **Figure 4.18** Confocal microscopy images of DLD-1 cells in medium supplemented with FCS treated with A: $\text{K(FITC)} \text{DIPVSLRSK(RhB)}$ (4.5), B: $\text{DDDDK(FITC)} \text{DIPVSLRSK(RhB)}$ (4.8). Their respective bright field images are also shown. Incubation time was 4 hrs and the media was removed, the cells washed twice with PBS, and the media replaced for all images. Images of FITC derivatives were obtained in the green channel. Images of rhodamine B derivatives were obtained in the red channel. Scale bar represents 20 µm.
The resulting images are shown in Figure 4.18, where cellular uptake is visible in the red channel for cells treated with both K(FITC)DIPVSLRSK(RhB) (4.5) and DDDDK(FITC)DIPVSLRSK(RhB) (4.8), with the former showing a higher amount of intracellular fluorescence. This is most likely due to a greater proportion of the compound having undergone cleavage in the media, consistent with the results described in sections 4.9.2.1 and 4.9.2.2. However, the addition of exogenous MMPs in the media through supplementation with FCS had influenced the presence of colocalised fluorescence between the red and green channels observed in serum-free DLD-1 and A549 monolayers. The is particularly apparent for cells treated with K(FITC)DIPVSLRSK(RhB) (4.5), where Figures 4.16A and 4.17A show that the uncleaved peptide undergoes some degree of cellular accumulation prior to cleavage. In order to observe as much intracellular fluorescence as possible, the cells were washed twice with PBF solution to remove any background fluorescence from intact FITC remaining in the media after dosing. Figure 4.18 shows that there is essentially no intracellular fluorescence in the green channel, meaning that the intact peptide is not entering cells. As it has already been observed that the intact peptide does enter cells after 4 hrs, the absence of any FITC-containing species within cells is likely a result of K(FITC)DIPVSLRSK(RhB) (4.5) being cleaved more rapidly in the surrounding media by the additional MMPs and proteases supplied by the FCS. Of the two cleavage products, K(FITC)DIPVSLRSK(RhB) (4.5) and LRSK(RhB) (4.7), the cell-penetrable LRSK(RhB) (4.7) is then taken up by cells as seen in the red channel, and K(FITC)DIPVSLRSK(RhB) (4.5) remains outside cells, and is removed during washing with PBF.

The fluorescence distribution pattern DDDDK(FITC)DIPVSLRSK(RhB) (4.8) and its fragments, shown in Figure 4.18B, also shows no intracellular fluorescence in the green channel, and a slightly lower fluorescence intensity in the red channel when compared to K(FITC)DIPVSLRSK(RhB) (4.5). This follows the trend seen in other cell monolayers, which has been attributed to the slower rate of cleavage of the peptide containing the uptake-blocking moiety. The lack of FITC-related fluorescence was also expected, as even though the peptide remains intact for longer, the uptake-blocking group prevents its entry into cells, such that removal of the media and washing of the cells would have resulted in the removal of the intact peptide as well as the DDDDK(FITC)DIPVSLRSK(RhB) (4.9) fragment from the media.
4.10 Confocal microscopy of spheroids

4.10.1 Visualising MMP activity

Although previous work has demonstrated that there are sufficient levels of MMP-2 activity in the media which surrounds DLD-1 spheroids to cleave similar peptides\textsuperscript{228}, there have been no investigations into visualising the distribution of MMP activity within spheroids. In order to gain an understanding of where K(FITC)DIPVSLRSK(RhB) (4.5) and it’s tetra-aspartate analogue were being cleaved in relation to MMP activity, spheroids were dosed with the commercially available MMP substrate, MMPSense 680 (PerkinElmer\textsuperscript{TM}). MMPSense 680 is an optically silent substrate in it’s uncleaved, inactivated form and becomes highly fluorescent when cleaved and activated by MMP-2, -7, -9, -12 and -13\textsuperscript{241}. This particular reagent was chosen based on its near-infrared (NIR) fluorescence profile, requiring excitation by the 633 nm argon laser and emission collection > 650 nm, as shown by Figure 4.19.

![Figure 4.19](image)

\textit{Figure 4.19} Fluorescence excitation and emission spectrum for activated MMPSense 680. The excitation spectrum is shown in blue, emission spectrum in red and UV absorbance in grey.

This minimised the possibility of any bleed through from the green and red channels for FITC and rhodamine B respectively. In addition to avoiding any spectral overlap, the fluorophores were excited and images collected sequentially from longer to shorter wavelengths, as was done for all images contained in this chapter, and excitation for each fluorophore was performed with a different argon laser (488, 559 or 633 nm). One drawback of this reagent is its lack of selectivity for MMP-2, but given that it is selective for MMP’s which are implicated in tumour metastasis and progression it was still considered to provide valuable information.
regarding the cleavage of the intact peptides by MMP’s which are upregulated at the site of solid tumours.

The distribution of MMPs was first visualised in medium spheroids, which contain a significantly less well-defined necrotic core than larger spheroids. Figure 4.20A shows the fluorescence caused by MMP activation to be distributed throughout the spheroid in an irregular pattern, with some “patchy” areas of increased activity and some hotspots.

**Figure 4.20** Confocal microscopy images of medium DLD-1 spheroids treated with A: MMPSense 680 in the presence of FCS, B: MMPSense680 in the absence of FCS, C: MMPSense680 following incubation with 0.5 µM marimastat for 30 mins. Their respective bright field images are also shown. Incubation time was 4 hrs. Images of MMPSense 680 were collected in the NIR channel and are shown as magenta. Scale bar represents 100 µm.

As MMPs are secreted enzymes, it was expected that a fluorescence gradient may be observed radiating outwards from the periphery of the spheroid into the surround media. However, it is more probable that cleaved fragments of MMPSense 680 in the media were too
dilute to observe fluorescence, and the MMPs which are subjected to greater physical restrictions once secreted, such as within the core of the spheroid, are able to concentrate in a single location, thereby giving rise to a fluorescence signal strong enough to be visualised.

Some studies have indicated that cell surface localisation is required to activate the proteolytic activity of some MMPs, including MMP-2. The method by which MMP-2 is attached to the cell surface has been investigated by Brooks and co-workers\textsuperscript{242}, who discovered the interaction between the αvβ3 integrin and MMP-2. Within a spheroid, this could lead to the accumulation of activated MMPs in certain regions due to physical factors, such as dead cells and debris hindering paracellular movement. It is also possible that these hotspots of MMP activity are in fact areas where cleaved MMPSense 680 has pooled and concentrated.

Pro-MMPs are also substrates for active MMPs, meaning that MMPs are capable of proteolytically inducing the activity of more MMPs\textsuperscript{243}. In particular MMP-3 has been shown to induce a 12-fold increase in MMP-1 activity through conversion of pro-MMP-1, and MT1-MMP performs cleavage of the prodomain of pro-MMP-2 yielding fully activated MMP-2\textsuperscript{244,245}. The implication of these localised activational cascades has been hypothesised to be responsible for the significant and localised amplification of ECM proteolytic activity\textsuperscript{243}. These features of MMPs could potentially explain the non-uniform distribution of MMPSense 680-based fluorescence and the distribution of any of the MMP-2 cleavable peptides in this Chapter.

Treatment of spheroids which had been grown in FCS-supplemented media but had then been washed and transferred to serum-free media overnight gave a similar MMP activity profile, as seen in Figure 4.20B. This meant that spheroid growing media which contained FCS could be changed 12-16 hrs prior to dosing, such that the intact peptides would not be cleaved by exogenous MMPs in the media.

The effectiveness of marimatstat, a potent broad spectrum MMP inhibitor, for inhibiting MMP activity in tumour spheroids was also investigated, and the corresponding image taken following treatment with MMPSense confirms that MMP activity has been reduced significantly (Figure 4.20C). This indicated that treating tumour spheroids with marimastat would be an effective means of observing the biological activity of the two MMP-2 cleavable peptides, K(FITC)DIPVSLRSK(RhB) (4.5) and DDDDK(FITC)DIPVSLRSK(RhB) (4.8), in the absence of any MMP activity. Specifically, it would make obvious any non-specific cleavage by other proteases, which is a critical consideration in the design of enzyme-specific prodrugs.
Large DLD-1 spheroids were also grown in order to determine whether the pattern of MMP activity showed any deviation from that observed for medium-sized spheroids.

![Confocal microscopy images of large DLD-1 spheroid treated with MMPSense 680 in the absence of FCS.](image)

**Figure 4.21** Confocal microscopy images of large DLD-1 spheroid treated with MMPSense 680 in the absence of FCS. The bright field image is also shown. Incubation time was 4 hrs. Images of MMPSense 680 were collected in the NIR channel and are shown as magenta. Scale bar represents 100 µm.

As seen in Figure 4.21, a core region of MMPSense 680-based fluorescence is present, which was not seen in the smaller spheroids. The fluorescence in this region is diffuse, suggesting that the MMP activity is occurring in the interstitial space, as would be expected as MMPs are extracellular secreted enzymes, or that activation of the MMPSense 680 substrate is not actually occurring at this site, and the fluorescent substrate is merely pooling here post-activation. This region of fluorescence corresponds with the necrotic core of the spheroid, which has been well characterised previously\(^{246}\) and is generally not observed in tumour spheroids < 400 µm in diameter. There is a concentric band approximately 70 µM wide surrounding this core region which contains very little fluorescence and is possibly due to the presence of quiescent cells in this region, which have been shown to produce very low levels of MMPs\(^{247-249}\) and are discussed in detail in section 1.3.3.2. On the periphery of the tumour, MMP activity can be seen to increase again through a return of fluorescence. The reason for this may be the greater proportion of actively proliferating cells, which are undergoing the processes of growth and remodelling of the extracellular matrix in which active MMPs play an essential role.

Overall, these experiments have shown that MMPSense 680 is a useful reagent for observing the activity of MMPs within solid tumour models *in vitro*. This was confirmed by the addition of the broad spectrum MMP inhibitor marimastat, which effectively stopped all activation of MMPSense 680 by inhibiting active MMPs.
Chapter 4: Fluorophore-Peptide Substrates for Investigating MMP-2 Activity

4.10.2 In the presence of MMPs

As previously discussed in 4.10.1, active MMP-2 is found in low but significant levels in DLD-1 tumour spheroids. For this reason they were considered to be a suitable cell line with which to observe the behaviour of the MMP-2 cleavable peptides in 3D tumour spheroid models. Again, both medium and large spheroids were studied in order observe any differences in cleavage of the intact peptide, given the already established difference in MMP activity in section 4.10.1.

Initially, medium spheroids were grown and dosed in media containing FCS. The images are shown in Figure 4.22 along with images of spheroids dosed with the respective peptide for comparison. Figure 4.22A shows the diffusion profile of LRSK(RhB) (4.7) collected in the red channel, and shows diffusion of 50 – 100 µm from the spheroid periphery towards the centre with some punctate fluorescence, indicative of cell uptake. Given that the cellular uptake of LRSK(RhB) (4.7) was observed to be very effective in cell monolayers, the lack of diffusion into the core of the spheroid was expected. It is likely that the rapid uptake of LRSK(RhB) (4.7) resulted in sequestration by the peripheral cells, an effect which would have been enhanced by the binding of rhodamine B to the mitochondria. This diffusion pattern suggests that penetration of the spheroid might be enhanced by conjugation of a unit which inhibits immediate cellular uptake.

The two peptide fragments intended to remain outside the cell, K(FITC)DIPVS (4.6) and DDDDK(FITC)DIPVS (4.9) exhibited a more diffuse pattern of fluorescence(Figures 4.22B and 4.22C respectively), extending much further towards the core of the spheroid than LRSK(RhB) (4.7). This correlated well with the observations made in 2D fluorescence studies, where the FITC alone was sufficient to inhibit the uptake of this peptide fragment and this effect was enhanced by the presence of the tetra-aspartate group. There was a small amount of cell uptake of K(FITC)DIPVS (4.6) present within the very peripheral rim of cells in the spheroid, but the majority appeared to have diffused towards the core of the spheroid, consistent with the fragment remaining mobile in the interstitial space and being capable of paracellular transport.
Figure 4.22 Confocal microscopy images of DLD-1 medium spheroids treated with A: LRSK(RhB) (4.7), B: K(FITC)DIPVS (4.6), C: DDDDK(FITC)DIPVS (4.9), D: MMPSense 680, E: K(FITC)DIPVSLRSK(RhB) (4.5). Incubation time was 4 hrs and the media was replaced for all images. Images of FITC derivatives were obtained in the green channel. Images of rhodamine B derivatives were obtained in the red channel. Images of MMPSense 680 were obtained in the NIR channel. Scale bar represents 100 µm.
Figure 4.22E was taken after dosing medium-sized spheroids with the uncleaved peptide K(FITC)DIPVSLRSK(RhB) (4.5), as well as the corresponding image taken following treatment with MMPSense 680. The pattern of fluorescence seen in the red channel for K(FITC)DIPVSLRSK(RhB) (4.5) is similar to that of the free LRSK(RhB) (4.7) fragment in 4.22A in that there is diffusion from the tumour periphery toward the centre. However, for the full peptide, this red fluorescence extended further into the spheroid than the 100 µm observed for LRSK(RhB) (4.7), with some punctate fluorescence observed as far in as 150 µm, as well as on the spheroid border. These results suggested that a significant degree of cleavage by active MMPs readily occurred in the surrounding media, which was supported by the increased level of fluorescence in the surrounding media in the NIR channel. The greater diffusion distance could be attributable to the uncleaved peptide following a paracellular pathway as it distributes throughout the spheroid until it is cleaved, thereby allowing the RHS fragment to penetrate cells. Cleavage of the peptide was confirmed by the low amount of co-localisation with fluorescence in the green channel, which showed an uneven distribution towards the spheroid, and was very different to the free K(FITC)DIPVS (4.6) fragment, shown in 4.22B. Co-localisation with fluorescence in the red channel, while minimal, was still present and suggested that the two fluorophores were still contained within the same species in those locations.

Most interesting is the correlation between fluorescence in the green channel and that resulting from MMP activity in the near infrared channel. While this could appear to be the result of spectral overlap, the images were collected sequentially and emission ranges chosen to avoid overlap. As the MMPSense 680 was excited first using the 633 nm laser, and its image collected prior to excitation with the 488 nm laser for FITC, it is unlikely that any fluorescence observed in either channel is due to spectral bleed-through. The images for rhodamine B-containing species were also collected in this fashion; however as considerably less co-localisation is observed it does suggests that fluorescence bleed-through is not an issue. In order to prove this, spheroids dosed with FITC- or rhodamine B-containing peptides did not show any fluorescence emission when imaged in the NIR channel, meaning that any fluorescence in the NIR channel was the result MMPSense 680 activation alone. These control images are shown in Appendix A7.1.

It is possible to explain this effect by considering the return in fluorescence observed for both peptide fragments, K(FITC)DIPVS (4.6) and LRSK(RhB) (4.7) following cleavage of the full K(FITC)DIPVSLRSK(RhB) (4.5) peptide. Figure 4.10 shows that the K(FITC)DIPVS (4.6) fragment
is approximately 20-fold more fluorescent when on its own. Therefore, the hotspots of fluorescence visible in the green channel may be the result of cleavage in the vicinity of active MMPs, as shown in the NIR channel. The transient nature of these hotspots is shown in Figure 4.23, where a medium-sized spheroid was dosed with K(FITC)DIPVSLRSK(RhB) (4.5) and images collected every 15 mins. It should be noted that the high level of background fluorescence in the media is unavoidable, as the spheroid must be imaged in situ, in the solution in which it was dosed. Within 15 mins, fluorescence hotspots within the spheroid can be seen, and by 120 mins more hotspots have appeared closer to the core of the tumour consistent with diffusion on the intact peptide inwards, prior to cleavage. This trend continues until 180 mins. While still visible at 330 mins, at this time point some of the intense regions of fluorescence have begun to disperse, until almost none are visible in the final images collected.

![Confocal microscopy images of a DLD-1 medium spheroid treated K(FITC)DIPVSLRSK(RhB) (4.5).](image)

**Figure 4.23** Confocal microscopy images of a DLD-1 medium spheroid treated K(FITC)DIPVSLRSK(RhB) (4.5). Incubation time was 4 hrs and the media was replaced for all images. Images of FITC derivatives were obtained in the green channel. Scale bar represents 100 µm.

In order to better observe the diffusion of peptide fragments following cleavage by MMP-2, the intact peptides were also tested in larger spheroids with better defined necrotic cores. Previous work has shown that compounds which are not readily taken up into the cells tend to accumulate in the necrotic core, a result of gradual transport through the interstitial space. The treatment of large spheroids with the intact peptides is shown in Figure 4.24. Here, the spheroids were dosed for 24 hrs, in order to allow sufficient time for cleavage and potential accumulation in the core of the spheroid to occur.
The fluorescence distributions in the green channel of Figure 4.24A and B both show accumulation in the spheroid core which is not co-localised with any fluorescence in the red channel. This is most likely due to cleavage of the peptide, and subsequent diffusion of K(FITC)DIPVS (4.6) and DDDDK(FITC)DIPVS (4.9) towards the centre of the spheroid, as they are not readily internalised by cells. The mottled, diffuse fluorescence may be a result of the decreased cell density in the core of spheroids \(^{250}\), with obstructed regions of necrotic cells causing media to pool and concentrate the compounds in interstitial spaces.

Fluorescence in the red channel is again to be found predominantly around the perimeter of the spheroid, which supports the hypothesis that the LRSK(RhB) (4.7) fragment is largely sequestered by cellular uptake at the periphery, restricting tumour penetration. There is a significant difference in spheroid penetration observed between the peptides with and without the uptake-blocking group, Figure 4.24A and B respectively. There was very little co-localisation of red and green fluorescence observed for spheroids dosed with K(FITC)DIPVSLRSDK(RhB) (4.5), suggesting that the majority of the peptide has been cleaved, thus giving rise to two separate fluorescence signals. However, a higher degree of colocalisation was present for spheroids treated with DDDDK(FITC)DIPVSLRSDK(RhB) (4.8), as well as more extensive diffusion of red fluorescence into the tumour model. This correlates with the observations made in section 4.9.2.2, that cleavage of the peptide containing the...
uptake-blocking moiety occurs less readily in vitro than its asparate-free analogue. By remaining intact in the media for longer, the peptide has been able to diffuse further into the spheroid before it is cleaved, and the LRSK(RhB) undergoes cellular uptake. It is also possible that since this experiment was conducted over 24 hrs, compared to 4 hrs previously, the improved diffusion is a result of transcellular movement of the LRSK(RhB) (4.7) fragment. However, since this is not observed for K(FITC)DIPVSLRSK(RhB) (4.5), it is much more likely to be the result of the polyanionic group hindering both cleavage and uptake.

**4.10.3 In the absence of MMPs**

As the results described in section 4.10.1 had already proved that marimastat could effectively block MMP activity in vitro, spheroids which had undergone pretreatment with marimastat were dosed with the two intact sequences, K(FITC)DIPVSLRSK(RhB) (4.5) and DDDDK(FITC)DIPVSLRSK(RhB) (4.8). The images, collected after 4 hrs incubation, are shown in Figure 4.25A and B respectively.

![Figure 4.25](image)

*Figure 4.25* Confocal microscopy images of a DLD-1 medium spheroid treated with A: K(FITC)DIPVSLRSK(RhB) (4.5), B: DDDDK(FITC)DIPVSLRSK(RhB) (4.8), following incubation with 0.5 µM marimastat for 30 mins. Incubation time after dosing was 4 hrs and the media was replaced. Images of FITC derivatives were obtained in the green channel. Images of rhodamine B derivatives were obtained in the red channel. Scale bar represents 100 µm.

A significant amount of overlap exists between the fluorescence captured in the red and green channels, which suggests that the majority of the peptides have remained intact after 4 hrs.
Chapter 4: Fluorophore-Peptide Substrates for Investigating MMP-2 Activity

The punctate distribution of the co-localised fluorescence also suggests that some uptake by the cells within 1-5 cell diameters of the periphery had also occurred, which is conceivable given the small amount of cellular uptake of the intact peptide in monolayer cell culture (Figure 4.15A). The extent of penetration into the spheroid was determined to be in the range of 50 – 70 µm for K(FITC)DIPVSLRSK(RhB) (4.5), and the faintness of fluorescence compared to images collected in the presence of MMP-2 is likely due to the reduced fluorescence observed for the intact peptide, as described in section 4.7.1. For DDDDK(FITC)DIPVSLRSK(RhB) (4.8), the distance that the intact peptide has penetrated the spheroid approximately double that of the aspartate-free sequence, at 100 – 120 µm.

There were also regions where the fluorescence due to FITC and rhodamine B is not colocalised, despite the inhibition of active MMPs. It is probable that some non-specific cleavage by endogenous proteases is occurring. However the cleavage profile, including the rate of hydrolysis and site of cleavage, may differ to that enacted by MMP-2.
4.11 Summary

This study has demonstrated the potential in employing the MMP-2 peptide cleavage sequence for the selective activation of chemotherapeutic agents in the vicinity of solid tumours. The principle behind the rational design of this class of compounds was tested methodically through the synthesis of a small family of peptide-fluorophore conjugates. These conjugates included dipeptide-fluorophore compounds in order to model changes in the fluorescence spectra of the free fluorophores induced upon peptide conjugation. Additionally, fluorophore-modified peptides which contained tetra-aspartate residues were synthesised and tested in order to assess the effectiveness of the polyanionic moiety at inhibiting cellular uptake. Finally, the two target compounds K(FITC)DIPVSLRSK(RhB) (4.5) and DDDDK(FITC)DIPVSLRSK(RhB) (4.8) were synthesised in order to observe how the properties of the peptide changed in the presence of the uptake-blocking group.

The FITC and rhodamine B fluorophores retained their fluorescence upon peptide attachment, however the intensity was diminished and a blue-shift in the excitation and emission maxima was observed for both fluorophores. This loss of fluorescence was attributed to a shift in the pH-dependent equilibrium which exists between the “open”/fluorescent and “closed”/non-fluorescent spirolactam forms of both peptides, a result of HPLC purification. The effectiveness of the tetra-aspartate group at blocking cellular uptake was confirmed by fluorescence confocal microscopy of the DDDDK(FITC)D (4.3) and DDDDK(RhB)D (4.4) peptides, which showed that the intrinsic uptake blocking property of the FITC fluorophore was augmented by the tetra-aspartate residue.

In order to monitor the cleavage of the target peptides K(FITC)DIPVSLRSK(RhB) (4.5) and DDDDK(FITC)DIPVSLRSK(RhB) (4.8) by MMP-2, two methods were investigated. FRET activity was successfully observed between the two fluorophores in water, however this was not conveyed when fluorescence measurements were conducted in the TCN buffer required for MMP-2 cleavage assays due to quenching of the rhodamine B fluorophore. Instead, LC-MS studies showed that in the absence of the uptake-blocking group K(FITC)DIPVSLRSK(RhB) (4.5) is almost entirely cleaved into its respective fragments K(FITC)DIPVS (4.6) and LRKS(RhB) (4.7) after 24 hrs. LC-MS results also showed that the uptake-blocking group slows cleavage of the peptide, a trait which was also observed in fluorescence confocal microscopy.

The LRSK(RhB) (5.7) fragment was observed to enter cells and showed a similar localisation pattern to rhodamine B in the mitochondria. The two fragments K(FITC)DIPVS (4.6) and
DDDDK(FITC)DIPVS (4.9) were found to remain outside the cell, as intended, which in the absence of the tetra-aspartate group was attributed to the presence of FITC.

The target compounds K(FITC)DIPVSLRSK(RhB) (4.5) and DDDDK(FITC)DIPVSLRSK(RhB) (4.8) were tested in DLD-1 cell monolayers in the absence of FCS to ensure no MMP-2 activity. After 4 hrs K(FITC)DIPVSLRSK(RhB) (4.5) was found to have entered the cells intact and undergone a small degree of cleavage, possibly by non-specific proteases. DDDDK(FITC)DIPVSLRSK(RhB) (4.8) did not enter cell intact, and instead only a very minor amount of intracellular fluorescence in the red channel was visible, also indicative of non-specific cleavage.

With MMP-2 present in A549 cell monolayers, less uptake of the intact K(FITC)DIPVSLRSK(RhB) (4.5) peptide was observed and instead discreet localised fluorescence was observed in the red and green channels, suggesting that cleavage was occurring in the extracellular space. This result was also observed, but to a lesser degree, for DDDDK(FITC)DIPVSLRSK(RhB) (4.8). These results confirmed that extracellular cleavage of the peptides by MMP-2 resulted in the selective delivery of the payload fragment LRSK(RhB) (4.7) to tumour cells in the presence of elevated MMP-2 levels.

The behaviour of these peptides in 3D tumour models, spheroids, was also investigated using fluorescence confocal microscopy. Free LRSK(RhB) (4.7) readily enters cells on the spheroid periphery, whereas the free K(FITC)DIPVS (4.6) and DDDDK(FITC)DIPVS (4.9) peptides penetrate further into the spheroid, as they do not readily enter cells and thus adopt a paracellular pathway towards the spheroid core. The intact peptide K(FITC)DIPVSLRSK(RhB) (4.5) underwent cleavage in the surrounding media, resulting in sequestration of the LRSK(RhB) (4.7) fragment by the outermost cells, preventing it from penetrating further into the spheroid. Contrastingly, the slower cleavage of DDDDK(FITC)DIPVSLRSK(RhB) (4.8) resulted in a different distribution of fluorescence in the red channel, having allowed the intact peptide to diffuse further into the spheroid before cleavage and cellular uptake of the LRSK(RhB) (4.7) fragment. Pivotal future work will involve measuring the stability of the DDDDK(FITC)DIPVSLRSK(RhB) (4.8) prodrug in whole blood or serum, to ensure that it is stable en route to the tumour. These results confirmed the importance of the uptake-blocking group for ensuring delivery of the payload to the less accessible regions of solid tumour.

Co-incubation of DLD-1 spheroids with marimastat inhibited all MMP activity, and treatment with K(FITC)DIPVSLRSK(RhB) (4.5) saw colocalised fluorescence in the spheroid periphery, a result of the intact peptide being taken up by cells. Again, this result supported the need for an
additional moiety to inhibit cell uptake, in order to allow diffusion of the payload into tumour regions distal from the blood supply.

Finally, this chapter also investigated the use of the MMPSense 680 reagent for the simultaneous visualisation of MMP activity and compound activation. The results showed that MMP activity is not uniform throughout spheroids, instead there are hotspots of activity which correlate with cleavage of the intact peptides.

Overall, this work confirmed the potential of using an MMP-2 specific cleavage sequence for the selective delivery of chemotherapeutic agents to solid tumours. Both K(FITC)DIPVSLRSK(Rhb) (4.5) and DDDDK(FITC)DIPVSLRSK(Rhb) (4.8) underwent cleavage in both 2D and 3D cell systems in regions that demonstrated increased MMP activity. The presence of the tetra-asparate uptake-blocking group contributed to improved diffusion of the LRSK(Rhb) (4.7) fragment towards the core of the spheroid, symptomatic of its lower rate of cleavage by MMP-2 as confirmed by LC-MS.
Chapter 5

Metal Complex-Peptide Conjugates
5.1 Background and strategy

This work builds upon the results for the fluorophore-peptide conjugates described in Chapter 4. The MMP-2 specific sequence elucidated by Turk et al.\textsuperscript{138} was employed as a means of selectively activating cytotoxic Pt(IV) and Ru(II) complexes following cleavage by MMP-2. Again, this was based on the knowledge that a variety of tumours show an increase in MMP-2 expression due to the increased rate of cellular growth and ECM remodelling during the metastatic process\textsuperscript{130}, thereby potentially allowing for selective activation in solid tumour regions.

![Diagram showing enzymatic cleavage of potential prodrug containing an MMP-2 specific peptide cleavage motif. Top: the intact peptide contains a cytotoxic metal complex, plus an uptake blocking group to prevent influx into cells. Bottom: after cleavage by MMP-2, the primed side (RHS) is free to enter cells, while the unprimed side retains the uptake blocking group, preventing cell penetration.]

The results described in section 4.5.2 showed, by attaching the highly cell-penetrating rhodamine B fluorophore, that the tetra-aspartate blocking group was effective at preventing cell uptake, even in the absence of the uptake-blocking FITC. As it was expected that the presence of a bulky, lipophilic fluorophore such as FITC would have a significant effect on the properties of the peptide-fluorophore conjugate, it was omitted from these compounds. In particular, previous work has shown that the K(FITC)DIPVS fragment analogue K(FITC)IPVS has an IC\textsubscript{50} of 18.6 ± 1.8 µM in DLD-1 cells\textsuperscript{228}, meaning that any cytotoxicity measurements of peptides which included the K(FITC)DIPVS fragment as well as a metal complex would not be a direct measurement of the effect of conjugation on the metal complex’s cytotoxicity.
In adding an enzymatic-cleavage sequence for selective activation to the axial site of a platinum(IV) complex, the greatest design concern is to ensure that the peptide sequence is not lost before the intact metal-peptide conjugate has reached the reduction site. This could occur via one of two methods. Firstly, and of greatest likelihood, is by reduction to a platinum(II) species by extracellular reducing agents such as glutathione and ascorbate and secondly, the intact sequence could undergo cleavage by non-specific serum proteases. In order to overcome the first of these problems, the platinum(IV) complex was chosen based on resistance to biological reductants. Table 5.1 shows the correlation between reduction potential and the rate of reduction by ascorbate for Pt(IV) derivatives of cisplatin and oxaliplatin.

Table 5.1 Relationship between chemical structure, reduction potential and rate of reduction by ascorbate (1 – 3: slowest – fastest) for Pt(IV) complexes. §: structure shown in Figure 5.2.

<table>
<thead>
<tr>
<th>Equatorial coordination sphere</th>
<th>Axial ligands</th>
<th>$E_p$ (V) (vs Ag/AgCl)</th>
<th>Relative rate of reduction (by ascorbate)</th>
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<tbody>
<tr>
<td>[N$_2$Cl$_2$]</td>
<td>(OH)$_2$</td>
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</tr>
<tr>
<td>[N$_2$Cl$_2$]</td>
<td>(OCOCH$_3$)$_2$</td>
<td>-0.64</td>
<td>2</td>
</tr>
<tr>
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<td>Cl$_2$</td>
<td>-0.26</td>
<td>3</td>
</tr>
<tr>
<td>[N$_2$O$_2$]</td>
<td>(OH)$_2$</td>
<td>-0.8</td>
<td>3</td>
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<tr>
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<td>-0.64</td>
<td>2</td>
</tr>
<tr>
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<td>(OCOCH$_3$)$_2$</td>
<td>-0.48</td>
<td>1</td>
</tr>
</tbody>
</table>

As described in Chapter 1, oxaliplatin derivatives exhibit an unusual reduction trend whereby the Pt(IV) complex with two acetato axial ligands is, despite having the least negative reduction potential, reduced most slowly. For this reason, a complex with the same equatorial coordination sphere and similar moieties in the axial positions was chosen. Figure 5.1 shows the complex described above, its azido- derivative and cis, cis, trans-acetato[(1R,2R)-cyclohexane-1,2-diamine-N,N’]succinatoxalatoplatinum(IV) which was ultimately used for successful peptide conjugation through the terminal carboxylic acid moiety on the succinato ligand. This will be discussed further in section 5.4.2.3.
The positive results obtained for the platinum(IV)-peptide conjugates, described below, led to the investigation into a similar strategy for improving the efficacy of a lipophilic ruthenium(II) bipyridyl complex with the goal of generating a prodrug which undergoes selective activation in tumour regions and high levels of accumulation in spheroid tumour models. For this purpose, only the peptide fragment intended for cell uptake was synthesised and investigated in vitro.

In the last decade, ruthenium(II) complexes have emerged as an interesting class of compounds with potential anti-cancer activity, but are still limited by their lack of selectivity for cancer cells. The high level of diversity in the structures of active ruthenium compounds leads to an extensive array of proposed interactions including albumin and transferrin binding in blood plasma, ECM collagens and cell surface actins, regulatory enzymes within the cell membrane and in the case of Ru-intercalator complexes, DNA. The variety of potential non-specific biological interactions made these complexes attractive candidates for conjugation to an MMP-2 selective peptide sequence in an effort to improve their tumour selectivity and cell uptake.

Ru(II) polypyridyl complexes have traditionally been used in a wide range of applications due to their stability, solubility, and the ease of tuning their spectroscopic and redox properties through simple ligand manipulation. Until recently, ruthenium tris(polypyridyl) complexes had not been investigated for their cytotoxic activity. However, a preliminary but comprehensive combinatorial study by Mulcahy and coworkers showed that complexes of the type $[\text{Ru}(t\text{Bu}_2\text{bpy})_2(X-X)^2]^{2+x}$ (where $t\text{Bu}_2\text{bpy} = 4,4'$-di-tert-butyl-2,2'-bipyridine and $X-X =$ bidentate ligand) have $IC_{50}$ values in the range of 2-50 µM in HeLa cells. In particular, the compounds...
Chapter 5: Metal Complex-Peptide Conjugates

[Ru(tBu₂bpy)₃] and [Ru(tBu₂bpy)₂(bpy)] (where bpy = 2,2'-bipyridine) had IC₅₀ values of 20 µM and 50 µM respectively, making them attractive candidates for further investigation.

![Figure 5.3](image_url)

**Figure 5.3** Complexes of the type [Ru(tBu₂bpy)₂(X-X)]^{2+} where (from l-r) X = tBu₂bpy, bpy and DEA-bpy.

Zava *et al.* also evaluated the behaviour of tris(bipyridyl) complexes in monolayer cell cultures, and found a correlation between the cytotoxicity and lipophilicity of the bipyridyl ligand substituents. The inherent luminescence of these compounds, responsible for their widespread use as dyes, also allowed for cellular localisation studies by confocal fluorescence microscopy, where a strong membrane-associated fluorescence pattern was observed for the compound [Ru(DEA-bpy)₃]^{2+} (where DEA-bpy = 4,4'-diethylamino-2,2-bipyridine) when exposed to 10 µM of the compound. The outcomes of these studies led to the rational design of a tris(bipyridyl)ruthenium(II) complex to act as the cytotoxic moiety tethered to the RHS fragment of the MMP-2 selective sequence. For conjugation to the peptide through a lysine residue, a bipyridine ligand containing a carboxylic acid moiety was incorporated as the third bidentate ligand, giving the compound [Ru(tBu₂bpy)₂(HOOC-4′-CH₃bpy)]^{2+}. Figure 5.4 shows a comparison of the logP values for the target compound ligands with DEA-bpy, used in the study by Zava *et al.*

![Figure 5.4](image_url)

**Figure 5.4** Ligands for Ru(II) complexes in this study and their logP values. Calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02.
By increasing the lipophilicity of the cytotoxin tethered to the peptide, it was hoped that an increase in cell uptake and spheroid accumulation would be observed compared to the more hydrophilic Pt(IV)-peptide conjugates. Concomitantly, the addition of a short peptide to the Ru(II) complex was intended to overcome the strong membrane-association observed for complexes of this type, and improve transport across the membrane and into the cytoplasm.

5.2 Methods

5.2.1 LC-MS

Liquid chromatography-mass spectrometry (LC-MS) was performed on a Shimadzu LC-MS 2020 system using a LC-20AD Pump and a SPD-20A detector. Separation was achieved using a Waters SunFire™ series C18 column (150 x 2.1 mm, 5 µm particle size), at a flow rate of 0.2 mL/min over a linear gradient from 0% to 100% solvent B over 30 minutes (solvent A: 100:0.1 v/v Milli-Q water/formic acid, solvent B: 100:0.1 v/v acetonitrile/formic acid).

5.2.2 Preparative HPLC

Preparative HPLC was performed on a Waters 600 HPLC system fitted with a Waters 2998 Photodiode Array Detector. Separation was achieved using a Waters Sunfire™ C18 (5 µm, 19 x 150 mm) preparative column at a flow rate of 7 mL/min. A linear gradient of 5 to 60% solvent B minutes (solvent A: 100:0.1 v/v Milli-Q water/TFA, solvent B: 100:0.1 v/v acetonitrile/TFA) over 70 mins was used. Signals were detected at 210 and 254 nm. Relevant fractions were collected manually.

5.2.3 Fluorescence Spectra

Spectra of Ru(II)-peptide conjugates were collected at room temperature using a Varian Cary Eclipse fluorescence spectrophotometer, with a 1 cm x 1 cm quartz cuvette (Starna). Scans were run as 70 µM solutions in H₂O adjusted to pH 7.4, at 120 nm/min with excitation and emission slit widths of 5 nm. All compounds were prepared as 1 mM stock solutions and stored at -20 °C, and diluted immediately prior to analysis.

5.2.4 Cell lines

DLD-1 human colon carcinoma cells and A549 human lung carcinoma cells were obtained from ATCC and used within 6 months of resuscitation. Cells were maintained as monolayers in
complete media: Advanced DMEM (Invitrogen) supplemented with 2% foetal calf serum (FCS), 1% L-glutamine and 1% antibiotic antimycotic (AA). Cells were incubated under standard culturing conditions (37 °C with 5% (v/v) CO\textsubscript{2} under humidified conditions).

5.2.5 Accumulation studies

The following procedure was carried out for A549 and DLD-1 cell lines. Approximately 6 x 10\textsuperscript{6} cells were seeded into 7 cm culture dishes and allowed to adhere overnight. The cells were incubated with the compounds (30 µM in 6 mL) for 24 and 48 hrs, and harvested by incubation with trypsin. The cells were pelleted by centrifugation, then washed three times with PBS and finally resuspended in PBS (500 µL). Aliquots were taken from the cell sample for platinum analysis (100 µL) and protein analysis (100 µL). The samples were stored at -20 °C until immediately prior to analysis. All experiments were repeated in triplicate.

Sample preparation for ICP-MS involved digestion of the cell samples (100 µL) with HNO\textsubscript{3} (67-70%, 500 µL) in an ultrasonic bath for 1 hr. The samples were mineralised at 80 °C under a stream of N\textsubscript{2} and then redissolved in 1500 µL of 2% HNO\textsubscript{3} (w/v) spiked with 50 µg/L \textsuperscript{193}Iridium. After treatment in an ultrasonic bath for 1 hr, samples were analysed for total platinum and concentration values were corrected with respect to the iridium signal. The platinum concentrations were normalised against the cellular protein concentrations.

The parameters for ICP-MS analysis are given in Table 5.2.

\textbf{Table 5.2} Parameters for ICP-MS analysis.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RF Power</td>
<td>1550 W</td>
</tr>
<tr>
<td>Cooling gas (Ar) flow rate</td>
<td>15 L/min</td>
</tr>
<tr>
<td>Carrier gas (Ar) flow rate</td>
<td>0.65 L/min</td>
</tr>
<tr>
<td>Make-up gas (Ar) flow rate</td>
<td>0.5 L/min</td>
</tr>
<tr>
<td>Sample depth</td>
<td>7.5 mm</td>
</tr>
<tr>
<td>Dwell time</td>
<td>0.3 secs; 6 replicates</td>
</tr>
<tr>
<td>Isotopes monitored</td>
<td>\textsuperscript{103}Rh, \textsuperscript{193}Ir, \textsuperscript{195}Pt</td>
</tr>
</tbody>
</table>
5.2.5.1 Protein Assay

Protein concentrations were determined using the Biorad protein assay, which is based on the absorption shift from 465 to 595 nm that occurs upon binding of protein to the Coomassie Brilliant Blue G-250 reagent. The stock Biorad solution was diluted 1 in 5 with MilliQ water before use. Standard protein solutions (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, 1 mg/mL) were prepared from bovine serum albumin (BSA) in PBS and used immediately. Cell samples were vortexed immediately prior to use.

The protein assay was carried out by placing the diluted Biorad reagent (200 µL) into the wells of a 96 well plate. To this, 10 µL aliquots of the standard protein solutions and cell sample were added in triplicate and shaken for 1 minute before being incubated at 37 °C for 30 mins. The absorbance of each well was measured at 600 nm. The protein concentrations in each sample were determined using the standard curve constructed from the results of the protein standard solutions.

5.2.6 Cytotoxicity assays

The MTT assay was employed to determine the cytotoxicity values (reported as IC$_{50}$ values) of compounds in this chapter. Described by Carmichael and co-workers$^{252}$, the colourimetric assay involves the reduction of tetrazolium-based (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to the purple formazan by mitochondrial dehydrogenase, found in viable cells.

Approximately 1 x 10$^4$ cells in 100 µL of media (without antibiotics) were seeded into the wells of a 96 well plate and left to adhere overnight. Stock compound solutions (made to 1 mM concentrations) were diluted with media (without antibiotics) to give 9 concentrations across the range of 0-200 µM. The media was removed from the wells, and each drug concentration (100 µL volume) was added in triplicate to the wells. The cells were left to incubate for 72 hrs.

MTT (20 µL, stock solution made to 2.5 mg/mL in H$_2$O) was then added to each well, and the cells incubated for 3 hrs (37 °C with 5% CO$_2$) before the medium was removed and replaced with DMSO (150µL). The plates were shaken to dissolve the purple crystals and the absorbance recorded immediately at 600 nm by a microplate reader (Victor$^3$V, PerkinElmer). The IC$_{50}$ values were determined as the drug concentration which reduced the absorbance to 50% of that in untreated, control wells.
At least three independent experiments were performed for each compound with triplicate readings on each experiment.

5.2.7 Confocal microscopy

5.2.7.1 Monolayer cell culture

Approximately $2.5 \times 10^5$ cells were plated onto 35 mm glass-bottom culture dishes (MatTek) and allowed to adhere overnight. Depending on the experiment, cells were either plated in complete media, or serum-free medium. Cells were treated with the Ru(II) compounds for 4 or 24 hrs under standard incubation conditions. The cells were subsequently imaged with either the compound still present in the media, or with the medium replaced with fresh medium of an equal volume.

Images were collected on an Olympus FV1000 inverted microscope using an Olympus UPLSAPO 60x/NA:1.2 water objective lens in a humidified live-cell chamber maintained at 37 °C (Solent Scientific). A scan rate of 4.0 µs/pixel and fluorescence was collected following excitation with a 405 nm argon laser and emission collection between 600-680 nm.

5.2.7.2 Spheroids

Spheroids were formed by coating the wells of a 96-well plate with agarose (sterile, 0.75% w/v in PBF) and plating with 100 µL of cell suspension ($1.5 \times 10^5$ and $2.5 \times 10^5$ cells/mL for medium and large spheroids respectively) in complete media. The cells were left to aggregate for 72 hrs without motion, after which a single spheroid had formed per well. 12 hrs prior to treating with the compound, the media was replaced with serum-free media. The spheroids were treated with 20 uM of compound for 4 or 24 hrs, and then transposed into a 35 mm glass-bottom culture dish (MatTek) containing 2 mL of serum-free medium.

Images were collected using the same instrumental parameters as in section 5.2.6.2, but using an Olympus UPLAPO 10x/NA:0.40 objective lens.

5.2.8 Preparation of spheroid sections

Medium spheroids were grown following the method described in section 5.2.7.2. Without changing the media, 24 spheroids were dosed with the respective platinum-containing compounds (30 µM to 100 µL final volume) and incubated (37 °C with 5% CO₂) for 2, 6, 12 and 24 hr intervals.
When required, a minimum of 20 spheroids for each time point and compound were collected and transferred to a 1.5 mL centrifuge tube and washed with PBF (3 x 1 mL). The spheroids were transferred to a plastic mould and embedded in Shandon M-1 Embedding Matrix, then cryo-fixed by immersion in liquid nitrogen. The cryo-fixed, embedded spheroids were then stored at -80 °C until required for sectioning.

For SRIXE and XANES analysis, spheroid sections (20 µm thickness) were sliced at -12 °C onto formvar films, kept frozen on dry ice and freeze-dried as soon as possible at -50 °C for >12 hrs. After freeze-drying, the samples were stored in a closed container, under dessicant at room temperature until analysis.

5.2.9 SRIXE mapping

Spheroid sections were selected for analysis based on their diameter and the presence of an observable necrotic core. The sections with the greatest diameters were typically chosen for analysis as it was more likely that they contained a necrotic region.

Micro-SRIXE was performed at the Australia Synchrotron. The X-ray fluorescence spectra were collected using a monochromatic X-ray excitation beam (11.65, 13.9 or 16 keV), focussed to a spot size of 1 x 2 µm². A dwell time of 0.5-2 msec was used depending on the sample size and predicted concentration of the element of interest present. Mapped areas were typically 700 x 700 µm in size and the platinum concentrations were calibrated to a platinum foil standard. Spectra from SRIXE maps were fitted using GeoPIXE version 6.4.

5.2.10 XANES measurements

Following SRIXE mapping of the spheroid sections, smaller regions of interested (ROI) were chosen for XANES spectroscopic analysis. The fluorescence maps for the platinum L3 absorbance edge (energy range 11.674 to 11.504 keV) were obtained for the chosen ROIs, and the spectra obtained were batch processed using GeoPIXE version 6.4. A customised IDL program, written by Dr Martin De Jonge (Australian Synchrotron), was employed to stack the fluorescence maps according to the excitation energies to construct the XANES maps. The same IDL program allowed for the averaged XANES spectrum to be extracted from different regions of the XANES map.

Solid samples containing mixtures of \( \text{trans-}[\text{Pt(OAc)}_2(\text{ox})(R,R\text{-chxn})] \) and oxaliplatin, in known ratios, were prepared by grinding the two samples together to achieve a fine, uniform mixture, then forming a slurry with a H₂O:EtOH (1:1) solution. A drop of solution was placed onto a
piece of Kapton film. Once air-dried, a separate piece of Kapton film was used to cover the dried solution. A small homogeneous region was used for XANES analysis.

5.3 Synthesis

5.3.1 Platinum complexes

5.3.1.1 cis, cis-dichlorido[(1R,2R)-cyclohexane-1,2-diamine-N,N']-platinum(II), cis-[PtCl$_2$(chxn)]

To resolve the pure (1R,2R)-cyclohexane-1,2-diamine, (R,R)-cyclohexane-1,2-diamine tartrate (6.417 g, 24.28 mmol) was dissolved in H$_2$O (20 mL) and the pH adjusted to 14 with KOH (10 M). Liquid-liquid extraction was then performed with aliquots of DCM (5 x 50 mL). The organic fraction was retained and the DCM removed under vacuum at room temperature to a volume of ~10 mL. Diethyl ether (20 mL) was added along with Na$_2$SO$_4$ to remove residual H$_2$O. The Na$_2$SO$_4$ was removed by filtration under gravity and the filtrate dried under N$_2$ until the pure (R,R)-cyclohexane-1,2-diamine crystallised. It was then used immediately in the next step. Yield = 1.954 g (70%).

(R,R)-cyclohexane-1,2-diamine (1.954 g, 17.12 mmol) was dissolved in H$_2$O (~100 mL). K$_2$[PtCl$_4$] (7.0056 g, 16.88 mmol) was then added and the mixture stirred overnight at room temperature. The resulting bright yellow solid was collected under vacuum and washed with a small amount of H$_2$O and ethanol. Yield = 6.313 g (98%). IR (KBr): 3275 s, 3185 s, 3102 m (ν NH$_2$); 2933 s, 2865 m (ν CH); 1567 s (δ NH$_2$); 755 s (ρ NH$_2$). $^1$H NMR (DMSO-d$_6$): δ 5.525 (d, 2H, NH$_2$); δ 5.012 (m, 2H, NH$_2$); δ 2.090 (m, 2H, H$_1$, H$_2$); δ 1.818 (d, 2H, H$_3'$, H$_6'$); δ 1.415 (d, 2H, H$_4'$, H$_5'$); δ 1.193 (m, 2H, H$_3$, H$_6$); δ 0.950 (m, 2H, H$_4$, H$_5$). $^{13}$C NMR (DMSO-d$_6$): δ 61.479 (cyclohexyl C$_1$, C$_2$); δ 31.645 (cyclohexyl C$_3$, C$_6$); δ 23.918 (cyclohexyl C$_4$, C$_5$). $^{195}$Pt NMR (DMSO-d$_6$): δ -2280.
5.3.1.2 cis, cis-[(1R,2R)-cyclohexane-1,2-diamine-N,N’]oxalatoplatinum(II), [Pt(ox)(chxn)], oxaliplatin, (5.1)

\[ \text{[PtCl}_2\text{(chxn)] (6.313 g, 16.60 mmol) was suspended in H}_2\text{O (~ 75 mL). To this, AgNO}_3 \text{(5.499 g, 32.38 mmol) was added in the dark with stirring. The reaction was left to stir for 2 days in the dark, occasionally washing the sides of the vessel down with a small amount of H}_2\text{O. The solid AgCl was removed by filtration, and to the filtrate oxalic acid dehydrate (4.297 g, 32.38 mmol) was added and the pH adjusted to 5 with KOH (10 M). The solution was stirred overnight at room temperature. The solid was collected by vacuum filtration and washed with a small amount of cold H}_2\text{O, ethanol and diethyl ether. Yield = 4.242 g (63%). IR (KBr): 3211m, 3161m, 3092br (ν NH}_2 \text{); 2930m (ν C-H); 1703s, 1663s, 1612s (ν C=O); 1382s (ν C-O). 1H NMR (D}_2\text{O): δ 2.42-2.32 (br, m, 2H, H}_1\text{, H}_2 \text{); δ 2.09-2.05 (d, 2H, H}_3'\text{, H}_6' \text{); δ 1.64-1.53 (br m, 2H, H}_4'\text{, H}_5' \text{); δ 1.37-1.26 (br m, 2H, H}_3\text{, H}_6 \text{); δ 1.23-1.13 (br m, 2H, H}_4\text{, H}_5 \text{). 13C NMR (D}_2\text{O): δ 168.73 (C}_7\text{, C}_8 \text{); δ 62.56 (cyclohexyl C}_1\text{, C}_2 \text{); δ 32.02 (cyclohexyl C}_3\text{, C}_6 \text{); δ 24.29 (cyclohexyl C}_4\text{, C}_5 \text{. 195Pt NMR (D}_2\text{O): δ -1999.}\]

5.3.1.3 cis, cis, trans-[(1R,2R-cyclohexane-1,2-diamine-N,N’]dihydroxidooxalato-platinum(IV), [Pt(OH)_2(ox)(chxn)]

\[ \text{[Pt(ox)(chxn)] (100 mg, 0.25 mmol) was suspended in H}_2\text{O (5 mL). 30% H}_2\text{O}_2 (573 μL, 25.19 mmol) was added with stirring. After 2 hrs, the sides of the vessel were washed down with a further 30% H}_2\text{O}_2 (500 μL) and the reaction was stirred overnight. The white solid was isolated by vacuum filtration and washed with a small amount of cold H}_2\text{O, ethanol and diethyl ether. Yield = 0.057 g (52%). IR (KBr): 3523m (ν OH); 3167br, 3072br (ν NH}_2 \text{); 1703s, 1664s (ν C=O); 1380s, 1338br (ν C-O). 1H NMR (D}_2\text{O): δ 2.91-2.81 (br m, 2H, H}_1\text{, H}_2 \text{); δ 1.68-1.55 (br m, 2H, H}_4'\text{, H}_5' \text{); δ 1.36-1.20 (br m, 2H, H}_3\text{, H}_6 \text{). 13C NMR (D}_2\text{O): δ 172.87 (C}_7\text{, C}_8 \text{); δ 61.91 (cyclohexyl C}_1\text{, C}_2 \text{); δ 31.32 (cyclohexyl C}_3\text{, C}_6 \text{); δ 23.85 (cyclohexyl C}_4\text{, C}_5 \text{. 195Pt NMR (D}_2\text{O): δ 1312.}\]
5.3.1.4 cis, cis, trans-[(1R,2R)-cyclohexane-1,2-diamine-N,N’]hydrido-
methoxido-oxalatoplatinum(IV), \([\text{Pt}(\text{OCH}_3)(\text{OH})(\text{ox})(\text{chxn})] \)

\([\text{Pt}(\text{ox})(\text{chxn})] \) (3.007 g, 7.573 mmol) was suspended in dry methanol (50 mL). To this, 30% \( \text{H}_2\text{O}_2 \) (859 \( \mu \text{L}, 7.573 \text{ mmol} \)) was added and stirred for 7 days at room temperature. The solid was isolated by filtration under vacuum, then recrystallised in a minimum volume of hot \( \text{H}_2\text{O} \). The sample was further purified by preparative HPLC to afford pale yellow crystals. Yield = 1.248 g (37%). IR (KBr): 3549m (\( \nu \text{ OH} \)); 3185m (\( \nu \text{ NH}_2 \)); 2949m, 2868m, 2806m (\( \nu \text{ CH} \)); 1761s, 1605s (\( \nu \text{ C}=\text{O} \)); 1372s (\( \nu \text{ C-O} \)).

\(^1\text{H} \text{NMR (D}_2\text{O): } \delta 2.93-2.87 \text{ (t, 3H, } \text{H}_9); \delta 2.83 \text{ (br m, } 2\text{H, } \text{H}_1, \text{H}_2); \delta 2.32-2.28 \text{ (d, 2H, } \text{H}_3', \text{H}_6'); \delta 1.67-1.54 \text{ (br m, 4H, } \text{H}_4', \text{H}_5', \text{H}_3, \text{H}_6); \delta 1.35-1.17 \text{ (br m, 2H, } \text{H}_4, \text{H}_5). \(^{13}\text{C NMR(D}_2\text{O): } \delta 166.32 \text{ (C}_7, \text{C}_8); \delta 62.91, 62.72 \text{ (C}_1, \text{C}_2); \delta 56.14 \text{ (C}_9); \delta 31.26 \text{ (C}_3, \text{C}_6); \delta 23.85 \text{ (C}_4, \text{C}_5). \(^{195}\text{Pt NMR (D}_2\text{O): } \delta 1276. \text{ MS-ESI (+)}: 467.60 \text{ [M+Na}^+]. \) Elemental analysis: \( \text{C}_{9}\text{H}_{18}\text{N}_{2}\text{O}_{6}\text{Pt.2H}_{2}\text{O} \) calculated C 22.46, H 4.61, N 5.82; found C 22.45, H 4.59, N 5.72. Crystal structure: see Appendix A2.1.

5.3.1.5 cis, cis, trans-[(1R,2R)-cyclohexane-1,2-diamine-N,N’]methyldio-
oxalatosuccinato-platinum(IV), \([\text{Pt(OH)}(\text{OSuc})(\text{ox})(\text{chxn})] \)

\([\text{Pt(OCH}_3)(\text{OH})(\text{ox})(\text{chxn})] \) (75 mg, 0.1684 mmol) was suspended in DMF (7.5 mL). To this, succinic anhydride (16.85 mg, 0.1684 mmol) was added and the reaction stirred at room temperature. After 3 hrs, another equivalent of succinic anhydride was added and the reaction stirred overnight. The next day, another equivalent of succinic anhydride was added and stirred for 6 hrs, until the solution had turned clear. The DMF was removed by vacuum distillation at 70 °C until a pale brown residue remained. To this, acetone (10 mL) was added to precipitate a pale brown solid. This was stirred overnight and then filtered under gravity and washed with acetone. Yield = 46 mg.
(50%). IR (KBr): 3155br, 3067br (v NH); 2947m, 2867w (ν CH); 1720s (ν C=O); 1364s, 1314w (ν C-O). 1H NMR (D2O): δ 2.90-2.77 (m, 3H, H9); δ 2.64-2.52 (m, 6H, H1, H2, H11, H11', H12, H12'); δ 2.39-2.22 (d, 2H, H3', H6'); δ 1.64-1.52 (m, 4H, H4', H5', H3, H6); δ 1.32-1.15 (m, 2H, C4, C5).

13C NMR (D2O): δ 182.80 (C10); δ 178.07 (C13); δ 166.38 (C7, C8); δ 62.29, 61.35 (C1, C2); δ 57.61 (C9); δ 31.31, 31.16 (C3, C6); δ 31.92, 30.48 (C11, C12); δ 23.84 (C4, C5). 195Pt NMR (D2O): δ 1358. MS-ESI (+): 567.80 [M+Na]+, MS-ESI (-): 543.00 [M-H]−.

5.3.1.6 cis, cis, trans-acetato[(1R,2R)-cyclohexane-1,2-diamine-N,N’]hydroxido-oxalatoplatinum(IV), [Pt(OAc)(OH)(ox)(chxn)]

The following procedure was based on one established by Zhang et al88. [Pt(ox)(chxn)] (2.00 mg, 5.03 mmol) was dissolved in acetic acid (300 mL). To this, 30% H2O2 (2860 µL, 25.2 mmol) was added and stirred overnight. The remaining H2O2 was deactivated by heating at 60°C, and the acetic acid evaporated to dryness under a stream of N2. Yield = quantitative. 1H NMR (D2O): δ 2.88-2.86 (br s, 2H, H1, H2); δ 2.30-2.26 (d, 2H, H3', H6'); δ 2.06 (s, 3H, H10); δ 1.65-1.59 (br m, 4H, H4', H5', H3, H6); δ 1.28-1.25 (br m, 2H, H4, H5). 13C NMR (D2O): δ 182.02 (C10); δ 166.15 (C7, C8); δ 61.60, 61.30 (C1, C2); δ 30.90, 30.74 (C3, C6); δ 23.43 (C4, C5); δ 22.67 (C10). 195Pt NMR (D2O): δ 1422.

5.3.1.7 cis, cis, trans-acetato[(1R,2R)-cyclohexane-1,2-diamine-N,N’]succinato-oxalatoplatinum(IV), [Pt(OAc)(OSuc)(ox)(chxn)], (5.2)

[Pt(OAc)(OH)(ox)(chxn)] (2.00 g, 4.23 mmol) was suspended in DMF (150 mL), to which 4 equivalents of succinic anhydride (1.692 g, 16.92 mmol) and triethylamine (2360 µL, 16.92 mmol) were added. The suspension was left to stir for 3
hrs, after which another 4 equivalents of anhydride were added. After being left to stir for 12 hrs, the DMF was removed under a stream of N₂ and the pale brown residue dissolved in MeOH for purification by HPLC. Yield = 1.440 g (59%). IR (KBr): 3075br, 3022br (ν NH); 2947m, 2873w (ν CH); 1721s (ν C=O); 1365s, 1291w (ν C-O).

1H NMR (D₂O): δ 2.82-2.78 (m, 2H, H₁, H₂); δ 2.60-2.51 (m, 4H, H₁₂, H₁₂', H₁₃, H₁₃'); δ 2.23-2.19 (d, 2H, H₆', H₆); δ 1.98 (s, 3H, H₁₀); δ 1.57-1.51 (m, 4H, H₄', H₅', H₃, H₆); δ 1.20-1.13 (m, 2H, C₄, C₅).

13C NMR (D₂O): δ 181.87 (C₉); δ 177.03 (C₁₁, C₁₄); δ 166.32 (C₇, C₈); δ 61.61, 61.30 (C₁, C₂); δ 30.81, 30.65 (C₃, C₆); δ 29.56, 28.91 (C₁₂, C₁₃); δ 23.40 (C₄, C₅); δ 22.15 (C₁₀).


5.3.1.8 cis, cis, trans-bis(acetato)[(1R,2R)-cyclohexane-1,2-diamine-N,N’] oxalato-platinum(IV), [Pt(OAc)₂(ox)(chxn)], (5.3)

[Pt(OAc)(OH)(ox)(chxn)] (100 mg, 0.211 mmol) was dissolved in acetonitrile (50 mL). To this, acetic anhydride (48 µL, 0.422 mmol) was added and stirred at reflux for 16 hrs. The acetonitrile and acetic acid by-product was evaporated to dryness under a stream of N₂ to yield a white solid. Yield = quantitative. 1H NMR (D₂O): δ 2.82-2.73 (br m, 2H, 2 x CH); δ 2.21-2.17 (d, 2H, CH₃); δ 1.97 (s, 6H, 2 x CH₃); δ 1.56-1.47 (m, 4H, 2 x CH₂); δ 1.18-1.15 (t, 2H, 2 x CH₂). 13C NMR (D₂O): δ 181.28 (C₉); δ 166.66 (C₇, C₈); δ 61.48 (C₁, C₂); δ 30.76 (C₃, C₆); δ 23.43 (C₄, C₅); δ 22.11 (C₁₀).

195Pt NMR (D₂O): δ 1454. Elemental analysis (C₁₄H₂₀N₂O₈Pt.1.5H₂O) calculated C: 26.57, H: 4.27, N: 5.16; found C: 26.26, H: 4.00, N: 5.21.
5.3.2 Ruthenium complexes

5.3.2.1 bis(4,4'-di-tert-butyl-2,2'-bipyridine)dichlororuthenium(II), [RuCl₂(tBu₂bpy)]

[RuCl₂(3H₂O) (250 mg, 0.955 mmol) and 4,4'-di-tert-butyl-2,2'-bipyridine (510 mg, 1.90 mmol) were suspended in dry DMF (5 mL) into which LiCl (270 mg, 6.35 mmol) was dissolved. The reaction mixture was refluxed for 6 hrs under N₂. The solvent was then removed under vacuum to yield a dark purple/black solid. The solid was then redissolved in DCM and purified on a neutral alumina column eluting with DCM:EtOAc (60:40) to give a dark purple, crystalline solid. Yield: 452 mg (67%). ¹H NMR (MeOD): δ 9.852-9.832 (d, 2H, bipyridyl); δ 8.630-8.624 (d, 2H, bipyridyl); δ 8.474-8.468 (d, 2H, bipyridyl); δ 7.784-7.757 (dd, 2H, bipyridyl); δ 7.395-7.375 (d, 2H, bipyridyl); δ 7.151-7.1245 (dd, 2H, bipyridyl); δ 1.519 (s, 18H, CH₃); δ 1.290 (s, 18H, CH₃). Elemental analysis (C₃₆H₄₈Cl₂N₄Ru.CH₃OH) calculated C: 59.99, H: 7.08, N: 7.56; found C: 60.01, H: 6.80, N: 7.56.

5.3.2.2 [(4-methyl-4'-carboxy-2,2'-bipyridine)bis(4,4'-di-tert-butyl-2,2'-bipyridine)]ruthenium(II) hexafluorophosphate, [Ru(tBu₂bpy)(HOOC-4'CH₃)₂PF₆] (5.6)

[Ru(t-Bu₂bpy)₂Cl₂] (250 mg, 0.35 mmol) and 4-methyl-4'-carboxy-2,2'-bipyridine (76 mg, 0.35 mmol) were dissolved in a mixture of MeOH:H₂O (140 mL, 21:7). The reaction was stirred at reflux in the absence of light for 18 hrs. Following this, MeOH was removed under vacuum and the remaining aqueous solution filtered under gravity. Aqueous NH₄PF₆ was then added dropwise to the filtrate, resulting in the precipitation of a red-brown crystalline solid which was then washed with cold EtOH. Yield = 164 mg (47%). ¹H NMR (MeOD): δ 9.03 (s, 1H, bipyridyl); δ
8.67-8.63 (d, 5H, bipyridyl); δ 7.97-7.89 (dd, 2H, bipyridyl); δ 7.65-7.59 (m, 5H, bipyridyl); δ 7.54-7.49 (t, 4H, bipyridyl); δ 7.36-7.35 (dd, 2H, bipyridyl); δ 2.59 (s, 3H, CH₃); δ 1.43 (s, 36H, CH₃). HR-MS: see Appendix A4.1.

### 5.3.3 General method for peptide synthesis

The general method for all amino acid peptide coupling is as described in section 4.3.2.

For conjugation of Pt(IV) and Ru(II) complexes, the mtt protecting group was removed from the C-terminal Lys residue by the method described in section 4.3.2. The resin was then swollen in DMF for 30 mins. The respective metal complex, HBTU, and HOBt (all 4 equivalents) were dissolved in DMF and DIPEA (6 equivalents) and shaken for 6 hrs.

Cleavage of the metal-peptide bioconjugates from the resin was performed under the same conditions described for resin cleavage in section 4.3.2, but were left to shake for only 30 mins. This was in order to minimise the length of exposure of the metal complexes to 95% TFA, which is capable of degrading the complexes after prolonged periods.

The solution containing the cleaved peptides was expelled through the fritted syringe and precipitated by the addition of ice-cold diethyl ether. The precipitate was collected by centrifugation, drying and lyophilisation. Purification was achieved by preparative HPLC (Linear gradient: 0 to 100% MeCN in 50 mins, eluents: H₂O and MeCN, both containing 0.1% TFA).

### 5.3.3.1 Attempted syntheses of platinum(IV)-peptide conjugates

For brevity, the attempted syntheses of platinum(IV) peptide conjugates via “click chemistry” is summarised in Table 5.3. Here, “click chemistry” refers to two reactions which differ in their catalysts and products, but in theory still meet “click chemistry” conditions, ie fast, mild conditions and high yielding with few side-products.

The reaction conditions including catalyst, solvent, method and result are shown, the details of which will be discussed further in section 5.4.1.
Table 5.3 Summary of small-scale click reactions. Pt(IV) complex 5.4: [Pt(OAz)(OCH₃)(ox)(chxn)], Pt(IV) Complex 5.5: [Pt(OAz)(OAc)(ox)(chxn)] (structures shown in Table 5.5). Method 1: Pt(IV) complex and amino acid/peptide were dissolved in 1.5 mL of solvent. 0.1 mol eq of catalyst (unless specified otherwise) was added and the reaction stirred for 1 hr. Method 2: Pt(IV) complex and amino acid/peptide were dissolved in 1.5 mL of solvent. In a separate vial, equimolar amounts of Cu(I)-I and TBTA were allowed to stir for 0.5 – 1 hr (or until solution turned orange). 0.1 mol eq of the Cu(I)-TBTA complex was then added to the reaction solution and the reaction stirred for 1 hr.

<table>
<thead>
<tr>
<th>Pt(IV) complex</th>
<th>Amino acid/peptide</th>
<th>Catalyst</th>
<th>Solvent</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.4</td>
<td>fmoc-Pra-OH</td>
<td>Cu(I)I</td>
<td>MeOH</td>
<td>1</td>
</tr>
<tr>
<td>5.4</td>
<td>LRSpg</td>
<td>Cu(I)I</td>
<td>MeOH</td>
<td>1</td>
</tr>
<tr>
<td>5.4</td>
<td>fmoc-Pra-OH</td>
<td>Cu(I)I</td>
<td>DMF</td>
<td>1</td>
</tr>
<tr>
<td>5.4</td>
<td>fmoc-Pra-OH</td>
<td>Cu(I)I</td>
<td>DMF</td>
<td>2</td>
</tr>
<tr>
<td>5.4</td>
<td>LRSpg</td>
<td>Cu(I)I</td>
<td>DMF</td>
<td>1</td>
</tr>
<tr>
<td>5.4</td>
<td>LRSpg</td>
<td>Cu(I)I</td>
<td>DMF</td>
<td>2</td>
</tr>
<tr>
<td>5.4</td>
<td>fmoc-Pra-OH</td>
<td>Cu(I)I</td>
<td>DMSO</td>
<td>1</td>
</tr>
<tr>
<td>5.4</td>
<td>fmoc-Pra-OH</td>
<td>CuSO₄/ascorbate (1 mol eq)</td>
<td>t-BuOH:H₂O</td>
<td>1</td>
</tr>
<tr>
<td>5.4</td>
<td>fmoc-Pra-OH</td>
<td>Cu(I)I</td>
<td>t-BuOH:H₂O</td>
<td>1</td>
</tr>
<tr>
<td>5.4</td>
<td>fmoc-Pra-OH</td>
<td>Cp*RuCl(PPh₃)₂</td>
<td>MeOH</td>
<td>1</td>
</tr>
<tr>
<td>5.4</td>
<td>fmoc-Pra-OH</td>
<td>Cp*RuCl(PPh₃)₂</td>
<td>DMF</td>
<td>1</td>
</tr>
<tr>
<td>5.4</td>
<td>fmoc-Pra-OH</td>
<td>Cp*RuCl(PPh₃)₂</td>
<td>DMSO</td>
<td>1</td>
</tr>
<tr>
<td>5.4</td>
<td>fmoc-Pra-OH</td>
<td>Cp*RuCl(PPh₃)₂</td>
<td>t-BuOH:H₂O</td>
<td>1</td>
</tr>
<tr>
<td>5.5</td>
<td>fmoc-Pra-OH</td>
<td>Cu(I)I</td>
<td>MeOH</td>
<td>1</td>
</tr>
<tr>
<td>5.5</td>
<td>fmoc-Pra-OH</td>
<td>Cu(I)I</td>
<td>DMF</td>
<td>1</td>
</tr>
<tr>
<td>5.5</td>
<td>fmoc-Pra-OH</td>
<td>Cu(I)I</td>
<td>DMF</td>
<td>2</td>
</tr>
<tr>
<td>5.5</td>
<td>LRSpg</td>
<td>Cu(I)I</td>
<td>DMF</td>
<td>1</td>
</tr>
<tr>
<td>5.5</td>
<td>LRSpg</td>
<td>Cu(I)I</td>
<td>DMF</td>
<td>2</td>
</tr>
<tr>
<td>5.5</td>
<td>fmoc-Pra-OH</td>
<td>Cu(I)I</td>
<td>DMSO</td>
<td>1</td>
</tr>
<tr>
<td>5.5</td>
<td>fmoc-Pra-OH</td>
<td>CuSO₄/ascorbate (1 mol eq)</td>
<td>t-BuOH:H₂O</td>
<td>1</td>
</tr>
<tr>
<td>5.5</td>
<td>fmoc-Pra-OH</td>
<td>CuSO₄/ascorbate (10 mol eq)</td>
<td>t-BuOH:H₂O</td>
<td>1</td>
</tr>
<tr>
<td>5.5</td>
<td>fmoc-Pra-OH</td>
<td>Cu(I)I</td>
<td>t-BuOH:H₂O</td>
<td>1</td>
</tr>
<tr>
<td>5.5</td>
<td>fmoc-Pra-OH</td>
<td>Cp*RuCl(PPh₃)₂</td>
<td>DMF</td>
<td>1</td>
</tr>
</tbody>
</table>

5.3.4 Conditions and characterisation of peptides

All peptides were synthesised following a similar protocol, purified by preparative HPLC and their purity established by LC-MS. Retention times using LC-MS (liner gradient: 0% to 100% solvent B over 30 minutes (solvent A: 100:0.1 v/v Milli-Q water/formic acid, solvent B: 100:0.1 v/v acetonitrile/formic acid).)

All peptide-fluorophore conjugates are > 95% purity. For novel metal-peptide compounds, additional HRMS data is presented. All LC-MS traces can be found in Appendix A3.

**Table 5.4** Synthetic parameters and characterisation of peptides.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Resin</th>
<th>Amino acids (C -&gt; N)</th>
<th>MS</th>
<th>HRMS</th>
<th>LC-MS t&lt;sub&gt;r&lt;/sub&gt; (min)</th>
<th>Yield</th>
<th>Molecular mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>LRSpg</td>
<td>fmoc-Pra-Wang resin</td>
<td>fmoc-Ser(tBu)-OH fmoc-Arg(pbf)-OH fmoc-Leu-OH</td>
<td>470.6 ([M+H]&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>n/a</td>
<td>n/a</td>
<td>40 mg (34%)</td>
<td>469.54</td>
</tr>
<tr>
<td>LRSK(Pt)</td>
<td>fmoc-Lys(mtt)-Wang resin</td>
<td>fmoc-Ser(tBu)-OH fmoc-Arg(pbf)-OH fmoc-Leu-OH Platinum(IV) complex</td>
<td>1058.4 ([M]&lt;sup&gt;-&lt;/sup&gt;), 529.9 ([M]&lt;sup&gt;2+&lt;/sup&gt;)</td>
<td>529.70954 (theoretical: 529.70964)</td>
<td>9.06</td>
<td>51 mg (19%)</td>
<td>1058.01</td>
</tr>
<tr>
<td>DIPVSLRSK(Pt)</td>
<td>fmoc-Lys(mtt)-Wang resin</td>
<td>fmoc-Ser(tBu)-OH fmoc-Arg(pbf)-OH fmoc-Leu-OH fmoc-Ser(tBu)-OH fmoc-Val-OH fmoc-Pro-OH fmoc-Ile-OH fmoc-Asp(OtBu)-OH fmoc-Lys(mtt)-OH FITC</td>
<td>1569.8 ([M]&lt;sup&gt;-&lt;/sup&gt;), 785.7 ([M]&lt;sup&gt;2+&lt;/sup&gt;)</td>
<td>785.34156 (theoretical: 785.34174)</td>
<td>12.22</td>
<td>28 mg (24%)</td>
<td>1569.58</td>
</tr>
</tbody>
</table>
| DDDDGDPVSLRSK(Pt) | fmoc-Lys(mtt)-Wang resin | fmoc-Ser(tBu)-OH  
|                   |                         | fmoc-Arg(pbf)-OH  
|                   |                         | fmoc-Leu-OH       
|                   |                         | fmoc-Ser(tBu)-OH  
|                   | fmoc-Val-OH             | fmoc-Pro-OH       
|                   | fmoc-Ile-OH             | fmoc-Asp(OtBu)-OH 
|                   | fmoc-Gly-OH             | fmoc-Asp(OtBu)-OH 
|                   | fmoc-Asp(OtBu)-OH       | fmoc-Asp(OtBu)-OH 
|                   | fmoc-Asp(OtBu)-OH       | fmoc-Asp(OtBu)-OH 
|                   | fmoc-Asp(OtBu)-OH       | Platinum(IV) complex  |
|                   |                         | 1044.3 ([M]^2+)   |
|                   |                         | 1043.90701 (theoretical: 1043.90639) |
|                   |                         | 12.43             |
|                   |                         | 14 mg (13%)       |
|                   |                         | 2086.98           |

| LRSK(Ru)          | fmoc-Lys(mtt)-Wang resin | fmoc-Ser(tBu)-OH 
|                  |                        | fmoc-Arg(pbf)-OH 
|                  |                        | fmoc-Leu-OH       
|                  |                        | Ruthenium(II) complex  |
|                  |                        | 668.80 ([M]^2+)   |
|                  |                        | 668.33864 (theoretical: 668.33875) |
|                  |                        | 19.25             |
|                  |                        | 2 mg (5%)         |
|                  |                        | 1336.67           |
5.4 Discussion of synthesis

5.4.1 Conjugation to platinum(IV) complexes

Two strategies were explored for conjugating the platinum(IV) complex to an integrated amino acid residue. Ideally, the method of attachment would employ SPPS-compatible reagents and mild reaction conditions, while also being high yielding to conserve the platinum(IV) complex. In this vain the copper(I)-catalysed and ruthenium-catalysed azide-alkyne Huisgen cycloaddition reactions, commonly referred to as “click” chemistry, were investigated as they were expected to provide an orthogonal and versatile method for the attachment of a range of cytotoxic moieties. Due to the commercial availability of fmoc-propargyl glycine which contains the necessary terminal alkyne moiety, it was decided that the peptide would contain the alkyne functionality while the platinum(IV) complex would contribute the azide group. Peptides were synthesised accordingly.

Concurrently, standard peptide coupling between the orthogonally deprotected amine group on lysine residues and the axial carboxylic acid on \([\text{Pt(OAc)}(\text{OSuc})(\text{ox})(\text{chxn})] \) (5.2) was attempted. The varying success of these strategies is discussed below.

5.4.2.1 Off-resin “click” chemistry

As shown in Table 5.3, azide-alkyne cycloaddition was attempted with various catalysts. For copper(I)-catalysed azide-alkyne cycloaddition (CuAAC) to proceed, a source of Cu(I) is required, including Cu(I) salts, most commonly copper iodide, or the in-situ reduction of Cu(II) salts, such as CuSO\(_4\) reduced by sodium ascorbate\(^{253}\). Both these catalysts give rise to the 1,4-disubstituted regioisomer of the 1,2,3-triazole, in contrast to the mixture of 1,4- and 1,5-regioisomers which is typically attained via the thermal cycloaddition pathway. This is shown in Scheme 5.1.

![Scheme 5.1](image_url)

Scheme 5.1 Different disubstituted 1,2,3-triazole products of "click" reactions performed with different catalysts.
The exact mechanism by which CuCAAC occurs is believed to be stepwise, rather than a concerted mechanism as in thermal cycloaddition reactions. Beginning with the formation of a Cu(I) acetylide species via π-complexation with the alkyne, a thermodynamically favourable process in aqueous solutions, a second Cu atom is believed to activate the azide functionality through a dimeric Cu species to form a 6-membered metallocycle intermediate, which then contracts to the 1,4-disubstituted 1,2,3-triazole as shown in Scheme 5.2.

![Scheme 5.2](image)

Scheme 5.2 Mechanisms of the "click" chemistry reaction using a Cu(I) catalyst (left) and ruthenium catalyst (right). Images taken from [251] and [254] respectively.

Table 5.3 summarises the extensive number of small-scale reactions carried out in an effort to achieve a high yielding, high purity “click” reaction under mild conditions. The experimental parameters which were varied were the solvent, catalyst, presence of Cu(I)-stabilising ligand, as well as both the azide- and alkyne-containing molecules and the reaction progress was monitored by ESI-MS (+ve). Also, it should be noted that due to the preliminary nature of this work, two Pt(IV) complexes, [Pt(OAz)(OCH₃)(ox)(chxn)] (5.4) and [Pt(OAc)(OAz)(ox)(chxn)] (5.5) were investigated. These complexes were chosen based on the redox properties of analogues with identical coordination spheres, shown in Table 5.5. It was expected that the more electrochemically stable [Pt(OAc)(OCH₃)(ox)(chxn)], with the more negative reduction potential would be more resistant to reduction by Cu(I) during the click reaction. However, due to the desirable resistance to reduction by ascorbate shown by [Pt(OAc)₂(ox)(chxn)] (5.3),
it was also investigated as it should yield a Pt(IV)-peptide conjugate more resistant to reduction *in vitro*.

**Table 5.5** Platinum(IV)-azide complexes, their reduction potentials, and reduction half life in ascorbic acid. Data collected by Dr Jenny Zhang.

![Platinum(IV)-azide complexes](image)

<table>
<thead>
<tr>
<th></th>
<th><a href="OH">Pt(OAc)</a>[(oa)(chm)]</th>
<th><a href="em">Pt(OAc)</a>(chm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_{\text{ox}}$ (mV)</td>
<td>-568</td>
<td>-452</td>
</tr>
<tr>
<td>$t_{\text{1/2}}$ (h)</td>
<td>0.6</td>
<td>9.4 days</td>
</tr>
</tbody>
</table>

Initially, different solvents were investigated because of the difference in solubility between the LRSpg peptide, fmoc-Pra-OH and Pt(IV) complexes, in order to achieve optimum solvation of the reactants and CuI catalyst. Alcohol/water mixtures are commonly used due to their ability to dissolve lipophilic molecules while retaining the advantage of water for the final protonation step shown in Scheme 5.2, so a 50:50 mixture of H$_2$O:tert-butanol was investigated. Also chosen were methanol, dimethylformamide and dimethylsulfoxide for their ability to dissolve all the reactants. Of all these reactions, that in DMF proceeded the most effectively, confirmed by the presence of a peak at m/z 998 after one hr. Although ESI-MS can not give full quantitative information about a sample, it was expected that the ionisation properties of all species containing the LRSpg peptide would be comparable, so the relative intensities of the “clicked” platinum-peptide conjugate peak and the free peptide peak were compared over time and showed no change in relative peak heights after 1 hr. This suggested that the reaction was no longer proceeding after this time point, which could be due to three possible issues.
The first of these problems could be that the Cu(I) is being sequestered from the reaction through binding with the free -NH₂ groups on both the N-terminus amine and arginine guanidine side-chain, not regenerated as it should be in the final step in Scheme 5.2, thereby suppressing any catalytic activity. Secondly, the Cu(I) cation, which has low thermodynamic stability may undergo oxidation to Cu(II), and thereby causing reduction of the Pt(IV) reactant. Lastly, the C-terminal carboxylic acid may be undergoing intramolecular cyclisation with the terminal alkyne to form an enol lactone.

Solutions were explored to overcome these potential problems, starting with the use of a Cu(I)-stabilising ligand, tris-(benzyltriazolylmethyl)amine (TBTA), to prevent binding of the Cu(I) catalyst to any other functionalities. Cu(I)I and TBTA were stirred in equimolar amounts in DMF for up to an hr before being added to the reactants, in order to allow for complete complexation. TBTA is considered to be more effective than other Cu(I)-stabilising ligands due to its tetradeionate binding nature allowing for complete envelopment of the Cu(I) centre. The combination of the tertiary amine and triazole functionalities are thought to simulatenously contribute to the electron density around the Cu(I) which accelerates the catalysis (amine), while being labile enough to temporarily detach from the Cu(I) in order to form the Cu(I)-acetylide-ligand complex (triazole)₂⁵⁴. A comparison of the mass spectra of reaction mixtures in the absence and presence of TBTA at 15 mins (Figure 5.5) does shows a significant increase in the relative peak height of the Pt(IV)-peptide conjugate compared to the free peptide. It also shows fewer reaction side-products, possibly due to a smaller degree of oxidation of the Cu(I) catalyst and consequently less reduction of any Pt(IV)-containing species.
As no improvement in the yield of the Pt(IV)-peptide conjugate was observed after 15 mins, it was reasoned that another process must be taking place, potentially through the formation of a “click” side-product, although this is rare. Mindt and coworkers have reported on the Cu(I)-catalysed intramolecular cyclisation of alkynoic acids\(^{255}\), with particular emphasis on the commercially available propargyl glycine residue which is located on the C-terminus of the LRSpg peptide in this work. In order to observe whether this was occurring, an FTIR spectrum of unreacted peptide which had been reclaimed via HPLC was obtained, and revealed the absence of the characteristic -C≡C- stretch in the 2260 – 2100 cm\(^{-1}\) range, see Appendix A5.1.

**Figure 5.5** ESI-MS (+ve) spectra of reactions with and without TBTA.
This indicated that the alkyne functionality was no longer present, consistent with the formation of an enol lactone, as shown in Scheme 5.3.

![Scheme 5.3 Formation of enol lactone via Cu(I)-catalysed intramolecular cyclisation.](image)

As off-resin click chemistry with propargyl glycine as the C-terminal amino acid inevitably involves the free carboxylic acid required for cyclisation, it was decided that another approach would be taken. The first approach involved the use of an alternative catalyst, and the second approach will be discussed in section 5.4.2.2.

Although the most commonly used catalyst is Cu(I), it can be replaced by pentamethycyclopentadienylbis(triphenylphosphine)ruthenium(II) chloride (Cp*RuCl(PPh$_3$)$_2$), which selectively gives the 1,5-regioisomer$^{256}$. As the exact configuration of the 1,2,3-triazole was unlikely to affect cleavage of the peptide sequence by MMP-2, or the activity of the Pt(IV) complex, whether the final compound contained the 1,4- or 1,5- regioisomer was not considered to be of concern. The mechanism for the reaction via a (Cp*RuCl(PPh$_3$)$_2$)-catalysed pathway is also shown in Scheme 5.3. It is believed that the neutral [Cp*RuCl] complex is the catalytically active species formed by displacement of the spectator triphenylphosphine ligands. After forming a Ru(II) acetylide species, oxidative addition of azide to alkyne yields a ruthenacycle which is responsible for controlling the regioselectivity of the reaction. Reductive elimination then releases the triazole from the Ru(II) complex, and regenerates the catalyst.

A significant decrease in purity and yield was observed for reactions in which (Cp*RuCl(PPh$_3$)$_2$) was employed as the catalyst, which was initially thought to be due to the poor solubility of the ruthenium complex. However, even in DMF which best solubilised the catalyst, no discernable peaks were seen in the ESI-MS (+ve) spectrum at the appropriate m/z 998. Following these results, an alternative Cu(I)-based approach was investigated.
5.4.2.2 On-resin “click” chemistry

As the results discussed in section 5.4.2.1 demonstrated, the free -COOH moiety on the propargyl glycine residue facilitated intramolecular cyclisation and prevented the progress of a viable “click” reaction between the peptide and Pt(IV) complex. Overcoming this problem by protecting the free carboxylic acid was investigated. This could be achieved simply by leaving the peptide resin-bound for the click reaction.

This strategy was first tested on the resin-bound propargyl glycine amino acid. The fmoc group was deprotected following standard protocol, and the resin shaken with the Pt(IV) complex in the presence of Cu(I), without the TBTA ligand, in DMF. This successfully generated the Pt(IV)-peptide conjugate, which was cleaved from the resin using the standard TFA cocktail, but with the cleavage time reduced to 30 mins to minimise degradation of the Pt(IV) complex. The formation of the 1,2,3-triazole was confirmed using $^1$H NMR, with the singlet at 7.91 ppm being attributed to the single proton on the triazole ring. The presence of the intact Pt(IV) complex was also confirmed by $^{195}$Pt NMR which showed a single peak, corresponding to a single Pt-containing species, with a chemical shift of 1363 ppm. Since the first coordination sphere of the Pt(IV) complex was not altered in the reaction, this value is consistent with the free Pt(IV) precursor, $\delta = 1350$ ppm$^{250}$.

Given this success, the reaction was then repeated on the RHS peptide fragment, H$_2$N-LRSpg-Wang resin. This reaction did not yield any of the desired product, which was ascribed to two possible factors; either the bulky protecting groups on the Ser(OtBu) and Arg(pbf) residues were sterically hindering the alkyne residue from reacting with the Pt(IV) complex, or the presence of the free $\alpha$-amino group on the propargyl glycine residue is required for reactivity. Through the addition of a lysine residue, as shown in Figure 5.6, the propargyl glycine residue a) was located further from the peptide backbone to overcome steric hindrance and b) contained the free $\alpha$-amine moiety.
Figure 5.6 Resin-bound propargylglycine derivatives. From l-r: NH$_2$-Pra-Wang resin, NH$_2$-Lys(Pra)-Wang resin and NH$_2$-Leu-Arg-Ser-Lys(Pra)-Wang resin.

Reaction of the dipeptide H$_2$N-Lys(Pra)-Wang resin afforded the desired product in moderate yield (m/z 769.87), with a significant peak at m/z 343.07 corresponding to the 1,2,3-triazole containing peptide following reduction of the Pt(IV) moiety (Figure 5.7), which was expected to occur to some extent.

Figure 5.7 ESI-MS (+ve) spectrum of successful click reaction with NH$_2$-Lys(Pra)-Wang resin.
When repeated with the modified RHS peptide analogue LRSK(pg)-Wang resin (Figure 5.6), no peak corresponding to the desired reaction product was observed. This suggested that the dominant factor affecting the progression of the on-resin click reaction is the length of the peptide and presence of bulky side-chain protecting groups. In light of this, the click chemistry strategy was abandoned as a means of tethering Pt(IV) complexes to the C-terminus of peptides, and alternative synthetic strategies were investigated.

5.4.2.3 Peptide coupling

Given the aforementioned difficulties associated with employing click-chemistry as a means of orthogonally conjugating Pt(IV) cytotoxins to peptides, on-resin peptide coupling was attempted. As susceptibility to reduction during the reaction process was no longer an issue, the [Pt(OAc)2(ox)(chxn)] (5.3) analogue [Pt(OAc)(OSuc)(ox)(chxn)] (5.2), was chosen as it was believed that its ability to withstand reduction by ascorbate would ensure that the MMP-2 cleavable sequence was retained in the axial position for longer, which is key to the effectiveness of this prodrug strategy. The succinato ligand, containing a free carboxylic acid moiety, allowed for peptide coupling to a free amine residue, such as that on the side chain of the C-terminal lysine amino acid. The final structures of LRSK(Pt) (5.7), DIPVSLRSK(Pt) (5.8) and DDDDGDIPVSLRSK(Pt) (5.9) are shown in Figure 5.8.

Following the reaction conditions described in section 5.3.3, the crude Pt(IV)-peptide conjugates were obtained in relatively high purity, and following purification by preparative HPLC were obtained in their TFA-salt form. In addition to the characterisation details presented in Table 5.4, high-resolution mass spectrometry data can be found in Appendix A4.
Figure 5.8 Structures of platinum(IV)-peptide conjugates.
5.4.2 Conjugation to ruthenium(II) complexes

As discussed in section 5.1, the Ru(II) complex investigated in this work was specifically designed for attachment via peptide coupling to the peptide moiety through the 4-methyl-4'-carboxy-2,2'-bipyridine ligand. It was expected that the on-resin coupling of the Ru(II) complex would be less efficient than for the Pt(IV) complex due to the electron withdrawing effect of the Ru(II) metal centre inhibiting formation of the HBTU-activated acid. This was observed, as the ESI-MS (+ve) spectrum of the crude peptide post-cleavage showed a significant proportion of unreacted peptide, see Appendix A6.1. Despite the reaction being low-yielding, the Ru(II)-peptide conjugate was isolated using preparative HPLC, and its structure is shown in Figure 5.9.

![Figure 5.9 Structure of final Ru(II)-peptide conjugate.](image)

5.5 Platinum-peptide conjugates

5.5.1 Cytotoxicity

The cytotoxicity of the intact MMP-2 selective Pt(IV)-peptide conjugates, their fragments and the unmodified Pt(IV) and Pt(II) precursors were investigated and the results are listed in Table 5.6. It was found that all compounds, with the exception of DDDDGDPVSLRSK(Pt) (5.9), are moderately cytotoxic but not to the degree of the parent drug [Pt(ox)(chxn)] (oxaliplatin).
The Pt(IV) analogue, \([\text{Pt(OAc)}_2(\text{ox})(\text{chxn})] \) (5.3) is approximately 25-fold less cytotoxic in DLD-1 cells than its Pt(II) precursor (5.1), possibly due to the increased stability conferred by oxidation to Pt(IV) as well as the unusually slow reduction kinetics observed when acetato ligands occupy the axial positions\(^{88}\). Previous work has also shown that extremely stable Pt(IV) compounds tend to enter and leave cells without undergoing reduction, thereby exerting a lower cytotoxic effect\(^{250}\). The cytotoxicity of this complex is much lower in DLD-1 cells than previously observed in the A2780 human ovarian carcinoma cell line\(^{250}\), which may be due to cell-line dependent differences in the expression of intra- and extra-cellular reductants, influx and efflux mechanisms and other variations. This is supported by the significant improvement in cytotoxicity of the same compound when tested in A549 cells, where \([\text{Pt(OAc)}_2(\text{ox})(\text{chxn})] \) (5.3) is only 2-fold less cytotoxic than its platinum(II) precursor oxaliplatin. The cell-line dependent cytotoxicity of platinum(IV) complexes is not unusual, with work by Varbanov and co-workers showing a difference of up to 50-fold between IC\(_{50}\) values determined for platinum(IV) compounds across ovarian, colon and non-small cell lung cancer cell lines\(^{257}\).

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC(_{50}) (µM) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DLD-1</td>
</tr>
<tr>
<td>([\text{Pt(ox)(chxn)}] ) (5.1)</td>
<td>5.3 ± 0.7</td>
</tr>
<tr>
<td>([\text{Pt(OAc)}_2(\text{ox})(\text{chxn})] ) (5.3)</td>
<td>117 ± 4</td>
</tr>
<tr>
<td>LRSK(Pt) (5.7)</td>
<td>36 ± 3</td>
</tr>
<tr>
<td>DIPVSLRSK(Pt) (5.8)</td>
<td>44 ± 3</td>
</tr>
<tr>
<td>DDDDGIDPVSLRSK(Pt) (5.9)</td>
<td>&gt; 200</td>
</tr>
</tbody>
</table>

Table 5.6 Cytotoxicity (IC\(_{50}\)) of DIPVSLRSK(Pt) (5.8), DDDDGIDPVSLRSK(Pt) (5.9) and their cytotoxin-containing fragment LRSK(Pt) (5.7) and relevant Pt complexes in DLD-1 and A549 cells. Cytotoxicity was measured following 72 hrs incubation. Values shown are the means of data taken from three independent experiments with triplicate readings in each experiment.
The fragment LRSK(Pt) (5.7) is approximately 3-fold more cytotoxic than the free Pt(IV) compound in DLD-1 cells. While still significantly less cytotoxic than oxaliplatin, these results suggest that peptide conjugation is capable of modifying the properties of established cytotoxic compounds. This could be the result of improved cellular uptake of the peptide-Pt(IV) conjugate prior to reduction by facilitating transport across the cell membrane, or the result of the LRSK peptide having a directing effect within the cell, perhaps playing a role in transporting the Pt(IV) cytotoxin towards the nucleus to exert its effect. While outside the scope of this work, this could be confirmed by using nuclear staining techniques for fluorescence microscopy in conjunction with single-cell µ-SRIXE elemental mapping. While it could also be considered that by using the [Pt(OAc)\(\text{2}\)\(\text{2}\)](ox)(chxn)] (5.3) analogue [Pt(OAc)(OSuc)(ox)(chxn)] (5.2) and conjugating a tetrapeptide could be increasing the rate of reduction to Pt(II) by changing the nature of the reduction mechanisms occurring, this is unlikely given previous work which has shown that Pt(IV) reduction to Pt(II) proceeds via an inner-sphere pathway. The intact peptide DIPVSLRSK(Pt) (5.8) is only slightly less cytotoxic than the fragment LRSK(Pt) in DLD-1 cells, while the IC\(_{50}\) of DIPVSLRSK(Pt) (5.9) in A549 cells is almost identical to that of the the LRSK(Pt) (5.7) fragment. The similarity of the IC\(_{50}\) values for DIPVSLRSK(Pt) (5.9) and LRSK(Pt) (5.7) in DLD-1 cells was surprising and could be indicative of some cleavage occurring, possibly by non-specific proteases.

![Figure 5.10](image)

**Figure 5.10** Comparison of cytotoxicity of DIPVSLRSK(Pt) (5.8) and DDDDGDPVSLRSK(Pt) (5.9) in DLD-1 and A549 cells.
The effectiveness of the uptake-blocking moiety and controlling the activity of these compounds is supported by the cytotoxicity observed for the two intact peptides DIPVSLRSLK(Pt) (5.8) and DDDGDIPVSLRSLK(Pt) (5.9), shown in Figure 5.10.

The IC$_{50}$ of DIPVSLRSLK(Pt) (5.8) is close to that of its post-cleavage fragment LRSK(Pt) (5.7), suggesting that it is either entering cells intact, or that extracellular cleavage is occurring in order to allow the compound to enter cells. If this is the case, it also suggests that in less than 72 hrs, a substantial fraction of the peptide has been cleaved. In the case of DDDGDIPVSLRSLK(Pt) (5.9), the tetra-aspartate group may be effectively inhibiting the uptake of the intact peptide into cells as intended, but may also be responsible for preventing cleavage of the peptide by MMP-2, thereby stopping uptake of the LRSK(Pt) (5.7) fragment.

It was therefore important to compare the cytotoxicity observed in DLD-1 cells to A549 cells, which have been shown to secrete higher levels of active MMP-2 in cell monolayers. The cytotoxicity of DIPVSLRSLK(Pt) (5.8) improved by approximately 3-fold, while for DDDGDIPVSLRSLK(Pt) (5.9), no change was observed. While a direct comparison between cell lines cannot be made, this result does indicate that the cytotoxicity of the Pt(IV)-peptide sequence is higher in regions where MMP-2 is expressed, which fits the objectives of this work.

5.5.2 Monolayer accumulation studies

The cellular accumulation of platinum following treatment with [Pt(ox)(chxn)] (5.1), [Pt(OAc)$_2$(ox)(chxn)] (5.3), LRSK(Pt) (5.7), DIPVSLRSLK(Pt) (5.8) and DDDGDIPVSLRSLK(Pt) (5.9) was determined in DLD-1 and A549 cell lines, the cell lines used for cytotoxicity measurements due to their low and high levels of MMP-2 activity respectively. The results are presented in Figure 5.11.
Figure 5.11 Platinum accumulation in DLD-1 and A549 cells following 24 hrs treatment (30 µM) with [Pt(ox)(chxn)] (5.1), [Pt(OAc)2(ox)(chxn)] (5.3), LRSK(Pt) (5.7), DIPVSLRSK(Pt) (5.8) and DDDDGDPVSLRSK(Pt) (5.9). Accumulation values were obtained from 3 independent experiments. Error bars represent ± S.E.

The unmodified platinum(II) compound oxaliplatin exhibits the greatest amount of accumulation after 24 hrs, with a higher level of intracellular platinum in A549 cells compared to DLD-1 cells (25.5 ± 0.9 and 17.7 ± 1.2 ng Pt/mg protein respectively) contrasting with the cytotoxicity data presented in section 5.5.1. The lack of a direct relationship between cytotoxicity and accumulation for oxaliplatin suggests that there are other biological mechanisms taking place which may be causing deactivation and sequestration of the platinum(II) complex in A549 cells such as increased thiol conjugation. Moreover, DLD-1 cells may be undergoing increased levels of drug efflux as a result of the increased expression of adenosine triphosphate-binding cassette (ABC) transporters observed in cells having a gastrointestinal origin\textsuperscript{258}. In particular, DLD-1 cells express high levels of the P-glycoprotein (Pgp) efflux pump, which functions as a xenobiotic pump on the cell membrane to remove chemotherapeutic agents from cells, and is widely implicated in multidrug resistance\textsuperscript{259}. The higher accumulation observed in A549 cells could then simply be the result of a lower level of efflux of platinum(II)-adducts.

This notion is supported by the changes in accumulation observed between 24 and 48 hr time points in DLD-1 and A549 cells, as shown in Figures 5.12a and 5.12b respectively.
Figure 5.12 Platinum accumulation following 24 and 48 hrs treatment (30 µM) with [Pt(ox)(chxn)] (5.1), [Pt(OAc)_2(ox)(chxn)] (5.3), LRSK(Pt) (5.7), DIPVSLRSK(Pt) (5.8) and DDDDGDPVSLRSK(Pt) (5.9) in a) DLD-1 and b) A549 cells. Accumulation values were obtained from 3 independent experiments. Error bars represent ± S.E.

There is a significant increase in accumulation for [Pt(OAc)_2(ox)(chxn)] (5.3) from 24 to 48 hrs in A549 cells from 8.2 ± 0.9 to 16.5 ± 0.8 ng Pt/mg protein. However, following the same treatment, DLD-1 cells demonstrate a decrease in accumulation from 9.0 ±1.2 to 8.5 ± 1.0 ng Pt/mg protein, consistent with a significant amount of efflux taking place. This low accumulation and efflux trend could be a factor in the low cytotoxicity of [Pt(OAc)_2(ox)(chxn)] (5.3) in DLD-1 cells, as reported in Table 5.6. It may be the case that a significant proportion of the Pt(IV) compound is exported from cells prior to reduction to its active Pt(II) analogue,
given its long reduction half life (see section 5.4.2.1); This could be confirmed by performing efflux studies.

Interestingly, this trend does not continue for the platinum(IV)-peptide conjugates, with an increase in accumulation between 4 and 48 hrs occurring for LRSK(Pt) (5.7) (81%, 33%), DIPVSLRSK(Pt) (5.8) (33%, 64%) and DDDDGDPVSLR(Pt) (5.9) (120%, 56%) in DLD-1 and A549 cells respectively. Overall, the level of platinum accumulation is higher for A549 cells than DLD-1s, which correlates with the observed cytotoxicities across the two cell lines. Of the three platinum(IV)-peptide conjugates, the fragment LRSK(Pt) (5.7) and the intact peptide show similar levels of cellular accumulation after 24 hrs, with the conjugate containing the uptake-blocking moiety exhibiting slightly lower accumulation.

Overall, while investigating the cellular accumulation of platinum in cell monolayers can assist in understanding the behavior of the novel platinum(IV)-peptide conjugates, 2D cell models do not display the tumour microenvironmental characteristics such as poor nutrient supply as well as hypoxic and necrotic regions. For this reason, the results presented in this section must be supplemented by observations made in 3D tumour models, as presented in section 5.5.3.

### 5.5.3 Mapping distribution with SRIXE

In the absence of any fluorescent moiety, visualising the distribution of platinum species across a spheroid tumour model requires more sophisticated techniques. SRIXE mapping is superior to fluorescence confocal microscopy for mapping platinum as it measures the X-ray fluorescence signal generated directly by the platinum metal centre, as opposed to inferring the position of the platinum moiety based on a fluorescent ligand, which may have undergone detachment from the platinum atom.

DLD-1 spheroids were treated with [Pt(ox)(chxn)] (5.1) and [Pt(OAc)₂(ox)(chxn)] (5.3) for 2 and 6 hrs, in order to observe the time-dependant nature of platinum accumulation within the spheroids. These compounds provide control studies for DLD-1 spheroids which were treated with LRSK(Pt) (5.7), DIPVSLRSK(Pt) (5.8) and DDDDGDPVSLR(Pt) (5.9), in order to observe whether the additional peptide functionality significantly altered the localisation of the platinum cytotoxin. When interpreting these results, it should be noted that a small degree of sample shrinkage occurs during the cryo-sectioning, Formvar mounting and freeze-drying procedures involved in sample preparation.

SRIXE maps of the distribution of copper, platinum and zinc in DLD-1 spheroids treated with [Pt(ox)(chxn)] (5.1) for 2 and 6 hrs are shown in Figure 5.13 and Figure 5.14 respectively. After
incubation with the Pt(II) complex for 2 hrs, some platinum is visible on the spheroid periphery, but platinum moieties have already reached both the hypoxic regions of the tumour and the necrotic core with a maximum concentration of 18 ppm. The presence and position of the necrotic core is suggested by the presence of increased levels of copper, which in previous work have been shown to exist in the necrotic regions of spheroid models. Although no single explanation for this pattern of copper accumulation has been proposed, it is possible that the increased levels of oxidative stress experienced by cells within the spheroid core causes the upregulation of the copper-zinc superoxide dismutase (Cu-Zn-SOD) enzyme which normally protects cells from oxidative damage through the detoxification of reactive oxygen species, hence the higher levels of copper seen in these regions. Additionally, a thin band of copper can be seen around the periphery of the spheroid which may be due to the recruitment of copper for cellular metabolism by the more active, proliferating cells found on the outer surface of the spheroid. The possibility of this peripheral distribution being an artifact or result of the sample preparation process was considered, but ruled out because no other elements exhibited this distribution pattern. The same copper distribution pattern was observed in control spheroids, as shown in Appendix A8.1, showing that it was not induced by treatment with the platinum drugs.

Increasing the incubation time to 6 hrs resulted in an approximately 2-fold increase in concentration in the core region of the spheroid, with less platinum accumulation seen in the surrounding regions compared to spheroids dosed for just 2 hrs. There are two possible mechanisms by which this pattern of distribution within spheroids may be reached, and it probably a combination of these mechanisms which ultimately at play.

**Figure 5.13** Representative SRIXE elemental maps of a DLD-1 spheroid treated with [Pt(ox)(chxn)] (5.1) (30 µM, 2 hrs). Map was taken from central sections of the spheroids. Scale bar represents 100 µm.
Mechanism 1 involves the platinum(II) drug undertaking a transcellular route initially, whereby the complex is taken up by cells on the periphery and metabolised, resulting in the formation of new platinum species. The formation of platinum adducts via interaction with glutathione (GSH), metallothioneins and other thiol-containing biomolecules is well-known\(^{262-264}\), and in particular platinum-GSH complexes tend to be more anionic than the precursor platinum complex. Consequently, these complexes can be readily exported from the cells by GS-X efflux pumps and other transporters, but due to their acquired anionic charge, are no longer readily able to reenter cells. The platinum adducts then undertake a paracellular route to the centre of the spheroid whereby they gradually diffuse through the extracellular space and pool in the necrotic core.

![Representative SRIXE elemental maps of a DLD-1 spheroid treated with [Pt(ox)(chxn)] (5.1) (30 µM, 6 hrs). Map was taken from central sections of the spheroids. Scale bar represents 100 µm.](image)

The second mechanism (Mechanism 2) by which platinum species may accumulate in the spheroid core is based upon the assumption that the drugs are able to reach all spheroid regions, but undergo subsequent redistribution to the more metabolically active cells, establishing an “equilibrium” and resulting in a non-uniform distribution. The accumulation observed at the periphery is therefore due to the presence of a greater proportion of actively proliferating cells with higher metabolic demands. In the spheroid core, platinum complexes may become bound to some of the biomolecules which also accumulate as waste products, causing inactivation and entrapment in the necrotic regions. As there is no initial cell-uptake process in this mechanism, it is more likely to be experienced by compounds with low cellular accumulation.

For platinum(IV) complexes such as [Pt(OAc)\(_2\)(ox)(chxn)] (5.3), there is likely to be a combination of the two proposed distribution mechanisms taking place. As can be seen in
Figure 5.15, there was no accumulation of platinum in either the periphery or core of the spheroid after dosing for 2 hrs.

**Figure 5.15** Representative SRIXE elemental maps of a DLD-1 spheroid treated with [Pt(OAc)$_2$(ox)(chxn)] (5.3) (30 µM, 2 hrs). Map was taken from central sections of the spheroids. Scale bar represents 100 µm.

As already discussed, [Pt(OAc)$_2$(ox)(chxn)] (5.3) is particularly resistant to reduction by biological reducing agents, and as such will remain in its inactive Pt(IV) form and resistant to complexation with intracellular thiol-containing compounds as well as waste products in the necrotic core. The complex is therefore free, after 2 hrs, to diffuse throughout the tumour via both trans- and para-cellular transport. It is possible that by not concentrating in any one region, the concentration of platinum throughout the spheroid is below the ppm detection limit of the SRIXE technique.

**Figure 5.16** Representative SRIXE elemental maps of a DLD-1 spheroid treated with [Pt(OAc)$_2$(ox)(chxn)] (5.3) (30 µM, 6 hrs). Map was taken from central sections of the spheroids. Scale bar represents 100 µm.

However, after 6 hrs incubation with [Pt(OAc)$_2$(ox)(chxn)] (5.3), a defined region of platinum accumulation within the necrotic core is visible, shown in Figure 5.16. The concentration of platinum is approximately 8-fold lower than that found within spheroids treated with its platinum(II) precursor [Pt(ox)(chxn)] (5.1) with an identical incubation time. The absence of
any platinum hotspots on the periphery suggests that intracellular transformation of the unreduced platinum(IV) complex is not the major mechanism of accumulation occurring. Instead, the platinum(IV) complex may be taken up and exported from cells without metabolism, and therefore accumulate in the core where reduction to platinum(II) occurs. The increased lability of the platinum(II) centre facilitates binding with other pooled biomolecules, hindering transport to other regions of the spheroid and causing accumulation in the core. These results correspond with the significant decrease in cytotoxicity for \([\text{Pt(OAc)}_2(\text{ox})(\text{chxn})]\) (5.3) compared to its platinum(II) congener, as shown in Table 5.6, although a direct comparison of uptake in cell monolayers and penetration through spheroids cannot be made definitively.

After investigating the behaviour of the platinum(II) and platinum(IV) complexes in 3D tumour models, spheroids were also incubated with the platinum(IV)-peptide conjugates.

When spheroids were treated with just the cytotoxic cleavage fragment LRSK(Pt) (5.7) for 12 hrs, the pattern and level of platinum accumulation was significantly different to what was expected. As can be seen in Figure 5.17, only a very low level of platinum was observed in the core at a concentration of 2-4 ppm. Moreover, no platinum had accumulated in the periphery of the spheroid. Contrastingly, incubation with DIPVSLRSK(Pt) (5.8) for 12 hrs resulted in significant levels of accumulation in the necrotic core (8-14 ppm, see Figure 5.18), which is comparable to the platinum distribution observed for the unconjugated platinum(IV) complex shown in Figure 5.16. One possible explanation for the increased platinum accumulation observed for DIPVSLRSK(Pt) (5.8) compared to LRSK(Pt) (5.7) is based on the lipophilicity of the
compounds. Unlike the fluorophore-peptide and the Ru(II)-peptide compound investigated in Chapters 4 and 5 respectively, the platinum(IV) moiety tethered to the LRSK peptide is hydrophilic, and this hydrophilicity is enhanced upon peptide conjugation. Compound lipophilicity can directly relate to its ability to cross cell membranes by passive diffusion through the phospholipid bilayer. A direct correlation between increasing lipophilicity of platinum compounds and uptake via passive diffusion has been established by measuring platinum levels of a library of compounds across different cell lines. The conjugation of the LRSK tetrapeptide, despite conferring some lipophilicity through the peptide backbone, ultimately increases the hydrophilicity of the platinum(IV) complex through the addition of amino acids which are charged at pH 7.4. Extending the amino acid chain as in DIPVSLRSK(Pt) (5.8) only adds one more charged amino acid while increasing the lipophilic peptide backbone by 5 amino acids. Therefore, the more hydrophilic LRSK(Pt) (5.7) may not be being taken up readily by peripheral cells, therefore distributing throughout the spheroid with on a small proportion of compound reducing to platinum(II) and interacting with pooled thiol-compounds and waste products in the core, as in the previously described Mechanism 2.

The increased lipophilicity of the intact peptide DIPVSLRSK(Pt) (5.8), without the uptake-blocking group, may improve cell entry such that some reduction to platinum(II) occurs intracellularly. However, the absence of platinum accumulation at the periphery suggests that this process, Mechanism 1, is either no longer occurring after 12 hrs, or is occurring to a lesser extent than Mechanism 2.

Finally, spheroids were incubated for 12 hrs with the intact peptide DDDDGIDIPVSLRSK(Pt) (5.9), with the resulting SRIXE maps shown in Figure 5.19. As in all other spheroid sections, a
necrotic core is discernable by the elevated levels of copper at the centre of the spheroid, therefore it can be seen that there is almost no platinum present in the spheroid core. This distribution is reminiscent of that observed for LRSK(Pt) (5.7) which, as already discussed, may be too hydrophilic for cell uptake via passive diffusion. In the case of DDDDGDIPVSLRSK(Pt) (5.9), cell uptake is also prevented by the presence of the uptake-blocking group, which may explain the similarly low level of platinum accumulation as seen for LRSK(Pt) (5.7).

These results, showing poor spheroid penetration of the target compound DDDDGDIPVSLRSK(Pt) (5.9) and LRSK(Pt) (5.7) do not correlate well with the cell monolayer cytotoxicity and platinum accumulation results discussed in sections 5.5.1 and 5.5.2. Given that the cytotoxicity, accumulation and SRIXE map data correlate well for the DIPVSLRSK(Pt) (5.8) conjugate it is unlikely to be an artefact of sample preparation, and more likely to be a result of the physical and biochemical differences between 2D and 3D cell models. These results further support the importance of investigating the behavior of novel drug compounds in tumour models which are more sophisticated than cell monolayers, such as multicellular tumour spheroids and \textit{in vivo} animal models.

\subsection*{5.5.4 Determining speciation with XANES}

While SRIXE platinum mapping can provide valuable information pertaining to the distribution of platinum drugs in tumour models, its main limitation is that it provides no information on the oxidation state or speciation of the platinum being detected. As discussed in section 1.8.2.2, previous work by Hall \textit{et al} has shown that the reduction of platinum(IV) drugs in cells can be observed using XANES\textsuperscript{214,215}. 

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure_5.19.png}
\caption{Representative SRIXE elemental maps of a DLD-1 spheroid treated with DDDDGDIPVSLRSK(Pt) (5.9) (30 \textmu M, 12 hrs). Map was taken from central sections of the spheroids. Scale bar represents 100 \textmu m.}
\end{figure}
The XANES spectra of platinum(II) and platinum(IV) exhibit significant differences in the height of the platinum-L3 edge height which is unrelated to the coordination sphere of the platinum centre. Exploiting this difference allowed for the construction of a calibration curve from standard mixtures of known Pt(IV):Pt(II) composition using the compounds [Pt(OAc)$_2$(ox)(chxn)] (5.3) and [Pt(ox)(chxn)] (5.1). The platinum-L3 edge XANES spectra were obtained for the mixed standards, and a method similar to that described by Hall et al was used to construct the calibration curve. The normalised XANES spectra and calibration curve are presented in Figure 5.20 and 5.21 respectively.

Figure 5.20 Normalised XANES spectra of standard mixtures prepared from different ratios of [Pt(OAc)$_2$(ox)(chxn)] and its platinum(II) precursor, oxaliplatin.
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Figure 5.21  Linear fit of peak height ratio (a/b) derived from the XANES spectra of standard mixtures of [Pt(OAc)$_2$(ox)(chxn)] and oxaliplatin ($R^2 = 0.9767$).

The XANES spectra of the mixed standards (Figure 5.18) reveal an absence of pre-edge features, with the peaks corresponding to platinum(II) and platinum(IV) being consistent with the results of previous XANES experiments$^{214,215}$. For the platinum(II) standard [Pt(ox)(chxn)] (5.1), with a N$_2$O$_2$ coordination sphere, the peak height ratio (a/b) was determined to be 1.63. This is higher than the values reported for platinum(II) compounds containing an N$_2$Cl$_2$ coordination sphere, but conforms to the trend observed for platinum(IV) complexes, where the number of “hard” oxygen donor atoms increases the L3 edge peak height. The reason for this is most like related to the electronic differences between platinum(II) and platinum(IV) centres that give rise to the increased peak height for platinum(IV) over platinum(II). The platinum-L3 edge is the result of electronic transitions from occupied 2p orbitals into the higher unoccupied 5d orbitals. While platinum(II) has a 5d$^8$ configuration, the two additional vacancies in the 5d$^6$ configuration of platinum(IV) increases the statistical probability of the 2p $\rightarrow$ 5d transitions, thereby increasing the L3 edge intensity$^{266}$.

The linear relationship between the peak height ratios of oxaliplatin-based standard platinum(II):platinum(IV) mixtures is shown in Figure 5.21, and can be used to deduce the extent of reduction of the platinum(IV)-based compounds which had occurred in the spheroid samples mapped by the SRIXE technique in section 5.5.3.

The platinum-L3 edge spectra were obtained from regions of interest (ROI) selected from the previously collected SRIXE maps. The regions selected were those which contained the highest concentrations of platinum, in order to acquire the highest quality XANES data possible, an
example of which is shown in Figure 5.22. XANES data were collected from similarly chosen hotspots in the necrotic core for spheroids treated with the platinum(II) complex [Pt(ox)(chxn)] (5.1), its platinum(IV) congener [Pt(OAc)$_2$(ox)(chxn)] (5.3), and the intact peptide DIPVSLRSK(Pt) (5.8). While this sampling technique provides the strongest possible XANES signal, it does not take into account the fact that the efficacy of the drug may not be related to concentration. The results reported here should be considered with this limitation in mind.

![Image](image.png)

**Figure 5.22** An example of a ROI selected from a SRIXE map of a spheroid treated with DIPVSLRSK(Pt) (30 uM, 12 hrs).

The spectra collected from these regions were averaged and normalized, and are shown in Figure 5.23, along with the normalised XANES spectra of the solid standards for comparison.
Figure 5.23 Normalised XANES spectra taken from platinum hotspots in spheroids treated with \([\text{Pt(ox)(chxn)}]\) (5.1), \([\text{Pt(OAc)}_2\text{ox}(\text{chxn})]\) (5.3), and DIPVSLRSK(Pt) (5.8). Normalised spectra of oxaliplatin (5.1) and \([\text{Pt(OAc)}_2\text{ox}(\text{chxn})]\) (5.3) are shown for comparison.

There are two salient features which emerge from the XANES spectra in Figure 5.23, the first of which is the high absorbance of the pre-edge region, even following normalisation. This could be the direct result of the low levels of platinum found in the samples, ranging from 5ppm to 30 ppm as shown in the SRIXE maps in section 5.5.3. In other studies, spheroid samples incubated with 30 µM of platinum compounds for 24 hrs resulted in concentrations in the range of 40 – 120 µM in the hotspots chosen for XANES analysis, and generated data of higher quality with improved signal to noise ratios. This ruled out the sample matrix as a contributing factor, and suggested that incubation for time periods greater than 6 hrs, as performed in this study, would generate higher quality spectra.

The second significant feature is the L3 peak height, which for all spheroid treatments was less intense than the peak for the platinum(II) standard. The peak height ratios for spheroids treated with platinum(II) (5.1), platinum(IV) (5.3) and DIPVSLRSK(Pt) (5.8) were 1.53, 1.68 and 1.32 respectively. These values, with the exception of 1.68 for the spheroid treated with the platinum(IV) compound \([\text{Pt(OAc)}_2\text{ox}(\text{chxn})]\) (5.3), are below the peak height ratio for pure platinum(II) \((a/b = 1.63)\). The linear calibration curve shown in Figure 5.21 is therefore unsuitable for determining the extent of reduction of platinum(IV) species in biological systems. Assuming that reduction is the only process taking place by which the platinum...
moieties are modified is evidently oversimplifying the processes which are occurring. Given
the time frame of treatment with the platinum compounds, it is likely that along with
reduction to platinum(II) some degree of interaction with DNA, intra- and extracellular thiols
and aquation has also taken place. The significantly lower peak height of the L3-edge for the
spheroid treated with oxaliplatin \((a/b = 1.53)\) compared to the peak for solid oxaliplatin \((a/b =
1.63)\) confirms that non-reduction-based modifications of the platinum centre are occurring.
The reduced peak height corresponds to platinum coordination spheres with a more covalent
nature, such as those generated by binding to thiol-based compounds. Platinum(II)
compounds have a high affinity for binding to sulfur donors such as glutathione and cysteine
over the nitrogen donor sites on DNA. Therefore with intracellular concentrations of cysteine,
glutathione and other sulfhydryl groups being as high as 10 mM\(^2\), nitrogen donor sites on
DNA strands cannot compete, resulting in sequestration and deactivation of the platinum(II)
compound through thiol and thio-ether adduct formation.

Previous work by Zhang has shown that the peak heights of sulfur-bound platinum(II) species
are significantly lower than for oxaliplatin, with \(a/b < 1.32\). This was attributed to the more
covalent nature of Pt-S bonds compared to Pt-O bonds, resulting in fewer vacant d-electron
states and therefore a lower statistical probability of \(2p \rightarrow 5d\) transitions. Additionally, a
shoulder peak was also observed around 11575 eV for platinum-thiol complexes, which is also
present in the post-edge region of the XANES spectra of all three spheroid treatments shown
in Figure 5.23. For the spheroid treated with \([\text{Pt(OAc)}_2(\text{ox})(\text{chxn})]\) \((\ref{5.3})\), the peak height \((a/b =
1.68)\) falls just within the range of the calibration curve (Figure 5.21), suggesting that some
platinum(IV) remains after 6 hrs incubation, as was expected, and the presence of the
shoulder is indicative of formation of platinum-thiol adducts. With the exception of spheroids
treated with DIPVSLRSK(Pt), the peaks of the treated spheroids in this study are higher than
pure platinum-thiol complexes, suggesting that a mixture of platinum(II)-based metabolites,
platinum-DNA adducts and platinum-thiol complexes is present in the spheroid core. It also
indicates that simplistic quantitative determination of platinum(IV) reduction is insufficient for
understanding the behavior of this class of compounds in solid tumour models, and that a
more sophisticated approach is required.
5.6 Ruthenium-peptide bioconjugates

5.6.1 Fluorescence spectroscopy

The inherent fluorescence of ruthenium-polypyridyl complexes has made them longstanding candidates for cellular imaging agents, but their inability to passively diffuse across the cell membrane has meant that electroportation, detergents and transfection reagents are often necessary to allow cell penetration.\(^{268-270}\)

The fluorescence spectra of the free Ru(II) complex \([\text{Ru}(t\text{Bu}_2\text{bpy})_2(\text{HOOC-4’-CH}_3\text{bpy})]2\text{PF}_6\) (5.6) and its tetrapeptide congener LRSK(Ru) (5.10) are shown in Figure 5.24. The large Stokes shift exhibited by the majority of ruthenium complexes is observed for both compounds, with excitation and emission maxima at 460 and 645 nm and 478 and 665 nm for \([\text{Ru}(t\text{Bu}_2\text{bpy})_2(\text{HOOC-4’-CH}_3\text{bpy})]2\text{PF}_6\) (5.6) and LRSK(Ru) (5.10) respectively.

![Figure 5.24](image-url) The fluorescence excitation and emission spectra of the free Ru(II) complex \([\text{Ru}(t\text{Bu}_2\text{bpy})_2(\text{HOOC-4’-CH}_3\text{bpy})]2\text{PF}_6\) (5.6) and its tetrapeptide congener LRSK(Ru) (5.10).

Conjugation to the peptide resulted in a bathochromic shift in both the excitation and emission spectra, possibly due to increased aggregation in solution by the peptide moieties.
through hydrogen-bonding interactions, thereby stabilising the ground state electronic configuration. However, the fluorescence intensity increased by approximately 4-fold, which is not consistent with the formation of peptide aggregates. Instead, it is probable that an improvement in solvation conferred by the hydrophilic peptide moiety is responsible for the ground state stabilisation and significant increase in fluorescence.

### 5.6.2 Cytotoxicity

Table 5.7 lists the results of the MTT cytotoxicity assays of the precursor ruthenium(II) complex \([\text{Ru(tBu}_{2}\text{bpy})_{2}(\text{HOOC-4'-CH}_{3}\text{bpy})]_{2}\text{PF}_{6}\) (5.6) and its peptide conjugate LRSK(Ru) (5.10). The unconjugated complex was found to have an \(IC_{50}\) value similar to those reported for related ruthenium(II)-bipyridyl compounds. However, conjugation to the LRSK peptide through the lysine residue doubled the cytotoxicity of the compound. When considered with the increased cellular uptake observed by fluorescence confocal microscopy and reported in section 5.6.3, it appears that the presence of the peptide facilitates the movement of the ruthenium(II) complex through the phospholipid bilayer and into the cytoplasm.

Table 5.7 Cytoxicity (\(IC_{50}\)) of \([\text{Ru(tBu}_{2}\text{bpy})_{2}(\text{HOOC-4'-CH}_{3}\text{bpy})]_{2}\text{PF}_{6}\) (5.6) and LRSK(Ru) (5.10) in DLD-1 cells. Cytotoxicity was measured following 72 hrs incubation. Values shown are the means of data taken from three independent experiments with triplicate readings in each experiment.

<table>
<thead>
<tr>
<th>Compound</th>
<th>(IC_{50}) ((\mu\text{M})) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>([\text{Ru(tBu}<em>{2}\text{bpy})</em>{2}(\text{HOOC-4'-CH}<em>{3}\text{bpy})]</em>{2}\text{PF}_{6}) (5.6)</td>
<td>70 ± 11</td>
</tr>
<tr>
<td>LRSK(Ru) (5.10)</td>
<td>33 ± 5</td>
</tr>
</tbody>
</table>

### 5.6.3 Confocal microscopy in monolayer cell culture

The intracellular localisation of the free Ru(II) complex (5.6) and LRSK(Ru) (5.10) was investigated in A549 and DLD-1 cell lines, the results of which are shown in Figure 5.25. Preliminary experiments which involved dosing the cells with 20 \(\mu\text{M}\) solutions of the compounds followed by incubation for 4 hrs did not show any intracellular fluorescence. Studies of Ru(II) complex interactions with the plasma membranes of cells by Zava and co-workers showed that complexes of this type required longer incubation periods for cellular
uptake. Furthermore, Ru(II) complexes of this type have been shown to possess “light-switch” activity whereby their inherent fluorescence is quenched by hydrogen-bonding in aqueous solutions, but returns once in more lipophilic environments. In light of these factors, it was decided that complexes would be incubated at 50 µM concentrations for 24 hrs to ensure cellular uptake for observation by confocal fluorescence microscopy.

The free Ru(II) complex (5.6) showed only faint intracellular fluorescence in A549 and DLD-1 cells, Figure 5.25A1 and 5.25A2 respectively. The fluorescence pattern in both cell lines is cytoplasmic, with no accumulation in the nucleus. This was somewhat unexpected given that this class of Ru(II) compounds are believed to exert their cytotoxic action through DNA intercalation. However, similar compounds have been shown to accumulate in small organelles in the cytoplasm with no nuclear localisation.

A considerable increase in fluorescence was observed when the cells were treated with an equivalent concentration of LRSK(Ru) (5.10) for 24 hrs. In A549 cells, a punctate cytoplasmic distribution is clearly visible within the cells (Figure 5.25B1). In the case of the DLD-1 cells shown in Figure 5.25B2, the beginning of apoptosis is visible, as cell rounding has begun to
occur. It is therefore not possible to conclude that all the fluorescence visible is the result of LRSK(Ru) (5.10) accumulation, as apoptotic autofluorescence may be contributing.

The improvement in apparent cell uptake is likely to be the result of attaching the LRSK tetrapeptide, which contributes to the cationic charge of [Ru(tBu₂bpy)₂(HOOC-4'-CH₃bpy)]²⁺ (5.6), making it 3+. Puckett and co-workers demonstrated that conjugation of the tetrapeptide RrRK (where r = D-arginine) to a highly conjugated Ru(II)-bipyridyl complex resulted in a 1.7-fold increase in cellular uptake compared to the free complex. While the LRSK peptide is not a sequence known for its cell-penetrating properties, it does exhibit some of the traits generally observed for cell-penetrating peptides (CPPs) including: amphipathy, the presence of positively charged amino acids within the peptide sequence, water-solubility while retaining some hydrophobic residues and a net positive charge at physiological pH. It is plausible that, to some extent, the LRSK peptide is exhibiting CPP-type behaviour, but investigating this further is beyond the scope of the present study.

### 5.6.4 Confocal microscopy in 3D tumour models

In order to investigate how the ability of [Ru(tBu₂bpy)₂(HOOC-4'-CH₃bpy)]²⁺PF₆ (5.6) to penetrate into solid tumours was affected by conjugation to a peptide in LRSK(Ru) (5.10), confocal microscopy of spheroids grown from DLD-1 tumour cells was performed. Figure 5.26 shows the distribution of the unconjugated Ru(II) complex (5.6) after being dosed with a 50 µM solution of the complex for 24 hrs, the same conditions which were employed in monolayer experiments.

![Confocal microscopy images of a DLD-1 spheroid treated with 50 µM [Ru(tBu₂bpy)₂(HOOC-4'-CH₃bpy)]²⁺PF₆ (5.6). Incubation time was 24 hrs and the media was replaced prior to imaging. The respective bright field image is also shown. Images were obtained in the NIR channel but are shown in yellow for clarity. Scale bar represents 100 µm.](image-url)
As evident in the yellow channel, the fluorescence signal is weak. As previously discussed, the fluorescence of Ru-bipyridyl complexes is quenched in aqueous environments, and returns upon reaching hydrophobic regions such as within the cell and cell membrane. Therefore, the faint fluorescence suggests that cellular uptake and membrane-association is not occurring to a significant degree. Despite this, a gradually decreasing diffusion gradient towards the core of the spheroid is visible, and regions of punctate fluorescence within 20-80 µM of the cell periphery indicate that there is a small amount of either cell uptake or membrane-attachment occurring. As this image was collected at 24 hrs, it is quite possible that the complex has penetrated further towards the core than it appears in the fluorescence image, but the higher proportion of active and proliferating cells on the periphery are more proficient in taking up the complex, as also seen in section 4.10.2.

Contrastingly, Figure 5.27 shows the fluorescence distribution of LRSK(Ru) (5.10) in medium-sized spheroids at 4 and 24 hrs, and large spheroids at 24 hrs. The increase in fluorescence intensity compared to the free ruthenium complex is immediately apparent, but should be considered with the results described in section 5.6.1 in mind. However, even despite the inherently stronger fluorescence of LRSK(Ru) (5.10) compared to [Ru(tBu2bpy)2(HOOC-4’-CH3bpy)]2PF6 (5.6), an improvement in cellular uptake and spheroid penetration is evident.
Figure 5.27 Confocal microscopy images of DLD-1 spheroids treated with 50µM LRSK(Ru) (5.10) for A: 4 hrs, B: 24 hrs, C: 24 hrs. The media was replaced for all images. The respective bright field images are also shown. Images were obtained in the NIR channel but are shown in yellow for clarity. Scale bar represents 100 µm.

After 4 hrs, punctate fluorescence is visible up to 50 µm in from the periphery of the spheroid, consistent with localised regions of cellular uptake. Given that this compound, as described in section 5.6.3, appears to localise in the intracellular cytoplasm and does not associate strongly with the cell membrane, fluorescence visible in the yellow channel of these images has been attributed to intracellular localisation. The image collected after 24 hrs (Figure 5.27B) shows a more consistent fluorescence distribution profile across the diameter of the spheroid compared to at 4 hrs, with punctate fluorescence visible within a 120 µm rim of the spheroid.
periphery. This suggests that the processes of diffusion towards to the core of the tumour and cell uptake are working harmoniously to deliver the Ru(II) cytotoxin to the less accessible regions of the spheroid. Moreso, as the fluorescence of LRSK(Ru) \( \text{(5.10)} \) and the free Ru(II) complex precursor \( \text{(5.6)} \) is only a property of the complex and not the free metal centre or ligands, the observation of fluorescence conveys the position of the Ru(II) complex.

As shown in section 4.10.2, fluorescence distribution patterns are greatly influenced by the size of the spheroid. This can be seen again in Figure 5.27C, where the behaviour of the LRSK(Ru) \( \text{(5.10)} \) compound was investigated in a large spheroid due to the presence of a more distinct active periphery, quiescent zone and necrotic core. As for the medium-sized spheroids, cellular uptake is observed around the spheroid periphery, correlating with the most active cells. However, a region of no fluorescence is observed in a concentric band between 100-200 \( \mu \text{m} \) inside the cell periphery, which corresponds with the quiescent, non-proliferating region of cells which remain viable but are not actively involved in cell growth. The absence of fluorescence in this regions suggests that they are not actively taking up the LRSK(Ru) \( \text{(5.10)} \) compound, and instead it is diffusing into the necrotic core of the spheroid. Intriguingly, although uptake of the compound by necrotic cells was not expected, some diffuse fluorescence is observed in the core. This suggests that the accumulation of waste products and debris in the necrotic core is either modifying the composition of the extracellular medium, for example increasing its lipophilicity, such that it can induce fluorescence of LRSK(Ru) \( \text{(5.10)} \). It is also possible that the complex is undergoing interaction with cell debris, thereby associating with a lipophilic environment and causing the compound to fluoresce.
5.7 Summary

Cytotoxic platinum(IV) and ruthenium(II) derivatives of the model peptides presented in Chapter 4 have been successfully synthesised and their properties investigated in vitro. The conjugation of platinum(IV) cytotoxins to peptides was explored using a variety of “click” chemistry strategies, which would have allowed for a great deal of synthetic flexibility, but was ultimately unsuccessful. Repeatable, high-yielding, on-resin conjugation was achieved through peptide coupling between the axial succinato ligand of [Pt(OAc)(OSuc)(ox)(chxn)] (5.2) and the side-chain amino group of a C-terminal lysine residue. Conjugation to the ruthenium(II) complex [Ru(tBu2bpy)2(HOOC-4’-CH3bpy)]PF6 (5.6) was also achieved by peptide coupling, this time to the free carboxylic acid moiety on the 4-methyl-4’-carboxy-2,2’-bipyridine ligand.

A small family of platinum(IV)-peptide conjugates was synthesised, and their biological behaviour was tested in DLD-1 and A549 cells which show no MMP-2 activity and significant MMP-2 activity respectively. The cytotoxicity data in monolayer cells showed that none of the peptide-Pt(IV) conjugates were as cytotoxic as the free platinum(II) precursor in either cell line, which was not unexpected. There was a difference between the cytotoxicities of the platinum (IV) compound [Pt(OAc)2(ox)(chxn)] (5.3) in the two cell lines, with the significant decrease in cytotoxicity in DLD-1 cells attributed to the increased expression of drug efflux pumps in cells which are derived from gastrointestinal origin. This result correlated with the decrease in platinum accumulation measured between 24 and 48 hrs in DLD-1 cells treated with [Pt(OAc)2(ox)(chxn)] (5.3), suggesting that the efflux process is occurring at a greater rate than cellular uptake. The intact DIPVSLRSK(Pt) (5.8) peptide is slightly less toxic than its post-cleavage fragment LRSK(Pt) (5.7) in DLD-1 cells, which may be due to reduced cleavage in the absence of MMP-2, or it may also be undergoing increased cellular efflux as seen for the free platinum(IV) complex. However, in A549 cells the IC50 of DIPVSLRSK(Pt) (5.8) is almost identical to that of LRSK(Pt) (5.7), suggesting that MMP-2 cleavage is contributing to the activation and subsequent cytotoxicity of the compound. In both cell lines, the conjugate which contains the uptake-blocking group DDDDGDPVSLRSK(Pt) (5.9) is not cytotoxic, either through cleavage inhibition by the tetra-aspartate group, low cellular uptake leading to poor accumulation, or other unknown factors. Given that the intracellular platinum accumulation is not significantly lower for DDDDGDPVSLRSK(Pt) (5.9) compared to DIPVSLRSK(Pt) (5.8), it is possible that another mechanism is taking place, which requires further investigation.

The comparative studies conducted in 3D tumour models, spheroids, using SRIXE mapping and XANES analysis gave results which in some instances did not correlate with observations made
in 2D cell monolayers. While platinum accumulation studies did not show a significant difference between the levels of intracellular platinum following incubation with LRSK(Pt) (5.7), DIPVSLRSK(Pt) (5.8), and DDDDGDPVSLRSK(Pt) (5.9), no detectable platinum species were observed in the hypoxic/necrotic regions or periphery of spheroids treated with with LRSK(Pt) (5.7) and DDDDGDPVSLRSK(Pt) (5.9). This was attributed to the extremely low lipophilicity of the compounds, with low cellular uptake resulting in paracellular diffusion at low concentrations throughout the spheroid, below the detection levels of the technique. However, in light of the 2D platinum accumulation studies demonstrating that LRSK(Pt) (5.7) and DDDDGDPVSLRSK(Pt) (5.9) do undergo cellular uptake to some extent, a spheroid-specific activity may be taking place, which requires further investigation. The XANES data collected showed that the metabolism of the administered platinum(IV) compounds was too complicated to model using a simple calibration curve based on standard mixtures of platinum(IV) and platinum(II) compounds, with the reduced peak height ratio and broadening of the post-edge features indicative of the formation of platinum-thiol adducts, consistent with previous studies.

After observing the poor spheroid penetration of the hydrophilic platinum(IV)-peptide compounds, the substitution of the cytotoxic platinum(IV) complex for a ruthenium(II)-bipyridyl complex saw an improvement in spheroid penetration over 4 and 24 hr time periods. Peptide conjugation of the ruthenium(II) complex effectively reduced its lipophilicity, such that the compound was able to traverse the cell membrane, as opposed to remaining attached to the phospholipid bilayer as is typically seen for complexes of this class. Fluorescence confocal microscopy of LRSK(Ru) (5.10) showed that the compound was localised in the cytoplasm, while little of the free complex [Ru(tBu2bpy)2(HOOC-4'-CH3bpy)]2PF6 (5.6) was observed intracellularly. This increase in uptake is likely to have contributed to the increased cytotoxicity of the compound, reducing the IC50 value by a factor of 2. These ruthenium(II)-peptide conjugates therefore present an opportunity for future work, including synthesis and testing of a peptide family similar to that of platinum(IV) peptide conjugates.
Chapter 6

CONCLUSIONS
The results presented in this study demonstrate that improving the selectivity of established cytotoxic compounds by exploiting the unique characteristics of the tumour microenvironment is achievable. In particular, the localised activation of prodrug compounds by exploiting the upregulation of particular enzymes was found to be a viable design rationale, and was investigated using a family of model compounds as well as target compounds. In order to provide better insight into the activation of these compounds, recombinant DNA techniques were employed in order to visualise the targeted enzymes and compare their distribution in 2D and 3D cell studies relative to the administered compounds.

For the purpose of prodrug activation, the overexpression of the MMP-2 enzyme across a range of solid tumours was exploited. In order to visualise the expression of MMP-2, and the other member of the gelatinase family MMP-9, in cell monolayers and tumour spheroids, plasmid vectors which included the MMP-2 or -9 DNA fused to AmCyan, a fluorescent protein, were constructed. Expression of these plasmids in mammalian cells was achieved by transfection, but the Amcyan fluorescent protein proved not to be ideal for in vitro fluorescence imaging due to interactions with riboflavin in cell culture medium. While AmCyan was chosen in order to avoid spectral overlap with the FITC and rhodamine B fluorophores employed in the model peptide family, a variety of other fluorescent proteins may prove to be more suitable. While this technique allows for the visualisation of the secreted MMP enzyme, it does not discriminate between active and inactive forms of the enzyme and as such a supplementary technique was required to observe MMP activity. In this vain, MMPSense 680 proved effective for observing MMP activity in tumour spheroids.

This work has formed the basis for future modification of the MMP-2-AmCyan and MMP-9-AmCyan plasmids through substitution of the fluorescent protein, or substituting the MMP-encoding DNA for that of another protein of interest. Ideally, a fluorescent protein which does not exhibit spectral overlap with FITC, RhB or the NIR dye MMPSense 680 would allow for simultaneous imaging of secreted MMP-2 and -9, MMP activity and the model compounds investigated in Chapter 4.

Investigations into the biological properties of K(FITC)DIPVSLRSK(RhB) (4.5) and DDDDK(FITC)DIPVSLRSK(RhB) (4.8) were conducted systematically through testing a library of peptide-fluorophore conjugates. The polyanionic tetra-aspartate moiety was found to be effective for inhibiting cellular uptake, but also played a role in slowing the rate of cleavage of DDDDK(FITC)DIPVSLRSK(RhB) (4.8) by MMP-2, compared to K(FITC)DIPVSLRSK(RhB) (4.5). This reduction in the rate of cleavage was advantageous, as it resulted in a greater proportion of
the intact peptide penetrating inwards towards the less accessible spheroid core so that upon cleavage, the LRSK(RhB) (4.7) fragment was delivered to cells in the quiescent and hypoxic regions. This was not observed for K(FITC)DIPVSLRSK(RhB) (4.5), as the inhibition of MMP activity by coincubation with marimastat resulted in the intact peptide undergoing sequestration by the healthy, normoxic cells on the periphery of the spheroid, meaning none of the LRSK(RhB) (5.7) payload could penetrate into the spheroid.

The uptake-blocking group was also proven to be effective in monolayer cell studies in the absence of MMP-2 activity, designed to mimic healthy tissue. After 4 hrs K(FITC)DIPVSLRSK(RhB) (4.5) was found to have entered the cells intact, and as such was probably undergoing non-specific cleavage both inside and outside the cell. DDDDK(FITC)DIPVSLRSK(RhB) (4.8) however did not enter the cell intact, with the minimal amount of red fluorescence visible inside the cell a result of extracellular cleavage. This result supports the use of an uptake-blocking moiety for minimising compound uptake by healthy cells.

In A549 cell monolayers, which mimic tumour tissue due to their significant level of MMP-2 activity, less uptake of the intact K(FITC)DIPVSLRSK(RhB) (4.5) peptide was observed and instead discreet localised fluorescence was observed in the red and green channels, suggesting that cleavage was occurring in the extracellular space. This result was also observed, but to a lesser degree, for DDDDK(FITC)DIPVSLRSK(RhB) (4.8). These results confirmed that extracellular cleavage of the peptides by MMP-2 resulted in the selective delivery of the payload fragment LRSK(RhB) (4.7) to tumour cells in the presence of elevated MMP-2 levels.

Overall, this work confirmed the potential of using an MMP-2 specific cleavage sequence for the selective delivery of chemotherapeutic agents to solid tumours. Both K(FITC)DIPVSLRSK(RhB) (4.5) and DDDDK(FITC)DIPVSLRSK(RhB) (4.8) underwent increased cleavage in 2D and 3D cell systems where MMP-2 activity was present, while only DDDDK(FITC)DIPVSLRSK(RhB) (4.8) demonstrated an ideal degree of selectivity for tumour over healthy tissues.

Following the success of the model fluorophore-peptide conjugates, cytotoxic platinum(IV) and ruthenium(II) derivatives were investigated in similar in vitro systems. The biological action of the platinum(IV)-peptide conjugate library was tested in DLD-1 and A549 cells in order to mimic healthy and tumour tissue respectively. The DIPVSLRSK(Pt) (5.8) peptide proved to be slightly less toxic than its post-cleavage fragment LRSK(Pt) (5.7) in DLD-1 cells,
which may be due to increased cellular efflux as seen for the free platinum(IV) complex, or due to a reduction cleavage in the absence of MMP-2, leaving it reliant on cleavage by non-specific proteases. However, in A549 cells the IC$_{50}$ of DIPVSLRSK(Pt) (5.8) essentially equalled that of its fragment, LRSK(Pt) (5.7), suggesting that the presence of MMP-2 is cleaving the peptide and contributing to the cytotoxicity of the compound. The conjugate which contains the uptake-blocking group DDDDGDIPVSLRSK(Pt) (5.9) reminained non-cytotoxic independent of the presence of MMP-2 activity. This may be the result of cleavage inhibition by the tetra-aspartate group, low cellular uptake leading to poor accumulation, or other unknown factors. Given that the intracellular platinum accumulation is not significantly lower for DDDDGDIPVSLRSK(Pt) (5.9) compared to DIPVSLRSK(Pt) (5.8), it is possible that another mechanism is taking place, which requires further investigation, particularly given the improved spheroid penetration exhibited by it’s peptide fluorophore analogue DDDDK(FITC)DIPVSLRSK(RhB) (4.8).

SRIXE mapping and XANES analysis again gave results which were not fully in agreement with the platinum accumulation data collected in 2D cell monolayer experiments. While platinum accumulation studies did not show a significant difference between the levels of intracellular platinum following incubation with LRSK(Pt) (5.7), DIPVSLRSK(Pt) (5.8), and DDDDGDIPVSLRSK(Pt) (5.9), platinum species were not observed in the hypoxic/necrotic regions or periphery of spheroids treated with with LRSK(Pt) (5.7) and DDDDGDIPVSLRSK(Pt) (5.9). This may be caused by the hydrophilic nature of the compounds, with low cellular uptake resulting in platinum concentrations below the detection levels of the technique. However, in light of the 2D platinum accumulation studies demonstrating that LRSK(Pt) (5.7) and DDDDGDIPVSLRSK(Pt) (5.9) do undergo cellular uptake to some extent, a spheroid-specific activity may be taking place, which requires further investigation. Additionally, further XANES experiments would need to be performed, perhaps with increasing the doses and exposure times, to maximise the concentration of platinum within the spheroid samples and thus obtain spectra of better quality.

Promisingly, the ruthenium(II)-peptide conjugate investigated in this work appeared to overcome some of the inherent drawbacks of ruthenium(II)-bipyridyl complexes upon attachment of the LRSK peptide. The improvement in cellular uptake, spheroid penetration and cytotoxicity make this compound an attractive candidate for future studies, particular as it may be monitored by simpler, more sensistive techniques such as fluorescence confocal microscopy.
Overall, these outcomes demonstrate that the delivery of cytotoxic compounds to tumours, and even specific regions of the tumour is possible through exploitation of the tumour microenvironment. However, the results presented in Chapter 5 emphasise the importance of choosing a cytotoxic compound that will undergo enhancement of its biological activity upon modification into a prodrug form. In this instance, the success of the fluorophore-peptide conjugates indicate that the properties of the cytotoxic moiety should resemble that of the rhodamine B fluorophore in terms of cell uptake, in order to ensure that the target compounds behave as similarly as possible to the model compounds. The optimisation of this type of prodrug could potentially improve tumour therapy significantly, by decreasing the systemic side effects of current chemotherapies through minimising the interaction of administered drugs with healthy tissue. As such, exploitation of the tumour microenvironment for achieving the targeted delivery of anticancer agents is a promising avenue for future drug development.
Appendix A1: $^1$H NMR Spectra

**Appendix A1.1** $^1$H NMR spectrum of [PtCl$_2$(chxn)]: (300 MHz, solvent: DMSO)

**Appendix A1.2** $^1$H NMR spectrum of [Pt(ox)(chxn)]: (300 MHz, solvent: D$_2$O)
Appendix A1.3 $^1$H NMR spectrum of [Pt(OH)$_2$(ox)(chxn)]: (300 MHz, solvent: D$_2$O)

Appendix A1.4 $^1$H NMR spectrum of [Pt(OCH$_3$)(OH)(ox)(chxn)]: (300 MHz, solvent: D$_2$O)
Appendix A1.5 $^1$H NMR spectrum of [Pt(OCH$_3$)(OSuc)(ox)(chxn)]: (300 MHz, solvent: D$_2$O)

Appendix A1.6 $^1$H NMR spectrum of [Pt(OAc)(OH)(ox)(chxn)]: (300 MHz, solvent: D$_2$O)
Appendix A1.7 $^1$H NMR spectrum of $[\text{Pt(OAc)}(\text{OSuc})(\text{ox})(\text{chxn})]$: (300 MHz, solvent: $D_2O$)

Appendix A1.8 $^1$H NMR spectrum of $[\text{Pt(OAc)}_2(\text{ox})(\text{chxn})]$: (300 MHz, solvent: $D_2O$)
Appendix A1.9 $^1$H NMR spectrum of [RuCl$_2$(tBu-bpy)$_2$]: (300 MHz, solvent: MeOD)

Appendix A1.10 $^1$H NMR spectrum of [Ru(tBu$_2$-bpy)$_2$(HOOC-4'-CH$_3$-bpy)]$^{2+}$PF$_6$$: (300 MHz, solvent: MeOD)
Appendix A2: Crystal Structure

Appendix A2.1 Crystal structure of [Pt(OCH$_3$)(OH)(ox)(chxn)].

Appendix A3: LC-MS Traces

Note: chromatographic peaks at ~3.5 mins and 8.2 mins are solvent front and LC-MS artifacts respectively.

Appendix A3.1 LC-MS trace of LRSK(RhB)
Appendix A3.2 LC-MS trace of K(FITC)DIPVS

Appendix A3.3 LC-MS trace of K(FITC)DIPVSLSKRhB
Appendix A3.4 LC-MS trace of DDDDK(FITC)DIPVS

Appendix A3.5 LC-MS trace of DDDDK(FITC)DIPVSLRSK(RhB)
Appendix A3.6 LC-MS trace of DDDDK(RhB)

Appendix A3.7 LC-MS trace K(RhB)D
Appendix A3.8 LC-MS trace of DDDDK(FITC)D

Appendix A3.9 LC-MS trace of K(FITC)D
Appendix A3.10 LC-MS trace of LRSK(Pt)

Appendix A3.11 LC-MS trace of DIPVSLRSK(Pt)
Appendix A3.12 LC-MS trace of DDDDGDPVSLRSK(Pt)

Appendix A3.13 LC-MS trace of LRSK(Ru)
Appendix A4: HR-MS Spectra

Appendix A4.1 HR-MS Spectrum of \([\text{Ru}(\text{tBu}_2\text{bpy})_2(\text{HOOC-4'-CH}_3\text{bpy})_2]2\text{PF}_6\)

Appendix A4.2 HR-MS Spectrum of LRSK(Pt)
Appendix A4.3 HR-MS Spectrum of DIPVSLRSLK(Pt)

Appendix A4.4 HR-MS Spectrum of DDDDGDPVSLRSLK(Pt)
Appendix A5: FT-IR Spectra

Appendix A5.1 FT-IR spectrum of cyclised LRSpg after “click” reaction.
Appendix A6: ESI-MS (+) Spectra

Appendix A6.1 ESI-MS (+ve) spectrum of crude LRSK(Ru). The peak at m/z 503.67 corresponds to the uncoupled LRSK peptide.

Appendix A7: Confocal Microscopy Images

Appendix A7.1 Confocal microscopy images of DLD-1 spheroids dosed with A: K(FITC)DIPVSLRSK(RhB) (4.5) and B: MMPSense 680. The images were collected using the following parameters. 1: Excitation: 488 nm, emission collection 500-540 nm; 2: excitation: 559 nm, emission collection: 580-660 nm; 3: excitation: 633 nm, emission collection 680-700 nm.
Appendix A8: SRIXE Maps

Appendix A8.1 SRIXE maps of control DLD-1 spheroid.
References


References


84. Hall, M. D. and Hambley, T. W. Platinum(IV) antitumour compounds: their bioinorganic chemistry, *Coordination Chemistry Reviews* 2002, **232**, 49.


99. Alessio, E., Mestrioni, G., Nardin, G., Attia, W. M., Calligaris, M., Sava, G. and Zorzet, S. Cis-dihalotetrakis(dimethyl sulfoxide)ruthenium(II) and trans-dihalotetrakis(dimethyl
References


109. Hartinger, C. G., Zorbas-Seifried, S., Jakupec, M. A., Kynast, B., Zorbas, H. and Keppler, B. K. From bench to bedside - preclinical and early clinical development of the
anticancer agent indazolium trans-tetrachlorobis(1H-indazole)ruthenate(III) (KP1019 or FFC14A), *Journal of Inorganic Biochemistry* 2006, 100, 891.


215. Hall, M. D., Foran, G. J., Zhang, M., Beale, P. J. and Hambley, T. W. XANES Determination of the Platinum Oxidation State Distribution in Cancer Cells Treated


226. Bogdanov, A. M., Mishin, A. S., Yampolsky, I. V., Belousov, V. V., Chudakov, D. M.,
Subach, F. V., Verkhssha, V. V., Lukyanov, S. and Lukyanov, K. A. Green fluorescent
227. Drossler, P., Holzer, W., Penzkofer, A. and Hegemann, P. pH dependence of the
absorption and emission behaviour of riboflavin in aqueous solution, *Chemical Physics*
228. Yamamoto, N., University of Sydney, 2011.
distribution of activatable cell penetrating peptides is superior to that of cell
of the American Chemical Society* 1963, 85, 2149.
231. Fields, G. B. and Noble, R. L. Solid-phase peptide synthesis utilizing 9-
fluorenylmethoxy carbonyl amino-acids, *International Journal of Peptide and Protein
Activation on the Distribution of Fluorescently Tagged MMP-2 Cleavable Peptides in
S. Investigating Rhodamine B-Labeled Peptoids: Scopes and Limitations of Its
234. Shelley, W. B. Fluorescent staining of elastic tissue with rhodamine-B and related
xanthene dyes, *Histochemie* 1969, 20, 244.
235. Reungpatthanaphong, P., Dechsupa, S., Meesungnoen, J., Loetchutinat, C. and
Mankhetkorn, S. Rhodamine B as a mitochondrial probe for measurement and
monitoring of mitochondrial membrane potential in drug-sensitive and -resistant cells,
*Journal of Biochemical and Biophysical Methods* 2003, 57, 1.
Belmont, CA, 2012.
237. Matsuura, S.-i., Itoh, T., Ishii, R., Tsunoda, T., Sakaguchi, K., Hanaoka, T. and Mizukami,
F. Encapsulation of fluorescent proteins in folded-sheet mesoporous materials: Effect
of pore size on energy-transfer efficiency, *Microporous and Mesoporous Materials*
2010, 131, 245.


