

Guanine Quadruplex Targeted Ruthenium(II) Polypyridyl
Luminophores for Imaging and Therapeutics

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A thesis submitted to Dublin City University for the award of Ph.D.

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Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Doctor of Philosophy is entirely my own work, and that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

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Abbreviations

3D	Three Dimensional
ATCUN	Amino terminal Cu(II) and Ni(II) binding motif
bpy	2,2'-bipyridine
CD	Circular dichroism
CPP	Cell penetrating peptide
ctDNA	Calf thymus DNA
DCE	Dichloroethane
DIPEA	N,N-Diisopropylethylamine
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DNA	Deoxyribose nucleic acid
ddr	DNA damage response
DPP	4,7-diphenyl-1,10-phenanthroline
dppz	dipyrido[3,2- <i>a</i> :2',3'- <i>c</i>]phenazine
ESI	Electrospray ionisation
FID	Fluorescent intercalator displacement
FLIM	Fluorescent lifetime imaging microscopy
G	Guanine
G4	Guanine quadruplex
GFP	Green fluorescent protein
GMP	Guanosine monophosphate
HATU	1-[Bis(dimethylamino)methylene]-1 <i>H</i> -1,2,3-triazolo[4,5- <i>b</i>]pyridinium-3-oxide hexafluorophosphate
HPLC	High-performance liquid chromatography
IC	Internal conversion
IC ₅₀	Half-maximal Inhibitory concentration
ICD	Induced circular dichroism
IR	Infrared
ISC	Inter system crossing
LC	Ligand centred charge transfer
LED	Light emitting diode
MALDI	Matrix assisted laser desorption ionisation
MCTS	Multicellular tumour spheroids
MeCN	Acetonitrile
mitoG4	Mitochondrial guanine quadruplex
MLCT	Metal-to-ligand charge transfer
MPP	Mitochondrial penetrating peptide
mtDNA	Mitochondrial DNA
NHS	Matrix assisted laser desorption ionisation
NLS	Nuclear localising sequence
NMR	Nuclear magnetic resonance
PACT	Photoactivatable chemotherapeutics

PBS	Phosphate buffer system
PCET	Proton coupled electron transfer
PDT	Photodynamic therapy
PI	Phototoxic Index
phen	1,10-phenanthroline
phendione	1,10-phenanthroline-5,6-dione
PIC-COOH	Phenylimidazo[4,5-f]1,10-phenanthroline carboxylic acid
PLIM	Phosphorescence Lifetime Imaging Microscopy
rG4	RNA guanine quadruplex
RBP	RNA binding proteins
RHAU	RNA helicase associated with AU-rich element
RNA	Ribose nucleic acid
RNAP	RNA polymerase
ROS	Reactive oxygen species
SG	Stress granule
STED	Stimulated emission depletion microscopy
TAP	1,4,5,8-tetraazaphenanthrene
TBAC	Tertbutylammonium chloride
TCSPC	Time correlated single photon counting
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TLC	Thin layered chromatography
tpphz	Tetrapyrido[3,2-a:2',3'-c:3'',2''-h:2''',3'''-j]phenazine
VR	Vibrational relaxation

Lorcan Holden

Guanine Quadruplex Targeted Ruthenium(II) Polypyridyl
Luminophores for Imaging and Therapeutics.

Thesis abstract

Ru(II) polypyridyl luminophores are often overlooked as candidates towards live cell imaging and sensing. Their promising photophysical attributes include environmentally sensitive ³MLCT states, large Stoke-shifts and photostability, allowing them to overcome some limitations of their organic fluorophore counterparts such as high background noise and photobleaching for applications in imaging. Further, these attributes can facilitate the multi-modal imaging and sensing such as phosphorescence lifetime imaging microscopy, resonance Raman mapping, and super resolution STED microscopy providing exciting opportunities to explore cellular environments and dynamics. Some of the most thoroughly studied Ru(II) complexes contain extended planar dppz ligands promoting DNA intercalation. More recently, derivatives of these complexes have targeted the non-canonical DNA and RNA secondary structure, guanine quadruplexes, though often a barrier to their exploration in a biological environment is poor cellular uptake or endosomal entrapment.

Presented in this thesis is the development of Ru(II) complexes designed to target guanine quadruplex nucleic acids while also promoting live cell uptake. Chapter 2 explores designing Ru-PDC3, a $[\text{Ru}(\text{phen})_2(\text{dppz})]^{2+}$ derivative, that contains the highly selective G4 targeting ligand, phen-DC3. This chapter examines the DNA binding properties and tests its potential as a multi-modal probe for G4 imaging across a series of cell lines. In Chapter 3 an analogue of Ru-PDC3 is synthesised, containing two TAP ligands with the aim of developing a G4 targeting probe capable of undergoing photoexcited redox chemistry in hypoxia, proving to be an excellent candidate for photodynamic therapy. Further, it was established that the complex was capable of mitochondrial localisation, providing the exciting prospect of targeting mitoG4s present in the mitochondrial genome. Chapter 4 investigated the bioconjugation of a $[\text{Ru}(\text{bpy})_2(\text{Pic-COOH})]^{2+}$ complex to a G4 binding peptide derived from the RHAU helicase. Conjugation of RHAU promoted DNA/RNA G4 binding and stability. Importantly, the conjugate was capable of cellular uptake where it was found to induce and localise to stress granules in a concentration and time dependent manner. This is the first reported probe of this type to image the formation of stress granules without the requirement for an external stressor.

Chapter 1 Literature Review of Guanine Quadruplex Targeted Molecules.

Abstract

The aim of this thesis is to generate novel luminescent Ru(II) probes that target guanine quadruplex nucleic acid secondary structures, for use in therapeutics and diagnostics (theranostics). Chapter 1 is a literature review that will introduce topics relating to the aims of the thesis by covering the scientific background and literature surrounding these topics. Firstly, DNA and its structure will be briefly introduced before a more detailed discussion on the structure and biological functions of guanine quadruplex DNA/RNA. This is followed by a review of some different approaches to target guanine quadruplexes using chemical probes. As developing diagnostics probes is a key aim of this thesis, the literature review will cover the visualisation of G4s after a brief primer on basic molecular photophysics. Ru(II) complexes that have been previously investigated for their guanine quadruplex binding capabilities will be introduced and discussed, due to their significant relevance to the central aim of this thesis. This literature review will conclude with reported strategies to achieve live cell uptake of Ru(II) complexes.

1.1 DNA and DNA Guanine quadruplexes as a biological targets

1.1.1 Deoxyribose Nucleic Acid

Deoxyribose nucleic acid (DNA) is an essential biomolecule that carries genetic information and is responsible for functionality, growth, and reproduction of cells. The first DNA form, B-form, was solved by Watson, Crick and Franklin 1953.¹ B-form DNA is a right handed double helix containing two complimentary strands of nucleotides that run antiparallel (Fig.1.1). Nucleotides contain a phosphate backbone, a five-membered sugar ring and a base. B-form DNA is comprised of Watson-Crick base pairs where guanine forms a base pair with cytosine and adenine forms a base pair with thymine. The double helix secondary structure is stabilised through hydrogen bonding, Van der Waals forces and π - π stacking (Fig. 1.1).

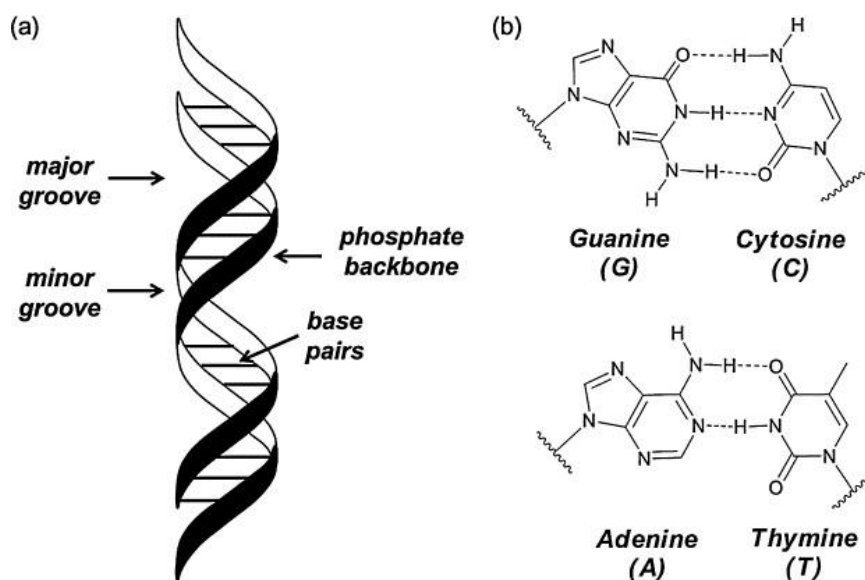


Figure 1.1 (a) Illustration of the B-DNA helix with grooves, phosphate backbone and base pairs. (b) Watson-Crick base pair bonding of guanine with cytosine and adenine with thymine. (Adapted from Ref. 2).

1.1.2 Guanine Quadruplex Structures

DNA and RNA structures that deviate from B-DNA can be classified as non-canonical. These structures include but are not limited to triplex DNA (H-DNA), i-motifs, Holliday junctions and guanine quadruplexes (G4s), as illustrated in Fig. 1.2.^{3,4} Interest in G4s has grown significantly with increasing links to several diseases including accelerated aging disorders, cancer, neurodegenerative disorders and rare genetic disorders such as fragile X syndrome making them an attractive target for therapeutics.⁵ In this section the structure and formation of guanine quadruplexes (G4s) is discussed followed by their biological roles.

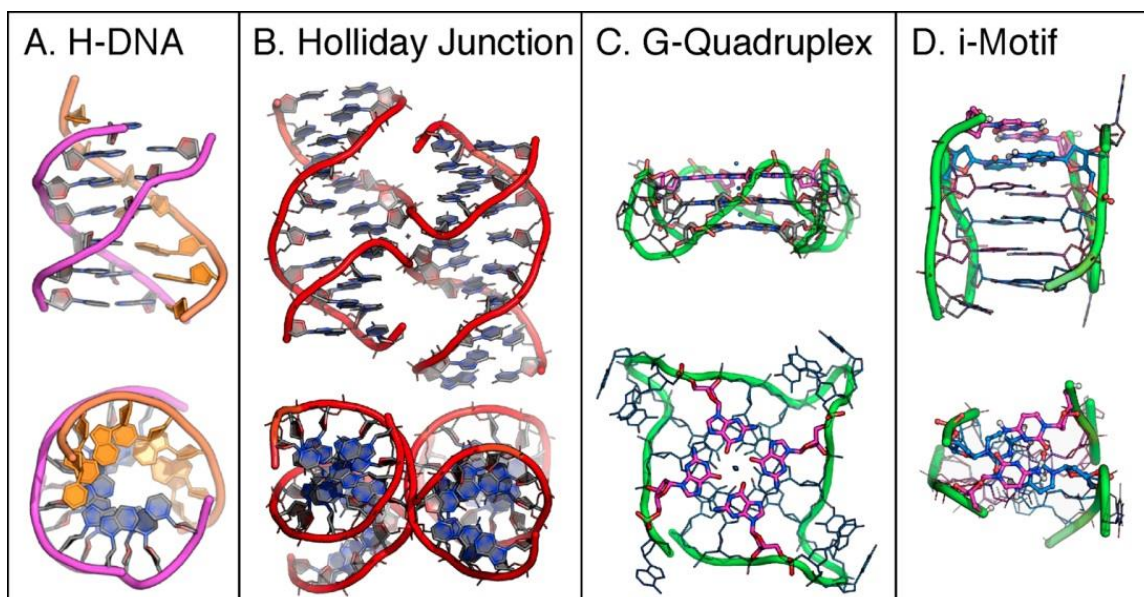


Figure 1.2 (a) The structures of triplex DNA (H-DNA), (b) the Holliday junction, (c) human telomeric G-quartet and (d) the i-motif, are viewed along and down their helical axes. (Adapted from Ref. 6).

The aggregation of guanine monophosphate was first observed over 100 years ago, followed 50 years later by the discovery that guanine monophosphate can form four-stranded helices.⁷ This discovery of four stranded helices was initially considered a

structural curiosity but it has since been shown that G4s can form from guanine rich sequences of DNA/RNA under physiological conditions. G4 formation is facilitated by the stabilisation of guanine bases into tetrads through Hoogsteen hydrogen bonding. These tetrads are further stabilised through stacking by π - π stacking and Van der Waals forces. Monovalent cations are located in the central channel between tetrads providing further stabilisation with a preference order of $K^+ > Na^+ > Li^+$ (Fig.1.3).^{7,8}

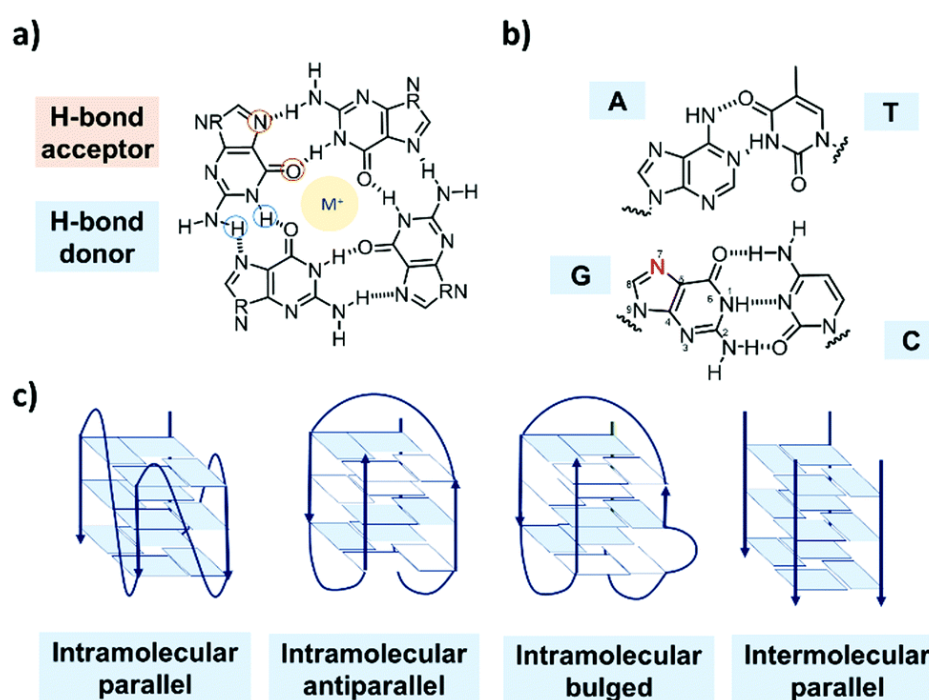


Figure 1.3 (a) Hydrogen bonding involved in tetrad stabilisation; (b) Base pair hydrogen bonding typically observed in B form DNA; (c) examples of different G4 topology, including parallel, anti-parallel and intermolecular parallel. (Adapted from Ref. 9).

G4s can form in a variety of topologies that can be categorised based on the orientation of their DNA strands. They can form from a single strand (intramolecular) or from multiple strands (intermolecular), within these classifications, strand orientation and loop location dictate if they are parallel, antiparallel or hybrid structures. Additionally, further topological variation in quadruplexes of the same orientation can occur through bulges and changes in loop length and base composition of loops.⁷ Intramolecular parallel G4s occur when strands have a loop connecting the bottom of one tetrad to the top of the adjacent tetrads,

referred to as propeller type loops. All parallel G4s have guanine glycosidic bonds which are in an anti-position. Intramolecular antiparallel structures form when at least one strand runs in the opposite direction, these quadruplexes can have lateral loops connecting adjacent G-strands or diagonal loops that connect opposite strands. Where an antiparallel G4 has three lateral loops, it can be referred to as a chair type, whereas an antiparallel G4 containing a diagonal loop, can be referred to as having a basket conformation. Antiparallel quadruplexes contain both anti and syn guanine glycosidic bond angles. To add an additional layer of complexity, some G4s report hybrid structures that have a combination of loop type and strand direction.¹⁰

In addition to DNA G4s, it has been reported that guanine rich sequences of RNA can also form quadruplexes. RNA guanine quadruplexes (rG4s) differ from their DNA counterparts in two important ways, the first is the presence of a 2'-hydroxyl group in the structure of their ribose sugar and secondly is the inclusion of the base uracil in place of thymine. The presence of the 2'-hydroxyl group has been linked to the increased stability of rG4s due to the increased intramolecular interactions it can introduce. rG4s are also typically parallel structures, this is attributed to the steric restraints introduced by the 2'-hydroxyl group causing a preference for the anti-conformation. Another important consideration when comparing G4s and rG4s in the context of cellular formation, is that DNA is typically double stranded whereas RNA has a more complicated structure including single stranded RNA, hairpins and loops.¹¹

1.1.3 Biological Functions of Guanine Quadruplexes

The earliest studies of G4s focused on telomeric DNA strands *in vitro*.^{12,13} Telomers are considered the aglet of DNA due to their location at the end of linear chromosomes and contain repetitive nucleotide sequences with relatively high guanine population. The functions of telomers are to prevent the degradation of chromosomes, maintain

chromosomal stability, prevent DNA end-joining and prevent accidental DNA recombination.¹⁴ Initial studies indicated formation of G4s in vitro but only over the last decade has evidence accumulated for their formation in cells.^{13,15} Difficulties in imaging occur due to low concentrations in telomers and their location in telomers are heavily sheltered from probe access by chromatin. The G-rich sequence of human telomeric DNA has the sequence (GGTTAG)_n which has been shown to form G4s under certain physiological conditions in vitro. An important feature of this sequence is the G overhang at its terminus, which is responsible for the formation of protective telomere structure and telomerase action. The telomere is protected by a protein complex called shelterin which prevents DNA degradation, the activation of DNA damage response (ddr) and non-homologous end joining.¹⁶ The protein telomerase is responsible for elongating telomeric sequences. This protein is highly expressed in many tumour cells and is implicated in cell proliferation. The abnormal processing of the G overhang by telomerase can confer immortality, leading telomeric G4s to be a potentially important drug target to inhibit telomerase activity in certain cancer cells. In fact, this has been shown to be the case when G4s are stabilised with small molecules preventing transcription.¹⁷

Telomers belonging to somatic cells undergo length shortening as they replicate. This limits the number of replications that can occur before the telomere is too short to divide without resulting in the damage of a coding region of DNA. When a cell can no longer divide, it has achieved senescence. In post-mitotic cells such as stem cells and neurons, telomerase is present. Telomerase functions by increasing the length of telomers thus facilitating infinite cell proliferation.¹⁸ While telomerase is not normally coded in all somatic cells, mutations can occur which code telomerase thus resulting in tumour growth. Small molecules such as telomestatin stabilise telomeric G4s preventing the function of telomerase resulting in rapid cell senescence.¹⁹

During replication, DNA is separated into two strands by replicative helicases. The leading strand provides the template for leading strand synthesis and the other strand is called the lagging strand. When undergoing replication, the leading strand undergoes replication continuously, but the lagging strand can undergo replication discontinuously, this provides an opportunity for G4s to form more readily in the lagging strand. It is still unclear, whether G4s have active roles in transcription, however they must be resolved to permit further replication of DNA.²⁰ Helicases specific for unwinding G4s resolve this issue, the existence of G4 helicases further supports the formation of G4s in *cellulo*. The intervention of small molecules here would stabilise the quadruplex and prevent a helicase enzyme from unwinding thus stalling replication.²¹

Transcription is the process in which DNA is copied into RNA by RNA polymerase (RNAP). This process is essential for gene expression. Bioinformatics studies suggest that over 40% of human genes contain putative guanine quadruplexes (pG4) at promoter sites when investigating the human genome *in silico*.²² Putative G4s are potential G4 forming sequences found in the genome containing a G-rich motif, usually in the form of $G_3+N_{1-7}G_3+N_{1-7}G_3+N_{1-7}G_3+$.²³ pG4s are enriched in promoter regions where it has been shown that >40% of human gene promoters contain at least one pG4. Further, when compared to the whole genome, sites that are both nuclease hypersensitive regions and within promoters contain an enhanced enrichment of pG4s.²²

There are several possible ways G4s can influence transcription. In Fig. 1.4, three examples are shown where G4s impede translation by blocking RNAP. These examples have been demonstrated through *in vivo* experimentation.²⁴ As shown in Fig 1. 4, G4s can form at multiple stages to prevent the function of RNAP, including intramolecular G4s before transcription and intermolecular G4 formation after the two strands of DNA have undergone annealing.

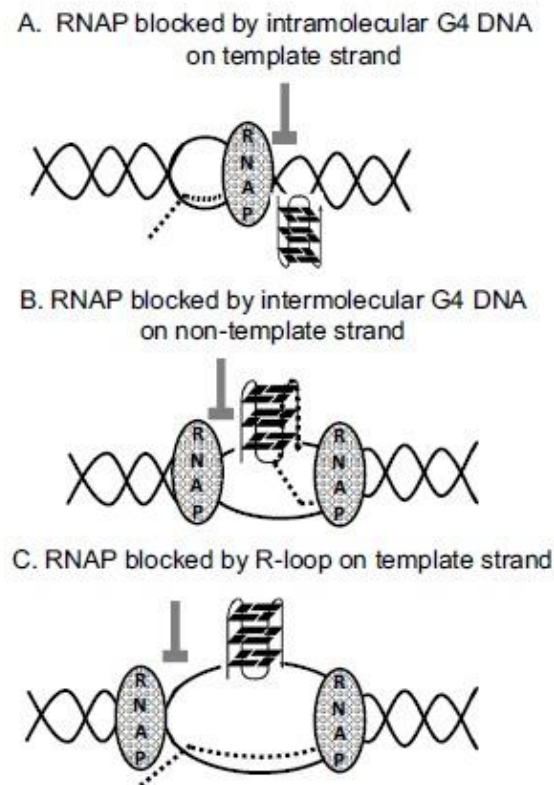


Figure 1.4 How the formation of G4s can impact and block RNAP. (Adapted from Ref. 22).

It is also postulated that G4 formation can result in higher expression levels of genes which would indicate that G4s could be responsible in transcription activation. This hypothesis could possibly be explained whereby the folding of non-coding, template strand keeps the strand single stranded, thus allowing further rounds of polymerisation. Du *et al.* suggest this claim by considering the fact that from 8,000 genes more than 5,000 genes contained G4 forming sequences 500 base pairs downstream of the transcription start site.²⁵

Transcriptional G4s of interest include c-MYC encoding G4s. The MYC is a family of genes that code for transcription factors. The transcription factors c-MYC encodes act as transcription activators and transcription repressor. Over expression of c-MYC is evident in a number of cancers, an effective treatment is the downregulation of the gene. The use

of G4 binding ligands contribute to the suppression of downstream regulation by G4 stabilisation.²⁶

Translation is also heavily impacted by the formation of G4s. Translation occurs when mRNA is decoded by ribosomes to produce an amino acid chain or peptide that can later fold into an active protein. Translation occurs over three phases: initiation, elongation and termination. There are three important sections of mRNA the 5' UTR (untranslated region), 3' UTR; and open reading frames (ORF). *In vitro* studies have shown the formation of G4s across these three regions of mRNA. In 5' UTRs, G4 formation can impede translational processes by inhibiting ribosome scanning function resulting in decrease of protein expression. The function of G4s in ORF contribute to elongation, translational folding and ribosomal frameshift.²⁷ The 3' UTR of mRNA facilitates protein binding which influences stability and transport of mRNA. The role G4s in 3' UTR is translational control as well as alternative splicing and mRNA targeting.²⁷ The occurrence of this inhibition was first reported for an rG4 in mRNA of fragile X mental retardation protein. Since then, numerous putative rG4s from the 5' UTR of mRNAs have been shown to inhibit the translation of proteins including NRAS, BCL2 and ADAM10.^{28–30}

More recently there has been an emerging link between cytoplasmic rG4s and the formation of stress granules (SGs).³¹ SGs are non-membranous biocondensates comprised of proteins and RNA formed by liquid-liquid phase separation (LLPS). They typically form as by cell stress response pathways from a number of stressors including extrinsic stressors like heat shock, high arsenic salt concentrations and exposure to UV-light or intrinsic stressors such as redox imbalances or proteostasis.³² It is currently thought that cells form SGs as a response to conserve energy and protect important biomolecules, thus promote cell survival. There have been studies investigating the contribution of G4s to both *in cellulo* and *in vitro* but the dynamics of their contributions is yet to be fully understood.³¹

In addition to DNA and RNA G4s, mitochondrial G4s (mitoG4s) also make for an exciting therapeutic target.³³ Although it is a somewhat reductive view, the mitochondria are commonly referred to as the powerhouse of the cell because of their role in the oxidative phosphorylation (OxPHOS) and the generation of adenosine tri-phosphate (ATP), an essential biomolecule for cellular functions.³⁴ It is important to note, mitochondria are also involved in apoptosis, calcium homeostasis, and heme synthesis. In order to maintain these functions, mitochondria maintain their own genome in the form of mitochondrial DNA (mtDNA) that is responsible for encoding 13 proteins. mtDNA is a circular nucleoid and consists of two strands, a heavy strand (H) and a light strand (L). The H strand contains a higher percentage of guanine and thymine whereas the L strand is composed with a lower guanine and thymine content. The majority of mitochondrial genomes can range between 14 and 17 kb in size.³⁵ Typically, in mammalian cells the length is 16.5 kb with approximate 170 putative G4s on the H strand and 5 putative G4s on the L strand. Considering the high guanine content within the H strand and the high K⁺ concentrations within mitochondria (150-180 mM), it is unsurprising that the formation of G4s is favourable. Mitochondria also contain the G4 unwinding proteins TWINKLE and DNA2. Knockdown experiments of TWINKLE results mtDNA depletion, indicating its necessity for replication. Similarly, mutations to DNA2 have been shown to impair mitochondrial function.³⁶ An important consideration for targeting mitoG4s is their lack of protective histones and reduced repair machinery, resulting in vulnerability to damage. Tan *et al.* have made recent efforts to target mitoG4s by through conjugation of triphenylphosphine (TPP) to the G4 ligands pyridostatin and ISCH-1 to generate MitoPDS and MitoISCH (Fig. 1.5).³⁷ MitoISCH was designed as a switch on fluorophore and found to localise in mitochondria where it was found that formation of mitoG4s were more prevalent in cell lines that rely on high glycolysis for energy production (cancer cells). The use of MitoPDS allowed further investigation and indicated that increased G4 folding resulted in enhanced

glycolysis. It should be noted that the authors did not run toxicity assays for mitoPDS which could have further probed mitoG4s as therapeutic targets for G4 stabilisation.

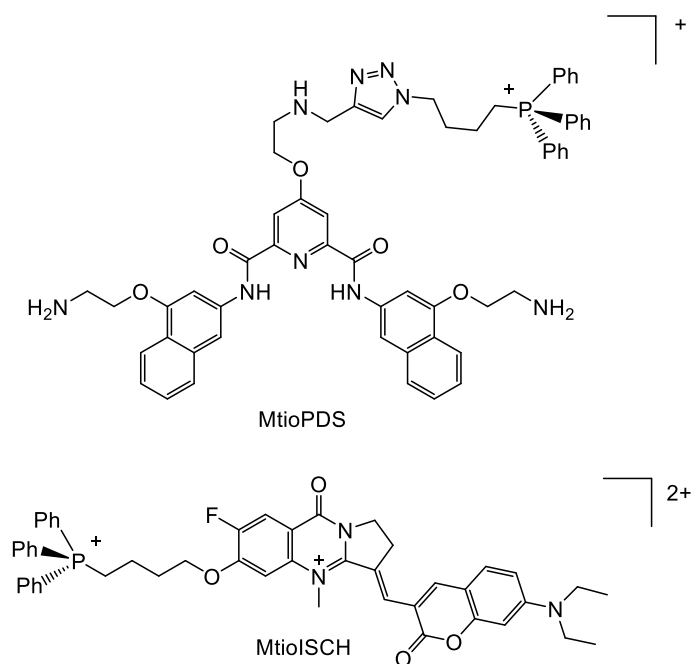


Figure 1.5 Structure of the mitoG4 targeted probes mitoPDS and mitoISCH.

1.2 Guanine Quadruplexes as Drug Targets

1.2.1 Guanine Quadruplex Binding Modes

To develop small molecule drugs targeting guanine quadruplex structures, it is essential to first consider their potential binding sites. There are multiple potential binding sites on every G4 structure that can often be sequence specific, these include binding through π -stacking/end-capping, loop recognition and intercalation within tetrads as illustrated in (Fig. 1.5).

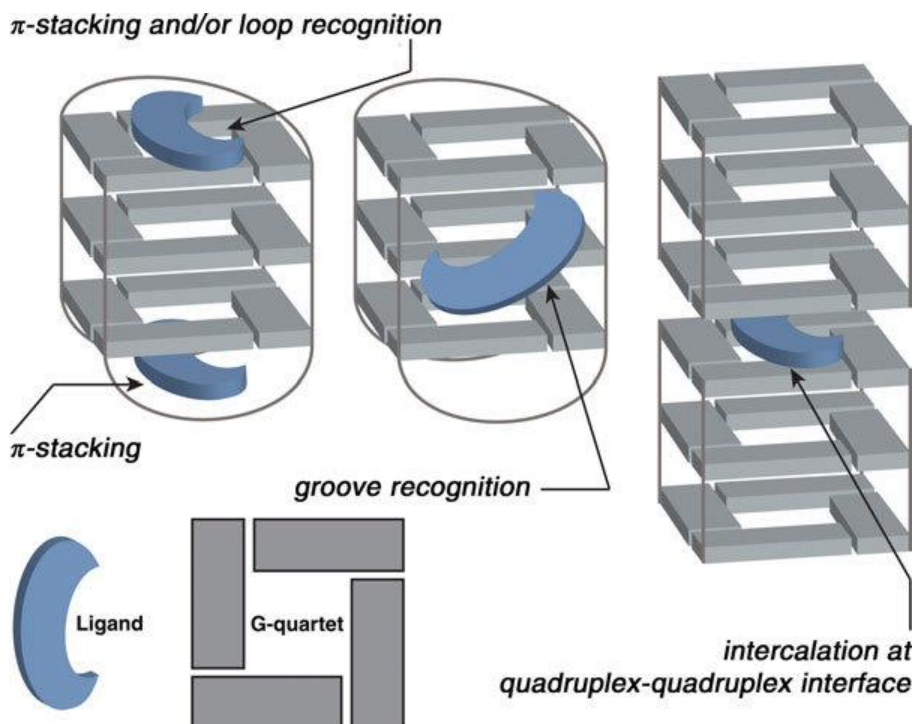


Figure 1.6 Possible binding modes present on a guanine quadruplex or a guanine-guanine quadruplex stack. (Adapted from ref. 38).

π -stacking/end-capping occurs over the aromatic surface of guanine tetrad where interactions with ligands are stabilised through Van der Waal interactions. As these interactions are usually facilitated by aromatic guanine surfaces, ligands capable of these binding interactions typically contain large polyaromatic surface areas that stack on top of a tetrad. Often, these large polyaromatic ligands contain flexible and protonatable side chains that facilitate additional cooperative binding to G4 like electrostatic interactions with protonated sites of a ligand and the negatively charged nucleic acid backbone, for example MMQ. These interactions can further be stabilised through the loop structures of guanine quadruplexes which is sequence and structure dependant.

While intercalation between guanine tetrads has been reported, it is significantly less common than other binding modalities and typically requires loss of a monovalent cation between tetrads.^{39,40} The porphyrin, TMPyP4 has been reported to show this interaction in

certain conditions including binding between tetrads of G4-DNA nanowires (Fig. 1.7). More recently, through extensive NMR investigation, Phen-DC3 has been reported as an intercalator of 23TAG and induces a conformational change to an antiparallel chair-type G4.⁴¹

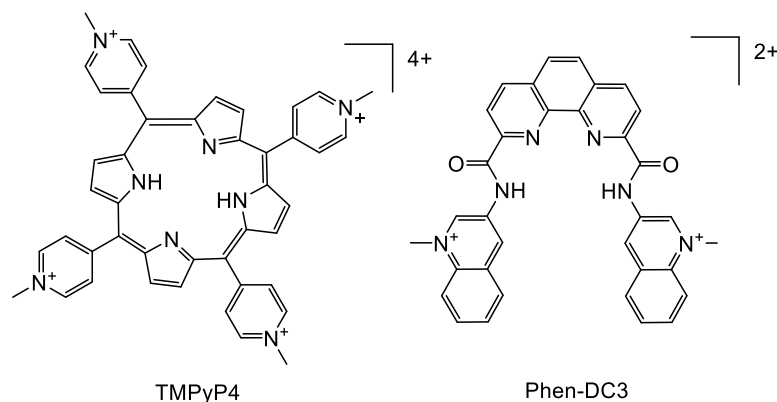


Figure 1.7 G4 binding molecules TMPyP4 and Phen-DC3.

Groove binders are less common than end-capping/stacking ligands but possess some key advantages. Notably, they are not required to be highly aromatic, which means such molecules can achieve better aqueous solubility. One key example of a G4 binder is Distamycin A, a well-known antibiotic, that has been reported to bind within the groove of the intermolecular G4 [d(TAGGGTT)₄].⁴² Distamycin A has provided a platform to design and investigate analogue groove binders further, achieving higher selectivity.⁴³ The complex Pt-MPQ combines an aromatic end-capping MPQ ligand to a platinum moiety where the ethyl linker is capable of groove binding (Fig. 1.8).⁴⁴ Selectivity issues can arise with this class of compounds as they commonly bind to helical DNA, for example, Distamycin A also binds to the minor groove of DNA and AT-rich sequences.⁴⁵

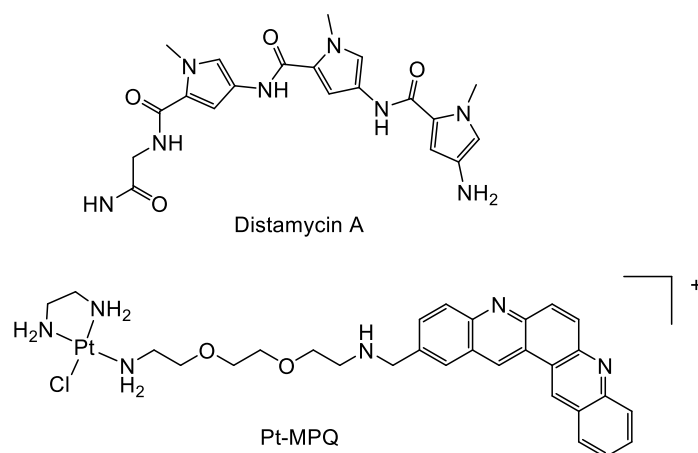


Figure 1.8 Guanine quadruplex groove binders

There are numerous ways to classify guanine quadruplex ligands such as structurally by focusing on functional groups or key scaffolds (methylated amines/porphyrins), quadruplex structural changes upon ligand binding (stabilising/destabilising/conformational change) or biological implications (anti-cancer therapeutic/gene regulation). The following section will focus on how guanine quadruplex ligands can bind and how binding can impact G4 structure.

1.2.2 Stabilising Ligands

Stabilisation is the most common effect G4 targeting ligands have on the G4 structure. Stabilising ligands function through telomerase inhibition and attrition, becoming a direct mechanism to target cancer cells through telomere-induced senescence and apoptosis. It has been suggested that cancer cells can be selectively targeted as many have deficiencies of DNA damage response apparatus.⁴⁶ The first G4 targeted therapeutic, reported in 1997, stabilised G4s to impair telomerase activity. This 2,6-diamidoanthraquinone caused almost complete inhibition of telomerase at 100 μM and resulted in an IC_{50} at 23 μM . Through ^1H NMR, stabilisation was shown by the 2,6-diamidoanthraquinone with authors suggesting the compound functions like a K^+ ion.¹⁷

Telomestatin, a macrocyclic heterocycle, is another notable telomere stabilising molecule. It is a natural product isolated from *streptomyces anulatus* 3533-SV4, that binds through end-capping and stabilises G4 through Van der Waal forces. The macrocyclic structure of telomestatin binds poorly to helical DNA resulting in excellent efficiency.¹⁹ Subsequently, stabilising ligands have been found to cause transcriptional regulation in proto-oncogenes like C-MYC resulting in anti-proliferation. Extensions to anthraquinones have been explored for C-MYC stabilisation resulting in specific anti-cancer activity.⁴⁷ The use of FRET melting curves is a common way to assess the stabilisation of G4s, where the denaturation temperature of a G4 is increased when bound to a ligand. For example, Phen-DC3 has been reported to increase the $\Delta T_{1/2}$ °C of a FRET labelled telomeric quadruplex (0.2 μ M) in a buffer of 10 mM lithium cacodylate buffer (pH 7.2) with 10 mM KCl/90 mM LiCl. Binding of phen-DC3 increased the $\Delta T_{1/2}$ °C 6.0 °C (1 mol. eq), 20.0 °C (2 mol. eq), and 37.2 °C (5 mol. eq).⁴⁸

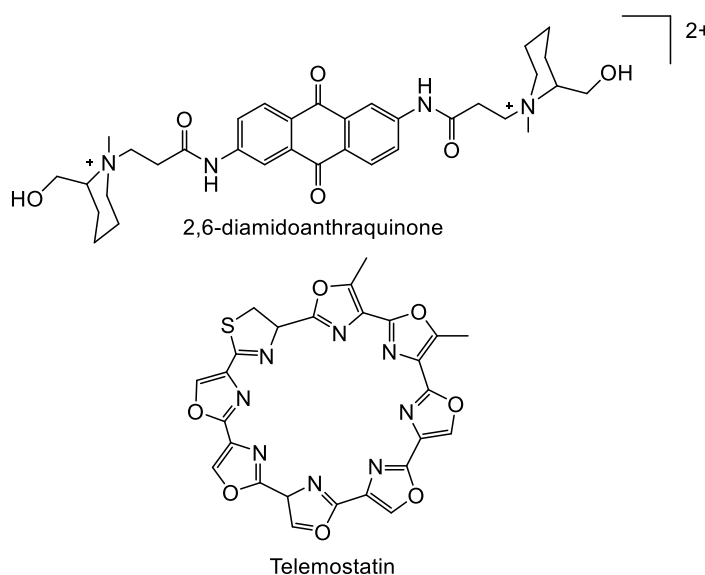


Figure 1.9 Guanine quadruplex stabilising ligands.

1.2.3 Alkylating Ligands

G4 ligands capable of alkylation have had great success in the specific targeting of quadruplexes, further they can offer the possibility for stimuli driven alkylation such as thermal or photochemical. The Freccero group developed a family of Naphthalene diimides (NDI) conjugated with alkyl linkers of varying length to quinone methide precursor alkylating groups capable of selectively targeting and alkylating a telomeric G4.⁴⁹ These NDI derivatives were capable of reversible alkylation when triggered by thermal digestion at 40 °C. The same group generated a new series of NDIs that contained an alcohol functional group in place of the quaternary amine to aid cellular uptake. They also implemented oxiranes as a new alkylating group to generate non-reversible covalent bond formation to the guanine quadruplex.⁴⁹ The group of NDIs targeting and alkylated an adenine residue located in the loop of the quadruplex (Fig. 1.10).

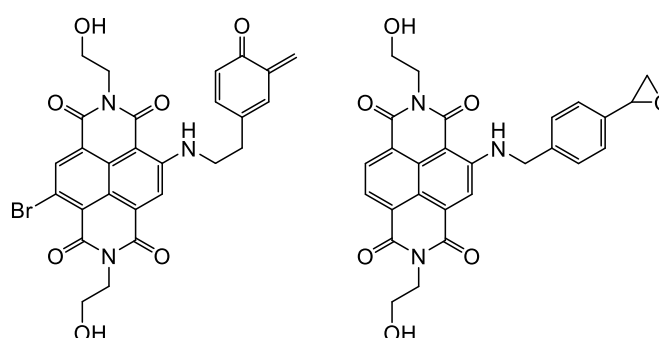


Figure 1.10 Guanine quadruplex NDI alkylating ligands.

1.2.4 Cleavage of G4s

Cleavage of telomeric DNA is a desirable therapeutic prospect, primarily due to the relationship shared between telomeric length, cell age and cancer proliferation.⁵⁰ G4 binders capable of selective telomeric cleavage can induce cell senescence.⁵¹ The Cowan group have produced the most notable work to date in this regard through the conjugation of selective G4 binders with the redox active Cu(II) moiety, ATCUN (Fig. 1.11). The group

first reported on a copper-acridine complex, where cleavage was investigated when bound to human telomeric DNA and MCF-7 cells. The group found the majority of cleavage at the sites A1-G7 and T6-A7 nucleotides. MALDI-MS experiments indicated hydrolysis and oxidative cleavage was occurring, both of which require co-reagents. Notably, both apoptosis and senescence were induced in MCF-7.⁵² The group developed two membrane permeable naphthalene diimide derivatives, the first containing a mono-substituted Cu complex and the second containing a disubstituted complex. These Cu-NDIs maintained better G4 selectivity, the disubstituted complex reported a dissociation constants of 0.15 μM and 63.8 μM when bound to a telomeric G4 and to ct-DNA respectively. The authors also report rapid cleavage of telomers inducing cell senescence.⁵³

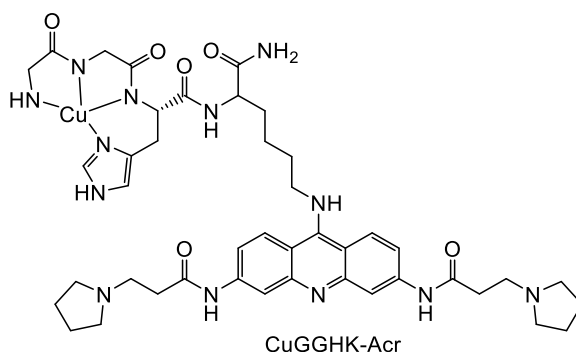


Figure 1.11 ATCUN containing Cu(II) moiety Guanine quadruplex cleaving ligand.

1.2.5 G4 Disrupting Ligands

Small molecules capable of destabilisation and disruption of guanine quadruplexes have garnered greater attention in recent years. This has occurred in parallel with a recent understanding of G4 helicases capable of unwinding fully formed G4s into single stranded DNA. Cells that don't express G4 unwinding helicases have been linked to numerous diseases such as Bloom syndrome and Hoyerlaal-Hreidarsson syndrome leading to recent

investigations into G4 destabilisers as potential therapeutics for such diseases.⁵⁴ To date only a small number of destabilising ligands have been reported.^{48,54} Furthermore, destabilisation can be reliant specific conditions, e.g. media or pH, resulting in an unclear picture of the relationship between disrupting ligands and their biological effects. This is the case for example for the porphyrin TMpyP4 which has been shown from extensive investigation to act as a G4 stabilising probe. Recently it was shown to destabilise RNA G4s that fold from (CGG)_n repeats implicated in Fragile X syndrome yet conversely the porphyrin is capable of stabilising telomeric G4s. More recently, Monchaud *et al.* developed a panel of assays to identify small molecules capable disrupting G4s.⁴⁸ Interestingly they identified the small molecule destabiliser PhpC through a combinatorial assay approach, testing the binding interactions through CD titrations, FRET melting assays, and fluorescence G4- unfolding assays. PhpC is a cytosine analogue that strongly binds to guanine quadruplexes and in doing so can trap and stabilise a flipping guanine disrupting a tetrad to induce destabilisation. Its binding mode can be described as a G-clamp (Fig. 1.12).

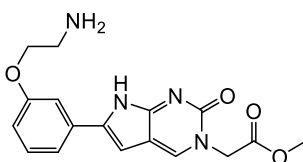


Figure 1.12 Chemical structure of PhpC.

1.2.6 Oxidation of Guanine Quadruplexes

Guanine has the lowest oxidation potential of the nucleobases and when multiple guanines are present in sequence in a DNA oligomer their oxidation potential is further reduced.^{55,56} When guanine undergoes oxidation, numerous products and pathways are available for the fate of the oxidised species, the major products are 8-oxo-7,8-dihydroguanine and dehydroguanidinohydantoin.⁵⁷ This can occur through the interaction

of ROS directly with guanine bases to yield 8-oxo-7,8-dihydroguanine. Alternatively, oxidation can occur at a remote site generating an electron hole in DNA where migration of the electron hole can induce a chemical reaction and oxidation of guanine.⁵⁸ This most commonly occurs at a 5' guanine of a G-rich sequences. Use of G4 ligands capable of ROS generation through photosensitisation, have been investigated as potential photoactivatable drug molecules capable of DNA damage and photocleavage. For example, TMPyP4 is reported to promote photocleavage of DNA and has been shown to have greater phototoxicity than dark toxicity, although it can bind to both duplex DNA and G4 DNA. The zinc(II) porphyrin, ZnP1, demonstrated binding to telomeric G4 DNA and light induced oxidation resulting in guanine cleavage.⁵⁹

Photoinduced oxidation through direct electron transfer at guanine quadruplex DNA is another pathway for guanine oxidation. Of particular interest in this regard, is Ruthenium(II) complexes bearing a tetraazaphenanthrene (tap) ligand, these complexes that have been specifically designed to bind G4s will be discussed in greater detail in later sections.

1.2.7 Peptide Based Ligands.

Another class of G4 targeted ligands are peptides, inspired from G4 binding domains of G4 binding proteins.⁶⁰ Peptides offer intriguing potential to target G4s, they can offer high selectivity by replicating biological interactions between proteins and nucleic acids to provide insight into biological functions. There are some noteworthy disadvantages of using peptides though, including their instability and capacity toward degradation in a cellular context, e.g. through proteolysis. Further, attempts to improve stability through modifications such as stapling can result in reduced G4 selectivity. The RNA helicase associated with AU-rich element (RHAU) or DHX36 has been reported to resolve both DNA and RNA G4s with high specificity.⁶¹ The RHAU protein has a specific G4 binding

domain called the RHAU specific motif (RSM) which has subsequently inspired investigations into RHAU based derivatives, that will be discussed in detail in chapter 4.⁶²

RGG and RG-rich based peptides have also provided interesting motifs to develop G4 ligands derived from G4-binding proteins. RGG peptides are derived from the FMRP RNA binding protein that contains an RGG box with the sequence RRGDGRRRGGGGRGQGGRGRGGGGFKG.⁶⁰ Initial NMR studies investigated an RGG peptide with a guanine rich RNA sequence (sc1) that forms a mixed quadruplex-duplex junction.⁶³ These studies indicated that the peptide binds along the major groove of the RNA duplex before the peptide is directed across the face of a quadruplex peptide. This interaction is illustrated in Fig. 1.13. Following this, the crystal structure of this binding between RGG and sc1 was determined.⁶⁴ The structure confirmed the binding mode of RGG to include both duplex region and quadruplex providing further validation. These interactions represent a notably different binding mode to RHAU derived peptides that bind through the end capping of G4s and highlight the requirement to investigate different motifs when designing G4 ligands for cellular applications.

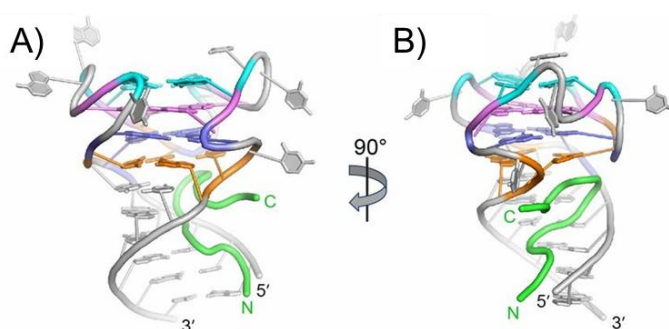


Figure 1.13 (a) Crystal structure of sc1 and RGG. RNA is in grey colour with guanine quartets in cyan, violet and blue, and the mixed tetrad in orange. The RGG peptide is highlighted in green. (b) 90° clockwise rotation of the crystal structure. (Adapted from ref. 62).

The RG-rich motif containing the sequence RGRGRGRGGGSGGSGGRGRG was first identified by examining the sequences of 77 G4 binding proteins and is similar to the RGG box sequence RRGDGRRRGGGGRGQGGGRGRGGGFKG. This sequence is also referred to as the Novel Interesting Quadruplex Interaction (NIQI) motif.⁶⁵

Most reported peptide studies have focused on solution based studies and have lacked cellular based studies to further probe their effectiveness as therapeutics or probes *in vivo*. This is largely attributed to poor stability and often poor uptake of the reported peptides. Recently the Pagano group investigated a series of peptides derived from the Rap1 protein, two of which showed improved G4 binding properties, but initial cellular studies showed poor cellular uptake.⁶⁶ Conjugation of these G4 binders to cell penetrating peptides (CPPs) promoted an increase in cellular uptake where γ H2AX indicated DNA damage in the nucleus of U20S cells. The CPP conjugate, R₇W-Myb^{397–415} had an IC₅₀ of 5 μ M.

1.3 Molecular Photophysics

Before the literature relating to the visualisation of guanine quadruplexes is reviewed, a primer on molecular photophysics will be addressed in this section. The photophysical fate of an excited molecule can be summarised by using a Jablonski diagram, as illustrated in Fig. 1.14, The processes (radiative and non-radiative) a chromophore can undergo include photon absorption, internal conversion (IC), fluorescence, intersystem crossing (ISC), vibrational relaxation (vr) and phosphorescence.⁶⁷

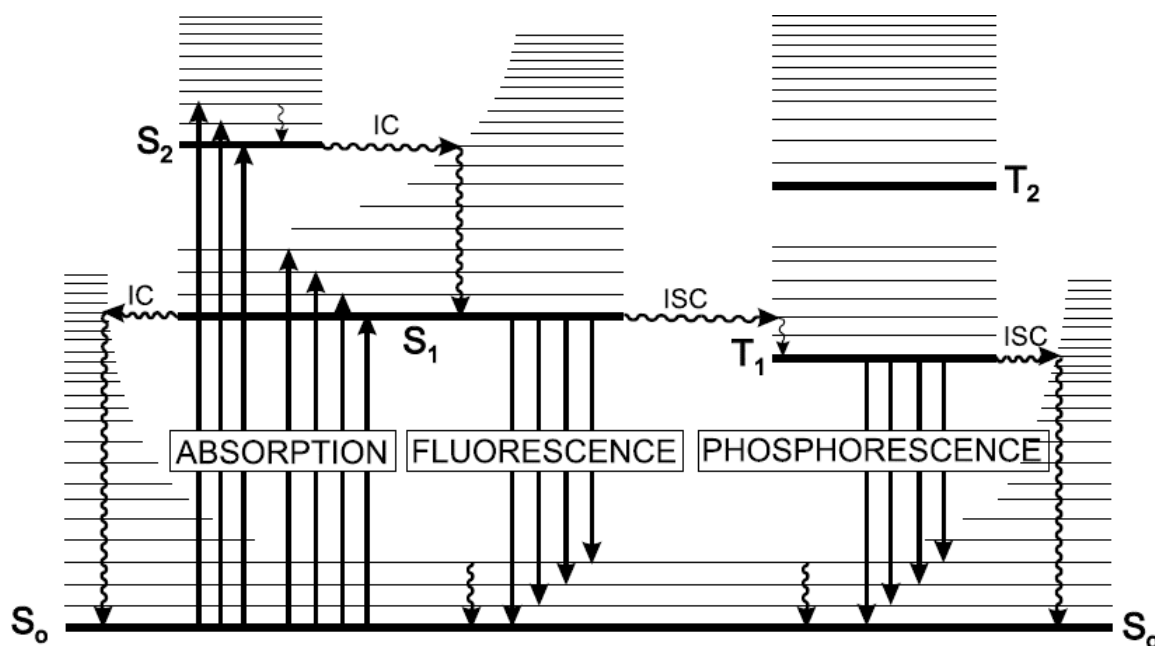


Figure 1.14 Jablonski diagram illustrating transitions between different excited and ground states. Where a straight line indicates absorption when directed upwards, a straight line directed downwards depicts an emissive process and a waved arrow represents a non-radiative process. (Adapted from Ref. 67).

When a molecule absorbs a photon of light, an electron is promoted from the low energy ground state orbital to a higher energy excited state unoccupied orbital. All excited states may have singlet or triplet multiplicity. This transition can be from S₀ (singlet ground state) to an excited orbital with the same multiplicity, S_n, where excitation from singlet spin to triplet spin, it is spin forbidden. Deactivation occurs through Internal conversion (IC), a non-radiative transition between to electronic states of the same multiplicity (S_n→S_{n-1}), isoenergetic crossover, followed by vibrational relaxation to the lowest vibrational energy of that state. Non-radiative processes occur when there is a change in excited state but without an associated emission of a photon.

The de-excitation process, Intersystem crossing (ISC), involves a spin forbidden transition between two isoenergetic electronic states of differing multiplicities (S₁→T₁). Although this

is classically forbidden, it may occur through spin-orbital coupling due to the presence of a heavy atom large enough to allow the transition.

Fluorescence is a radiative transition associated with excited state relaxation to a ground state ie, $S_1 \rightarrow S_0$. The fluorescent spectrum of a molecule occurs at higher wavelengths (lower energy) than the absorption spectrum. This is the result of energy loss from vibrational relaxation in the excited state. The gap between the absorption maximum and the emission maximum is referred to as the Stokes shift. The fluorescent lifetime of a molecule depends on the length of time excited molecules remain in the S_1 state before undergoing transition and is typically very short in the nanoscale range (10^{-11} - 10^{-9} s).

Phosphorescence occurs as a transition between states of differing multiplicity ($T_1 \rightarrow S_0$). Thus, phosphorescence is common in metal complexes, as spin orbit coupling causes singlet and triplet mixing, particularly in metal centred charge transfers and metal ligand charge transfers. As this transition is spin forbidden, the resulting lifetimes are longer for metal complexes than organic compounds. Phosphorescence typically occurs over long time-scales in the sub microsecond range (10^{-6} s) up to the order of seconds.

The luminescence lifetime of a molecule is controlled by the decay processes it undergoes and can be expressed by the following equation:

$$\tau = \frac{1}{k_r + k_{nr}}$$

Equation 1.1

Where k_r is the radiative decay constant and k_{nr} is the non-radiative decay constant

1.4 Visualisation of Guanine Quadruplexes

There are several difficulties to be considered when visualising G4s, including the requirement for high G4 density in order to form a fluorescent foci, and the presence of protective proteins *in cellulo*, that are capable of preventing an imaging probe from coordinating to a G4. Another barrier to visualisation is that the conditions under which G4s can form and unwind *in cellulo*, remain poorly understood, and the conditions rewired for their formation in solution may not be prevalent in the cell. There has thus been considerable effort made to develop probes for G4 imaging. Imaging probes developed can be divided into different classes: (i) switch on probes, that shows a measurable increase in luminescence on binding, (ii) switch off probes that decrease in luminescence upon binding and (iii) probes that do not vary in luminescence but bind with high specificity.

Antibodies are commonly employed in the latter class to due to their exceptional selectivity. In fact, the first reported G4 visualisation was a variable fragment of an antibody used to detect telomeric G4s in the macronuclei of a fixed ciliate.¹² The main drawback of such immunofluorescence approaches is their very poor permeability of cellular membranes, meaning cell fixation is usually required to allow for cell uptake, preventing dynamic studies. The most significant contribution has been the development of the BG4 antibody, by the Balasubramanian lab.^{68,69} BG4 is a single chain fragment

variable anti-body (scFv) that can be expressed in *E. Coli*. The antibody has shown nanomolar affinity for intra and intermolecular G4s with a slight binding preference for parallel G4s, impressively the antibody showed no binding towards other DNA and RNA structures, including RNA hairpin, dsDNA and ssDNA. Due to the high specificity and commercial availability of BG4, it has seen widespread adoption for immunofluorescent imaging of G4s. However, scFv antibodies often have do not fold correctly when expressed in mammalian cells, limiting their use to fixed cells. Recently the group have developed a new G4 antibody, SG4, a camelid heavy-chain-only that is capable of expression in human cells.⁷⁰

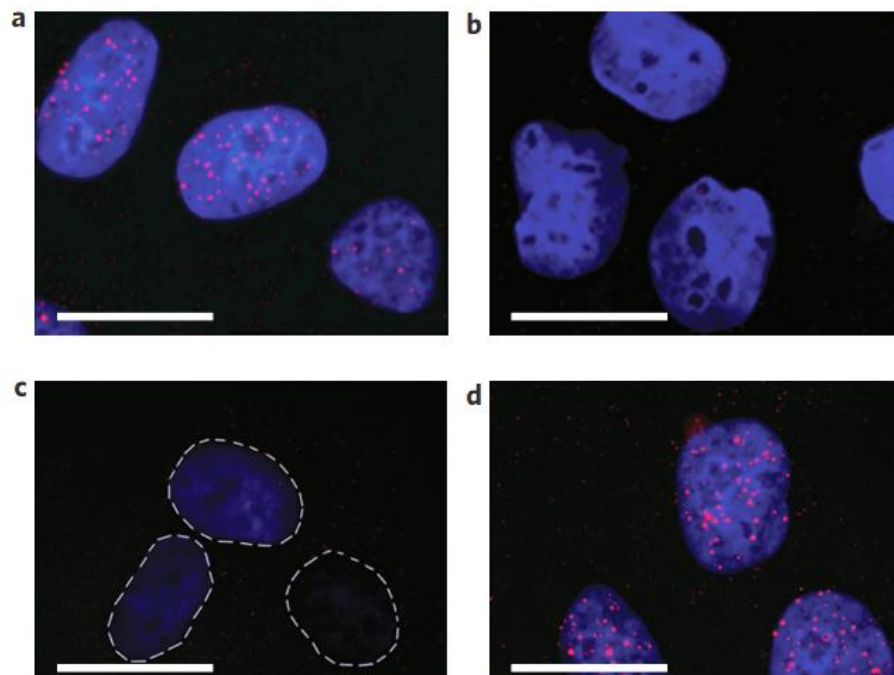


Figure 1.15 A) Immunofluorescence showing BG4 foci (red) in U2OS osteosarcoma cell nuclei. B) Loss of BG4 foci in U2OS cells after preincubation of the antibody with pre-folded G-quadruplex oligonucleotides. C) Loss of BG4 foci in U2OS cells after DNase I treatment. The dotted lines show the boundary of the nuclei. D) Increase in BG4 foci number after transfection with pre-folded G-quadruplex oligonucleotides. The nuclei are counterstained with DAPI (blue). (Adapted from Ref. 70).

A number of fluorescent probes that can be used with live cells have also been reported. Thiazole orange (TO) one of the first reported cyanine dyes and is regularly used as a staining agent for nucleic acids though it lacks specificity for G4 structures. TO is a typical example of a switch-on probe which has a notable increase in luminescence after nucleic acid binding, including G4 binding. This has spurred investigations to develop analogues with greater selectivity. Some effort has been made to form a derivative of TO with selective G4 binders such as bisquinolinium and Thiazole orange conjugates.⁷¹ These derivatives show a notable increase in luminescence upon binding to G4s due to enhanced selectivity as they show preferential binding to G4s over dsDNA. Another study investigated benzofuroquinolinium which presented a remarkable 30-fold increase in luminescence when bound to CMYC and telomeric oligonucleotides.⁷² Thioflavin T (ThT) is a small molecule capable of nuclear localisation in MCF7 cells and recognises nucleolar G4s. Competitive imaging with the G4 binding ligand, PDS, indicated displacement of ThT and the fluorescent foci of ThT dissipate, as illustrated in Fig. 1.16.⁷³

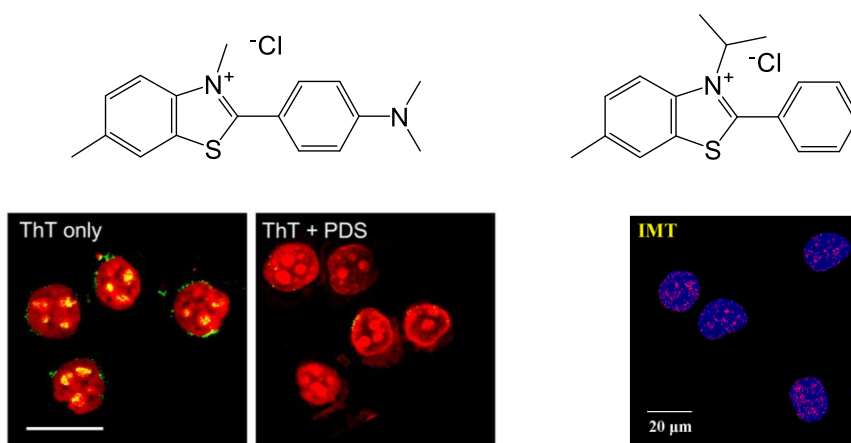


Figure 1.16 (a) Thioflavin T imaged in MCF7 before and after competition with pyridostatin. (b) IMT imaged in MCF-7 cells colocalised with Hoechst. (Adapted from Ref. 73) .

The benzothiazole derivative of thioflavin T, IMT, has recently been used to image G4s in cells.⁷⁴ IMT shows high selectivity for G4 DNA and G4 RNA over alternative DNA secondary structures and is another example of a light on probe. Selectivity was confirmed through fluorescence studies in vitro. The binding position of the probe with the G4 c-kit was investigated using solution NMR and fluorescent intensity experimentation with a labelled c-kit oligonucleotide. When the oligonucleotide was labelled at the 5' position, binding of IMT induced quenching. Conversely, when labelled in the 3' position binding of IMT to the oligonucleotide resulted in no quenching suggesting that the IMT probe binds to the top tetrad. In fixed cell experiments the probe was found to co-localise with the G4 binding antibody BG4. Validation of this localisation in live cells was achieved through introduction of PDS which reduced the fluorescent foci. Finally, when live HeLa cells were imaged using IMT the structural polymorphism was investigated during cellular progression by treatment with aphidicolin and hydroxyurea. A key outcome was a reported increase in foci during cell cycle progression from G₀ to S phase.

More recently there has been a push to image G4s using a combination of environmentally sensitive G4s using fluorescence lifetime imaging microscopy (FLIM), where variations in photophysical properties can distinguish DNA structural polymorphism.⁶⁸ The organic tripodal fluorescent probe, NBTE, was used to detect G4s in live cells based on photon counts from fluorescence lifetime imaging microscopy FLIM, facilitating distinction between G4 and alternative DNA topologies (Fig 1.17).⁷⁵ The probe was imaged in cancer and non-cancer cell lines using confocal microscopy. FRET analysis indicated that the probe has no significant binding selectivity for G4s over dsDNA. Variations upon DNA binding measured include an increase in fluorescence intensity between 3 and 82-fold, a bathochromic shift in absorption spectra ($\Delta\lambda_{\text{abs}} = 0\text{--}23$ nm) and a hypsochromic shift in emission ($\Delta\lambda_{\text{em}} = 20\text{--}68$ nm). When the ligand was bound to G4 oligonucleotides (parallel, antiparallel and hybrid) the fluorescence lifetime of its

second decay component, (t_2), was in the interval of 3.2 – 4.1 ns with a fractional fluorescence quantum yield of 0.21. Non G4 DNA secondary structures (single strand, double strand and i-motifs) had a t_2 interval of 1.7 – 2.5 ns with a fractional fluorescence quantum yield averaging 0.027. These results were used to distinguish G4s as probes bound to them possessed longer lifetimes and greater quantum yields. A key outcome of this paper was that cancer cells showed a notable increase in concentration of G4s such that in lung cells (A549) had a 3.7-fold increase in G4 DNA content vs HLF cells. In mammary cells (MCF-7 vs MCF-10A) a 5.4-fold increase in was observed and in hepatic cells (HepG2 vs LO2) a 3.6-fold increase was observed. These findings illustrate the potential use of G4 probes to obtain crucial biological information and moreover G4s provide an excellent basis for cancer therapeutics.

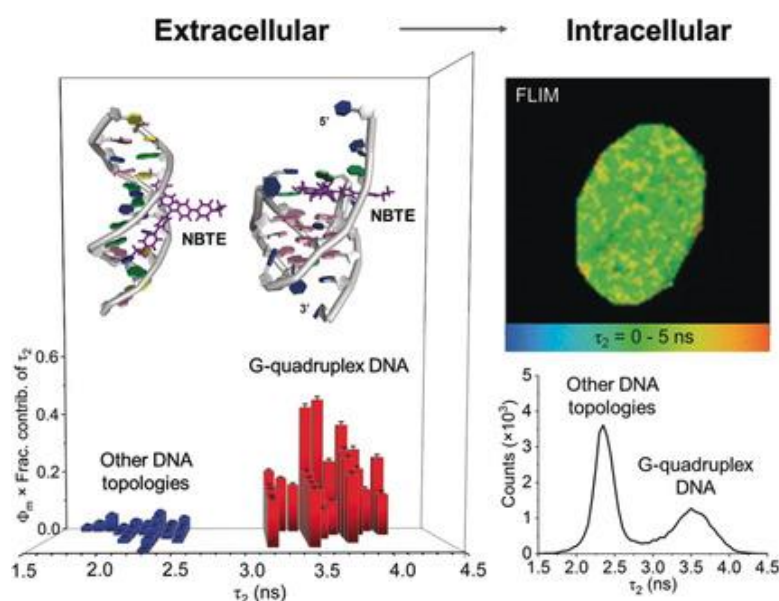


Figure 1.17 FLIM imaging of NBTE localised within the nucleus of MCF-7 cells. (Adapted from Ref. 75).

Although there have been significant advancements in recent years towards visualising the quadruplex, key barriers still remain, particularly selectivity, live cell uptake and localisation. Metal complexes offer unique advantages over common organic probes, such as long luminescent lifetimes, environmentally sensitive decays and environmentally

sensitive luminescent intensities. In combination, these proprieties can be implemented for a multi-modal imaging approach to visualisation.

1.5 Development of Ruthenium(II) Complexes Targeting Guanine Quadruplexes

There has been increasing interest of Ru(II) polypyridyl complexes and organometallics more generally as selective binders for guanine quadruplex DNA. This interest has been sparked through the multitude of possible development avenues available when designing such probes, particularly the possibility to design large, planar and aromatic complexes capable of end-capping guanine quadruplexes and binding in guanine quadruplex loops. Combined with their potential as therapeutics through ROS generation, and redox properties, their potential as therapy and diagnostic (thernaostic) becomes greatly compelling. First, the basic features of Ru(II) polypyridyl complexes will be introduced before a discussion on different classes of Ru(II) probes that have been investigated for their G4 binding capacity.

1.5.1 Photophysics of Ru(II) polypyridyl complexes

The photophysical properties of ruthenium(II) polypyridyl complexes can be introduced using $[\text{Ru}(\text{bpy})_3]^{2+}$ as a model. The absorption profile of $[\text{Ru}(\text{bpy})_3]^{2+}$ is illustrated in Fig. 1.18 and is comprised of multiple electronic transitions defined as metal centred (MC), ligand centred (LC), or charge transfer (CT).⁷⁶

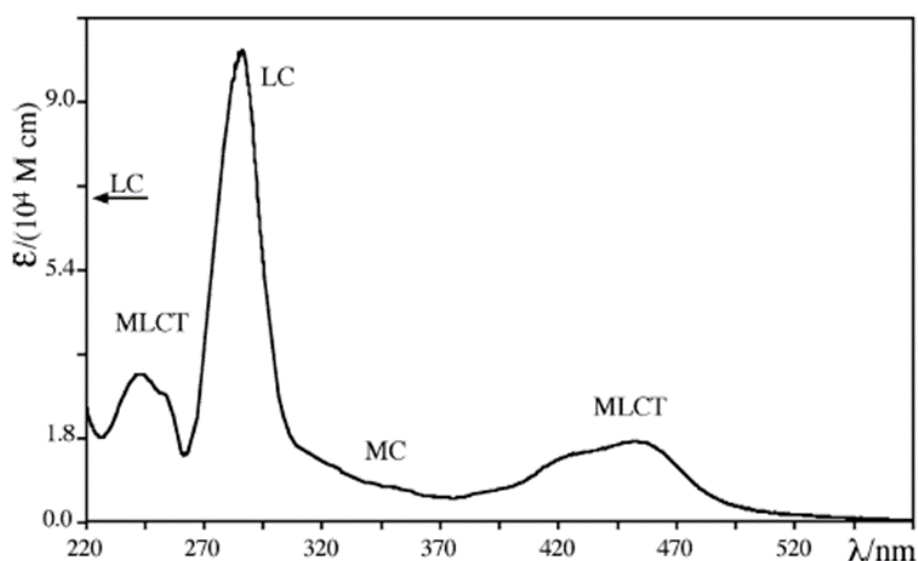


Figure 1.18 Charge transfers associated with the absorption spectra of $[\text{Ru}(\text{bpy})_3]^{2+}$ in an alcoholic solvent. (Adapted from Ref. 76).

$[\text{Ru}(\text{bpy})_3]^{2+}$ contains a broad metal to ligand charge transfer (MLCT) at 450 nm. Excitation into this band promotes an electron from the metal d orbital into π_L^* orbital where the $^1\text{MLCT}$ excited state is populated before ISC occurs populating the $^3\text{MLCT}$ (Fig. 1.18). ISC occurs efficiently due to the spin-orbit coupling promoted by the heavy atom of ruthenium. Consequently, emission from this state is formally phosphorescence and has an associated long-lived decay. Additionally, the $^3\text{MLCT}$ can also undergo the nonradiative process in terms of thermal activation, to an antibonding ^3MC that is accessible from the $^3\text{MLCT}$ state and its population to rapid non-radiative decay and ligand dissociation.^{77,78}

In this thesis, Ru(II) polypyridyl complexes containing dppz ligands are applied and introduce “light-switch” properties into the Ru(II) complex. For example, in a representative complex such as $[\text{Ru}(\text{bpy})_2(\text{dppz})]^{2+}$, it is emissive in aprotic solvent, but this emission is extinguished when in protic solvents.⁷⁹ These “light switch” properties arise because the dark phenazine state lies at lower energy than the emitting bpy state when the phenazine is protonated, as illustrated in Fig. 1.19.

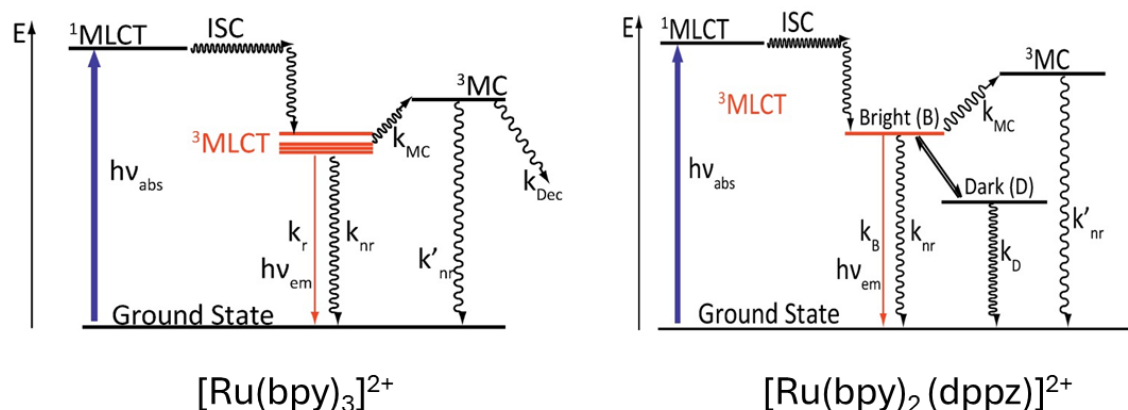


Figure 1.19 Photophysical process $[\text{Ru}(\text{bpy})_3]^{2+}$ and $[\text{Ru}(\text{bpy})_2(\text{dppz})]^{2+}$ undergo after the absorption into their excited states (Adapted from Ref. 79).

Not all dppz complexes elicit a light switch effect based on solvent environments, for example, $[\text{Ru}(\text{tap})_2(\text{dppz})]^{2+}$ is emissive in both protic and aprotic.⁸⁰ This is attributed to the greater π -accepting capability of the tap ligand in comparison to bpy causing localisation of the LUMO to the tap ligand instead of dppz even when dppz is protonated. Due to this the $^3\text{MLCT}$ of dppz is not populated when the complex is excited preventing the solvent dependent dynamics of the dark state and light state required for a light switch. This comparison is illustrated in Fig. 1.20. In the case of $[\text{Ru}(\text{tap})_2(\text{dppz})]^{2+}$ emission occurs in protic and aprotic solvent systems but emission lifetime is solvent dependent.⁸⁰

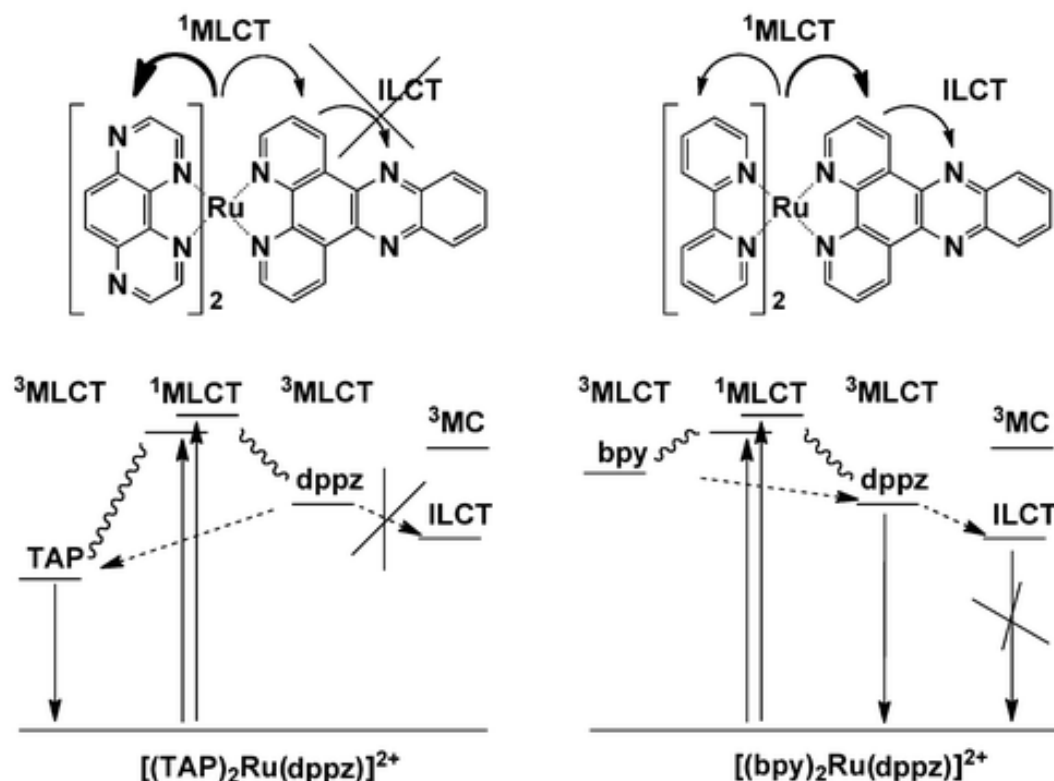


Figure 1.20 Photophysical process $[\text{Ru}(\text{tap})_2(\text{dppz})]^{2+}$ and $[\text{Ru}(\text{bpy})_2(\text{dppz})]^{2+}$ undergo after the absorption into their excited states. (Adapted from Ref. 80).

Ruthenium(II) complexes containing two or more tap ligands have an increased excited state reduction potential in comparison to their phen and bpy analogues, attributed to their high π -accepting capacity. Due to their excited state redox potential, they have the capacity to undergo photoinduced electron transfer (PET) with biomolecules making them interesting ligand systems for Ru (II) complexes in biological applications, this will be discussed in greater detail later in this thesis. Similarly, Ru(II) complexes containing 1,4,5,8,9,12-hexaazatriphenylene (HAT) ligands have increased redox potential. The oxidation and reduction potentials of these complexes depend on the number of ligands in the coordination sphere with π accepting properties, this effect is reflected in the ground and excited redox potentials shown in Table 1.1

Table 1.1 Oxidation and reduction potential of a series of ruthenium(II) complexes.

Complex	E _{ox}	E _{red}	E* _{red}	Ref
[Ru(HAT) ₃] ²⁺	+2.07	-0.62	+1.46	81
[Ru(tap) ₃] ²⁺	+1.94	-0.75	+1.32	81
[Ru(tap) ₂ (dppz)] ²⁺	+1.77	-0.80	+1.15	82
[Ru(bpy) ₂ (dppz)] ²⁺	+1.24	-1.02	+0.97	83

1.5.2 Ruthenium(II) Complexes Bearing a dppz Ligand

Ru(II) polypyridyl complexes bearing a dppz ligand have been a popular choice to probe quadruplex DNA given their “switch-on” functionality, and their ability to intercalate and stabilise helical DNA. Shi *et al.* initially investigated a racemic [Ru(bpy)₂(dppz)]²⁺ complex and its binding to a quadruplex and an i-motif (22AG and 22CT respectively).⁸⁴ A large luminescent increase was observed upon binding to 22AG in contrast to a weak luminescent intensity when bound to the i-motif, indicating a strong affinity for G4s over i-motifs. Melting curves studies showed a strong stabilisation of 22AG when bound to the ruthenium complex whereas no stabilisation was evident when bound to i-motifs, further confirming the strong affinity to G4 DNA over i-motifs. Shi *et al.* further investigated these interactions by examining [Ru(phen)₂(dppz)]²⁺ complex with 22AG and 22CT, where similarly strong affinity towards G4 DNA over i-motif was observed.⁸⁵

To improve selectivity for G4s and gain greater insights into binding modes, Shi *et al.* synthesised two novel complexes that extended dppz, the first was a bromination at the 12 position and the second being the synthesis of dppz with a phenyl ring on the 12 position. They determined that the binding stoichiometry of Ru:G4 was 1:1 through the generation of Job plots. Additionally, CD spectra of increasing Ru(II) concentration when

bound to a mixed hybrid quadruplex resulted in a negative band at 260 nm indicating a conversion of 22AG to an anti-parallel quadruplex.⁸⁶

The Glazer group synthesised the modified 3,6'-dimethyl-dppz complex, $[\text{Ru}(\text{bpy})_2(\text{dmdppz})]^{+2}$, where the complex can undergo photochemical substitution when bound to G4s and irradiated (Fig. 1.21).⁸⁷ Crystallography of the novel complex indicates that the introduction of the two methyl groups causes a distortion of a 15° bending out of plane from the normal octahedral complex. The complex binds strongly to both ctDNA and G4 DNA but photoejection of the dmdppz ligand is 3-fold quicker when bound to G4 DNA in contrast to ctDNA. The group synthesised a 11-bromo-derivative of the above-mentioned complex that would undergo luminescent quenching when intercalating in helical DNA. With the reasoning that substitution at the 11 position would result in a less symmetrical intercalation with helical DNA, allowing interaction of water molecules and a phenazine nitrogen resulting in population of the dark state. Data indicated across the methylated complexes vs the non-methylated complexes that two different binding modes were occurring. The first through intercalation and the second interaction of the distortion of the methylated complex caused interaction through end capping.⁸⁸

More recently the binding of dppz complexes to guanine complexes has been studied with an emphasis on advanced spectroscopic techniques such as ultrafast time-resolved infrared (TRIF) spectroscopy and transient absorption spectroscopy. The Quinn group reported on a racemic mixture of $[\text{Ru}(\text{phen})_2(\text{dppz})]^{2+}$ interactions with G4 DNA through the use of TRIF to provide insight on solution binding where crystallography cannot be achieved. This study revealed the significant role of thymine base interactions within the loop of a human telomeric G4 in a Na^+ environment.⁸⁹ The light switch luminescent properties of $[\text{Ru}(\text{phen})_2(\text{dppz})]^{2+}$ were further investigated using multiscale time-resolved spectroscopy methods, in this study the Ru(II) complexes as investigated with a Human telomere G4 and an *Oxyticha nova* G-quadruplex. Nanosecond luminescence lifetime

studies indicated a biexponential decay of the $^3\text{MLCT}$ emission and femtosecond transient absorption spectroscopy measurements indicate that the Ru(II) -G-quadruplex local binding environment is partially hydrophilic and partially hydrophobic. Interestingly, dark state formation of $[\text{Ru}(\text{phen})_2(\text{dppz})]^{2+}$ was recorded at approx. 10.5 ps where as in *Oxyticha nova* the dark state formation is slower at approx. 15.6 ps indicating that TTTT diagonal loop of *Oxyticha nova* reduces access of water to the ruthenium complex in comparison to the TTA loop of the Human telomere DNA.⁹⁰

There has been recent success in crystallising the Λ -enantiomer Ru(II) complex, $[\text{Ru}(\text{phen})_2(\text{qdppz})]^{2+}$, with a modified human telomeric sequence (htel21T_{18}) (Fig. 1.21). This is the first reported antiparallel quadruplex-ligand crystal structure and shows enantiospecific threading between the TTA loop and G-tetrad. To relate structural binding and solution behaviour, a replication assay was performed to compare the stalling of replication where Λ - $[\text{Ru}(\text{phen})_2(\text{qdppz})]^{2+}$ was the most effective.⁹¹

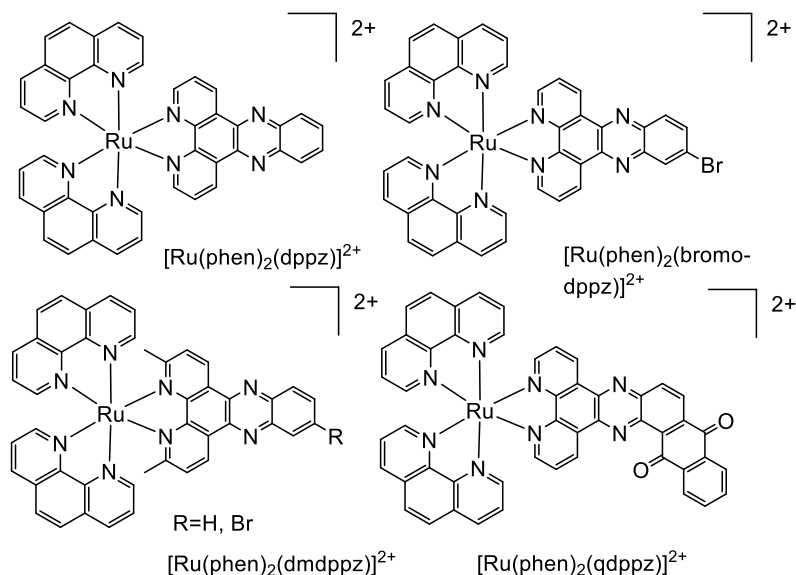


Figure 1.21 Ruthenium(II) complexes containing dppz or dppz modified ligands, investigated for their G4 binding capabilities.

1.5.3 Ruthenium(II) Complexes Bearing an Imidazo-phenanthroline

Ru(II) complexes bearing an imidazo-phenanthroline complexes are of interest because these ligands are easily modified to include multiple functional groups or aromatic extensions. The Liu group have synthesised and investigated a number of these complexes with a focus not only on binding activity but also their *in cellulo* activity, specifically for their ability to inhibit telomerase activity. For example, $[\text{Ru}(\text{phen})_2(\text{tip})]^{2+}$ and $[\text{Ru}(\text{bpy})_2(\text{tip})]^{2+}$ were investigated in vitro with ds26 and HTG21 through fluorescent titrations, FRET melting studies, CD and NMR studies it was determined that the complexes had a stronger affinity for G4s over helical DNA and that $[\text{Ru}(\text{phen})_2(\text{tip})]^{2+}$ had a higher affinity and stabilisation than $[\text{Ru}(\text{bpy})_2(\text{tip})]^{2+}$ (Fig. 1.22). It was also determined that the phen complex is a better telomerase inhibitor, the authors suggest this is due to the larger aromatic area of the phenanthroline ligand facilitating greater interaction with grooves and loops of the guanine quadruplex backbones.⁹² The group has had an emphasis on researching enantiopure complexes, for example they have successfully purified and investigated the Λ and Δ isomers of $[\text{Ru}(\text{phen})_2(\text{mopip})]^{2+}$, $[\text{Ru}(\text{phen})_2(\text{H-pip})]^{2+}$, and $[\text{Ru}(\text{bpy})_2(\text{H-iip})]^{2+}$ (Fig. 1.22). The in vitro cytotoxicity was reported for each of these enantiomers with HepG2 Cells, with $[\text{Ru}(\text{phen})_2(\text{mopip})]^{2+}$ having an IC_{50} of 4.4 ± 0.3 , 14.5 ± 1.3 and 7.2 ± 0.8 μM , for the Λ isomer, Δ isomer and racemic mixture respectively). $[\text{Ru}(\text{phen})_2(\text{H-pip})]^{2+}$ had an IC_{50} of 17.76 ± 0.89 and 28.51 ± 1.14 μM (Λ and Δ respectively) and $[\text{Ru}(\text{bpy})_2(\text{H-iip})]^{2+}$ having an IC_{50} of 135.7 ± 1.0 and 193.9 ± 0.8 μM (Λ and Δ respectively). With these respective inhibitions it is evident that enantiospecific binding plays a key role in the efficacy of ruthenium G-quadruplex binders.^{93–95}

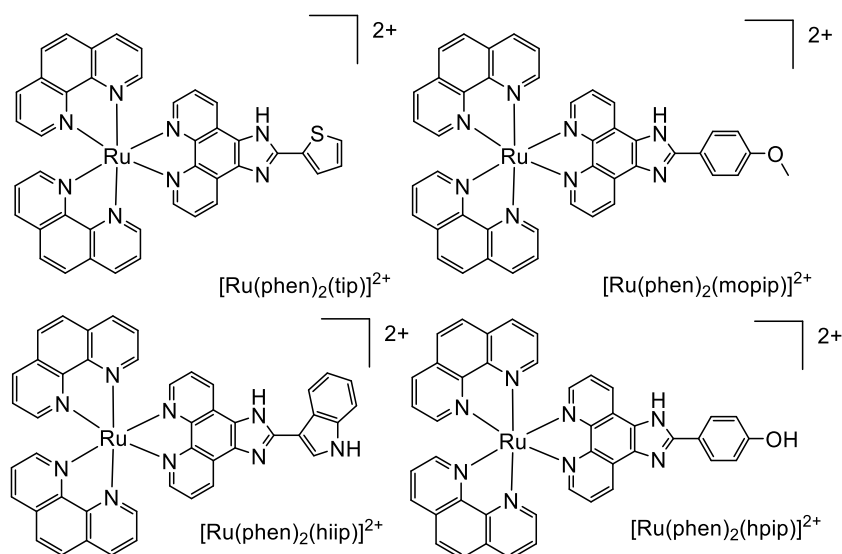


Figure 1.22 Ruthenium(II) complexes containing an imidazo-phenanthroline, investigated for their G4 binding capabilities.

1.5.4 Bimetallic Ruthenium(II) Complexes

Bimetallic tpphz bridged ruthenium complexes, have been extensively studied by the Thomas group, and are an interesting prospect for G4 selective phototherapeutics due to their good cellular uptake and photodynamic properties (Fig. 1.23). These complexes typically contain two ruthenium centres bridged through a tpphz ligand and possess bpy, phen or tap ancillary ligands. The first of these investigations illustrated significant emission enhancement by a factor of 2.5 when the bimetallic complex $[(\text{Ru}(\text{phen})_2)_2(\text{tpphz})]^{4+}$ was bound to a 22-mer guanine quadruplex in comparison to ctDNA and a 32 nm blue shift is observed for the peak maxima. Binding of this complex as investigated through both emission titrations and isothermal calorimetry, where it was concluded that the complex had a higher binding affinity to G4 DNA by approximately 5 kJ mol⁻¹, when compared to ctDNA.⁹⁶ Enantiomers of $[(\text{Ru}(\text{phen})_2)_2(\text{tpphz})]^{4+}$ and $[(\text{Ru}(\text{bpy})_2)_2(\text{tpphz})]^{4+}$, the $\Lambda\Lambda$ and $\Delta\Delta$ isomers were investigated on binding to ctDNA and HTS (G4). $\Lambda\Lambda$ - $[(\text{Ru}(\text{phen})_2)_2(\text{tpphz})]^{4+}$ showed a high binding affinity of 1.16×10^7 bound to

HTS compared to $6.73 \times 10^6 \text{ M}^{-1}$ when bound to ctDNA. NMR structural studies were then performed to gain insight into possible binding modes, it was determined that the $\Lambda\Lambda$ - $[(\text{Ru}(\text{phen})_2)_2(\text{tpphz})]^{4+}$ isomer bound mainly at the diagonal loop of HTS and is stabilised by steric fit. A distinct advantage of these bimetallic complexes is their good cellular uptake across multiple cell lines. The combination of lipophilicity and positive charge of bimetallic ruthenium complexes has often resulted in not only uptake into live cells but also nuclear localisation. It should be mentioned that to achieve these results, often very high concentrations of the probe is required which can present difficulties as a diagnostic tool, as the likelihood of off-target binding increases. Further it is evident that selectivity between helical and quadruplex DNA is not significant in certain studies and to develop a true G4 DNA targeting complex, greater selectivity is desirable.

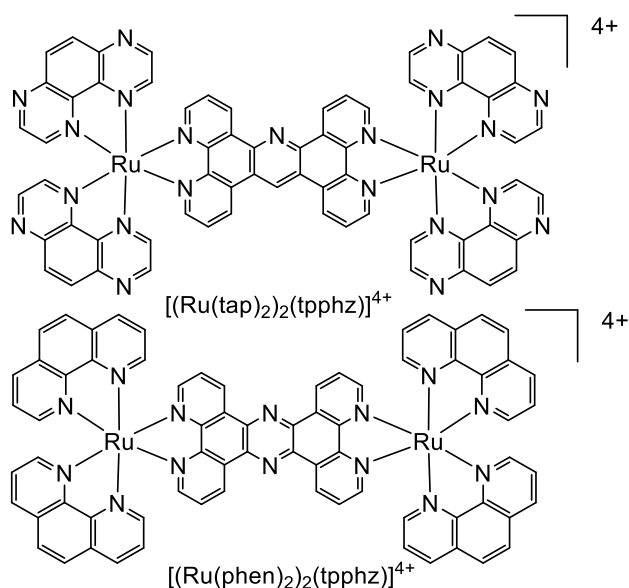
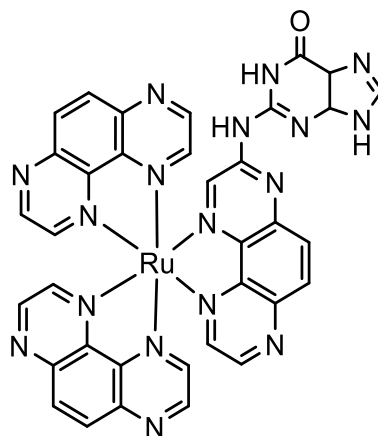
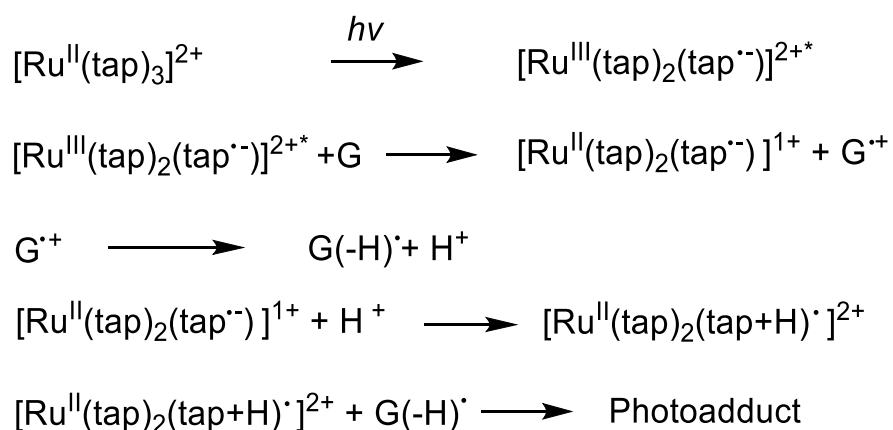


Figure 1.23 bimetallic ruthenium(II) complexes, investigated for their G4 binding capabilities.

1.5.5 Ruthenium(II) Complexes Bearing a Tap Ligand

Ru(II) complexes bearing two tap ancillary ligands possess desirable photo-redox properties with sufficient excited state energy to oxidise guanine for potential application in photodynamic therapy. These complexes have been thoroughly investigated for their redox potential in helical DNA environments where they have been demonstrated to oxidise guanine bases and form guanine-Ru(II) tap photoadducts (Fig. 1.24).



Photoadduct

Figure 1.24 Photoadduct formation of a Ru(II) tap-guanine complex.

Formation of a photolinked adduct between the complex $[(\text{Ru}(\text{tap})_2)_2\text{TPAC}]^{4+}$ and a four stranded intermolecular quadruplex was demonstrated through a combination of photoactivation studies using gel electrophoresis and mass spectrometry. The study

hypothesized that the bimetallic complex, is capable of photocrosslinking several guanine bases within the same quadruplex suggesting a high local concentration of photoadducts on the G4.⁹⁷

The Elias group has more recently focused on the development of ruthenium and iridium complexes for photodynamic therapy that include tap bearing ruthenium complexes to generate novel photoreactive complexes specifically targeting G4s (Fig. 1.25). The first of their reports compared four complexes, two of which contained tap ancillary ligands, $[\text{Ru}(\text{tap})_2\text{CPIP}]^{2+}$ and $[\text{Ru}(\text{tap})_2\text{CPITAP}]^{2+}$ and were compared to their phenanthroline analogues. Interestingly both $[\text{Ru}(\text{tap})_2\text{CPIP}]^{2+}$ and $[\text{Ru}(\text{phen})_2\text{CPIP}]^{2+}$ both showed exception photo-cytotoxicity when irradiated in U2OS cells which the authors attributed to a type II photoreaction in the case of the phen complex and a photo-induced charge transfer in the case of the tap complex.⁹⁸ The Thomas group reported a tap derivative of their bimetallic system, $[(\text{Ru}(\text{tap})_2)\text{tpphz}]^{4+}$, and investigated its binding towards ctDNA and a human telomer G4, evaluating it as a probe in imaging and in phototoxicity in human melanoma cells.⁹⁹ The complex showed binding to both types of secondary structure and binding affinities being reported as $2 \times 10^6 \text{ M}^{-1}$ and $5.1 \times 10^5 \text{ M}^{-1}$, indicating a preference in binding for dsDNA. The authors implemented two different methods to calculate the individual binding constants possibly leading to inconsistencies in reported values. It is hypothesised in the paper that the less favourable interaction with the G4 sequences is a result of loop destabilisation due to lone pairs on the aromatic nitrogen's of the tap ligand, although confirmatory experiments are not evident. Cellular imaging was performed in C8161 cells where the complex was incubated at a concentration of 100 micromolar, with localisation apparent throughout the cell though largely within the nucleus.

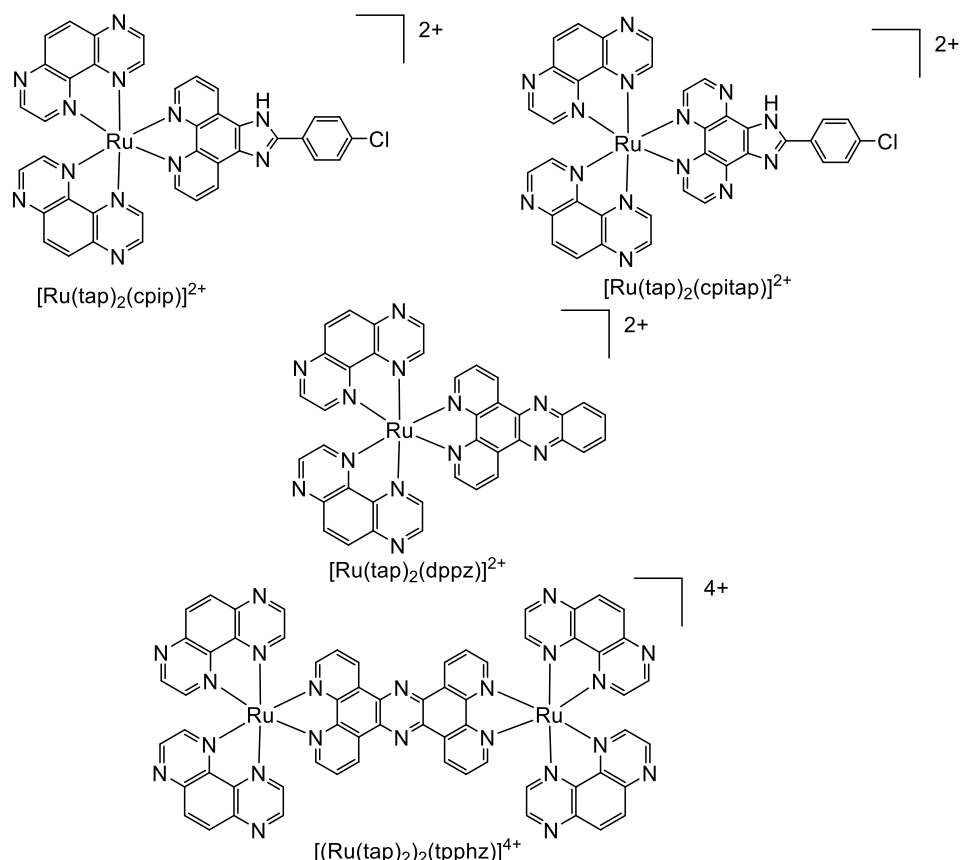


Figure 1.25 Guanine quadruplex binding ruthenium(II) complexes bearing a TAP ligand.

The Cardin Group have crystallised the lambda isomer of $[Ru(tap)_2(dppz)]^{2+}$ bound to a four stranded intermolecular G4.¹⁰⁰ This work suggests the complex can occupy multiple binding sites to the G4 with multiple binding modalities possible, intercalation, semi-intercalation and end capping. Unexpectedly the complex showed affinity towards thymine binding, particularly through intercalation via thymine base pairs. Interestingly, it did not appear to interact with the surface of any G-tetrad, instead interacting with the quadruplex through cavities formed by a mixture of T-T mismatching and A-T matching bases. This poses an interesting problem in the design of the probes for G4 specificity, in the case of complexes bearing tap or other electron transfer acceptor ligands, as interaction with guanine bases is desired to achieve redox chemistry in the case of an intermolecular guanine quadruplex.

Collectively, the work to date demonstrates that Ru(II) probes have great promise as imaging probes and theranostics towards quadruplexes and have the synthetic versatility to enable improvements in selectivity and photoredox properties. It should be stated that there is yet to be a highly selective Ru(II) probe capable of direct visualisation of G4s over alternative DNA structures or probes capable of selectively oxidising G4s over alternative DNA structures and in many of the examples mentioned, cell uptake and viability is often an issue. There are different approaches to achieve cell permeation for ruthenium(II) complexes, some of which are discussed in the following section and could possibly overcome these difficulties.

1.6 Cellular Uptake of Ruthenium(II) Complexes

Although ruthenium complexes possess several properties which make them ideal candidates for biological applications, they are yet to become common place when compared to the popularity of organic alternatives, this is in large part due to the unpredictable nature of their cellular uptake. However, through great efforts over the previous two decades, numerous different approaches have emerged exploiting the ruthenium complexes to achieve tuneable photophysics, cell uptake and localisation.

Cellular uptake can occur through several mechanisms including, passive diffusion, facilitated diffusion, active transport and endocytosis (Fig. 1.26).^{101,102} These uptake mechanisms can be categorized as energy dependant or independent. Passive diffusion occurs when a molecule passes through the phospholipid bilayer of the cell wall and then dissolves into the aqueous medium of the cytoplasm. This form of cell uptake is dictated by the molecules size and lipophilicity. This mode is not cell specific which can be an advantage when using a specific luminophore to study multiple types of cells. Facilitated diffusion utilises transport proteins within the cell membrane to transport small across a protein channel or pore. These processes are energy dependant and driven by a concentration gradient across the membrane. Additionally, these uptake mechanisms can

be driven by negative membrane potential. This is particularly useful for cationic Ru(II) complexes. Active transport mechanisms use energy provided by ATPase and internal ATP.¹⁰³ Many drug molecules have been found to utilise this mechanism including sugars, cations, anions and peptides. Active transfer mechanisms can be blocked by incubating cells at low temperatures (4 °C) or by ATP-depletion using metabolic inhibitors.

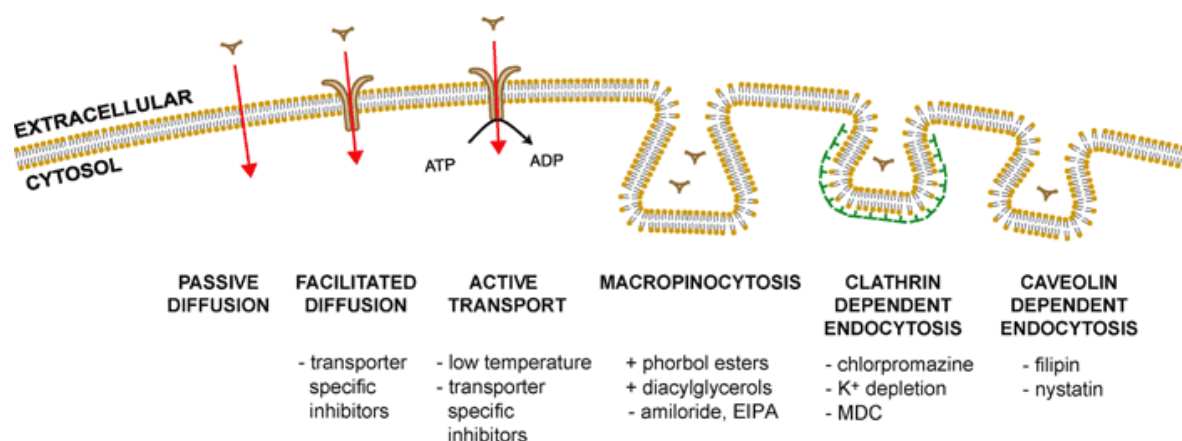


Figure 1.26 Cellular uptake mechanisms including passive diffusion, facilitated diffusion, active transport and endocytosis. (Adapted from Ref. 104).

Endocytosis is an energy independent process where vesicles form to wrap external molecules and transport them across the membrane. As previously mentioned, lipophilicity and charge are important parameters for cell uptake. Lipophilicity is measured as the logarithm of a molecule's partition coefficient between lipid and aqueous phases commonly between octanol and water. The partition coefficient is referred to as log P value where lipophilic molecules have a positive value and hydrophilic values have a negative value.

It is also important to note that by altering lipophilicity, vastly differing localisation can occur. The Barton group spearheaded investigations into the importance of lipophilicity while studying $[\text{Ru}(\text{N}^{\wedge}\text{N})_2(\text{dppz})]^{2+}$ complexes.¹⁰⁵ This study used a series of auxiliary ligands in ruthenium(II) dppz complex to achieve varying lipophilicity. The auxiliary ligands investigated include bipyridine, phenanthroline, diphenylphenanthroline (DIP) and mcbpy,

establishing a relationship between increasing the lipophilicity of auxiliary ligands with cellular uptake from passive diffusion mechanisms. A key result illustrating this point was the cell uptake recorded from the $[\text{Ru}(\text{DIP})_2(\text{dppz})]^{2+}$ complex where uptake was recorded at 0.5 μM whereas the alternative ligands required a concentration of 5 μM . The $[\text{Ru}(\text{DIP})_2(\text{dppz})]^{2+}$ complex had a log P value of +1.3 as a chloride salt compared to $[\text{Ru}(\text{bpy})_2(\text{dppz})]^{2+}$ has a log P value of -2.5. Later work from the Barton group determined that the cell uptake mechanism was energy independent occurred through passive diffusion.

Lipophilicity has also been attempted by means of cyclometallation. This process reduces the cationic charge on the complex by changing a coordinating nitrogen atom with an isoelectric carbon atom. Chao *et al.* have investigated this strategy to improve cellular uptake by synthesising an analogous complex to $[\text{Ru}(\text{bpy})_2(\text{dppz})]^{2+}$ where bipyridine is substituted for 2-phenylpyridine (ppy). This resulted in the rapid uptake of the complex into the nucleus causing the inhibition of transcription and apoptosis.¹⁰⁶

As mentioned, one approach to improve cell uptake of Ru(II) complexes is to exploit differing counterions. This includes recent work from Zhu *et al.* where pentachlorophenol was used to greatly enhance cellular and nuclear uptake of $[\text{Ru}(\text{bpy})_2(\text{dppz})]^{2+}$.¹⁰⁷ Active transport mechanisms were investigated by treatment of the cells with the metabolic inhibitor 2-deoxyglucose resulted in unaffected cellular uptake. Endocytosis was then investigated as a possible uptake mechanism by incubating the cells with known inhibitor which had no effect on uptake. Another uptake mechanism investigated was organic cation transporters. This was achieved by incubation with organic cation transporter inhibitor that also had no effect. Due to the above experiments the study suggests passive diffusion as the uptake mechanism. This initial study was expanded when Zhu *et al.* published a follow up paper researching a series of chlorophenolates as counterions. By studying a series of counterions, the lipophilicity and binding affinity were both determined

to be important physicochemical characteristics for cell and nuclear uptake. The findings conclude that the $[\text{Ru}(\text{bpy})_2(\text{dppz})]^{2+}$ has a positively correlated relationship with binding affinity and an inversely correlated relationship with the lipophilicity of the ion paired complexes.¹⁰⁸ While this group has multiple papers on this subject as outlined, this approach is yet to be replicated by other groups.

Cell penetrating peptides (CPPs) have been utilised to great effect with ruthenium complexes to provide enhanced cell uptake, organelle specific localisation, improved DNA binding and can mediate toxicity.^{109,110} CPPs are typically short cationic sequences typically containing less than 30 amino acid residues. Some advantages include their ease of synthesis through solid state building blocks. Additionally, the use of specific organelle targeting sequences can provide sub cellular localisation. The first generation of CPP metal conjugates contained polyarginine sequences. In general, polyarginine peptides conjugates transport their cargo into cells but their localisation is not predictable and often results in cytoplasmic localisation. Following these initial studies, peptides capable of sub cellular transfection were investigated, including mitochondrial penetrating peptides (MPP) that result in mitochondrial localisation and nuclear localising sequences (NLS) that transport their cargo across the nuclear membrane. Conjugation of these sequences to Ru(II) probes has provided organelle specific localisation for both imaging and therapeutic probes.^{111–113}

1.7 Conclusion and Project Scope

The formation of G4s in solution and in cells has only been established over the previous two decades, through the combination of various biochemical techniques, including immunofluorescence, CHIP sequencing and biological probing with selective G4 binding ligands. There still remains many outstanding questions regarding the impact of their formation on the fate of biological process and their potential as therapeutic targets. In order to gain a greater understating, novel probes are required, particularly those capable of multiple imaging modalities. The ligands designed as therapeutics reviewed in this chapter, still require more development to improve their efficacy and increase their toxicity. The use of metal complex as molecular imaging probes has gained some interest but often their poor cellular uptake or solubility can be a barrier to achieve meaningful cellular studies. This is similarly the case for G4 targeted probes, where studies are primarily solution based and require more detailed biological probing to determine their efficacy as therapeutics. This thesis aims to exploit the beneficial properties of Ru(II) polypyridyl complexes in combination with G4 targeting moieties to develop both therapeutic and diagnostic probes capable of live cell uptake and G4 binding.

In chapter 2, the novel probe RuPDC3 is synthesised, and its photophysical properties are examined when bound to DNA. RuPDC3 indicated a distinct increase in lifetime upon G4 binding comparatively to duplex DNA. Additionally, the complex showed distinct changes in its Raman spectra when bound to G4s in contrast to the complex in buffer or when bound tp dsDNA. Interestingly, at high concentrations the complex showed significant disruption to G4 ellipticity when examined by circular dichromism. When applied to cells, RuPDC3 proved to be an effective multi-modal probe where it was capable of PLIM and also resonance Raman mapping of the nucleus of HeLa Cells showing similar photophysical properties to solution studies.

In chapter 3, Ru-TAP-PDC3 was synthesised. This therapeutic was designed to target G4s and undergo direct photoinduced electron transfer in hypoxic conditions, overcoming the limitation of type II photosensitisers that require molecular oxygen for cytotoxicity. This complex was found to undergo PET with guanine monophosphate. Significant luminescent quenching occurred when bound to mitochondrial G4s indicating PET occurs when bound and undergoing photoirradiation. Ru-TAP-PDC3 showed high affinity for mitoG4s when investigated using G4 fluorescent intercalator displacement assay. The complex was found to directly photodamage mtDNA and induce apoptosis in both normoxic and hypoxic conditions.

Chapter 4 explores Ru-RHAU, a Ru(II) polypyridyl peptide conjugate that strongly stabilises the RNA quadruplex, NRAS. Comparisons between binding studies of the conjugate and parent complex indicate that binding to NRAS occurs through the peptide. When studied in cells, the complex was capable of inducing and imaging stress granules.

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Chapter 2 RuPDC3 as a Multimodal Probe for G4 DNA

Contributions: TD-DFT calculations were performed by Prof. Conor Long. Cellular Uptake, imaging, and toxicity studies were completed by Dr Karmel S. Gkika. Raman Mapping was completed by Dr. Karmel S Gkika and Dr. Nirod Sarangi. Rhianne Curley performed luminescent lifetime imaging microscopy experiments.

2.1 Abstract

Sensors capable of transducing G-quadruplex DNA binding are important both in solution and for imaging and interrogation *in cellulo*. Ru(II) based light switches incorporating dipyridylphenazine (dppz) ligands are effective probes for recognition and imaging of DNA and its polymorphs including G-quadruplex, although selectivity is a limitation. While the majority of Ru(II) based light switches reported stabilise the quadruplex, imaging/theranostic probes that can disrupt G4s are of potentially enormous value in study and therapy for a range of disease states. We report here on a Ru(II) complex (Ru-PDC3) that assembles both light switch capability of Ru(II)-dppz complexes with the well-known G4 selective ligand Phen-DC3 into a single structure. The complex shows the anticipated light-switch effect and strong affinity for G4 structures. Affinity depended on the G4 topology and sequence but across all structures bar one, it was roughly an order of magnitude greater than for duplex or single stranded DNA. Moreover, photophysical and Raman spectral data showed discrimination between duplex DNA and G4 bound structures offering the prospect of discrimination in imaging as well as in solution. Crucially, unlike the constituent components of the probe, Ru-PDC3 is a powerful G4 disrupter. From circular dichroism, a reduction of ellipticity of the G4 of between 70 and 95% was observed depending on topology and in many cases was accompanied by induced CD signal for the metal complex. The extent of change in ellipticity are amongst the largest reported for small molecule ligand G4 binding. Cellular applications were investigated in HeLa, MCF7 and A549. Interestingly, localisation was cell line dependant. PLIM, Raman mapping and STED were explored in HeLa cells where nuclear uptake occurred.

2.2 Introduction

DNA exhibits diverse structural polymorphism in-cellulo beyond the double helix, including higher order structures such as triplexes, i-motifs, Y-shaped junctions and guanine quadruplexes (G4s).¹ The latter have the propensity to form when guanine-rich sequences are under physiological conditions. This occurs from stacked guanine tetrads stabilised through Hoogsteen base pair hydrogen bonding. This stacking is further stabilised through a central monovalent cation such as potassium, sodium, or lithium.² Although G4 existence in cells has been well established over the previous two decades through different chemical and biophysical methods, the prevalence and biological role of these structures are still emerging.³ G4s feature in crucial steps in DNA translation, oncogene transcription and telomere stability and are thus cogent sites for both interrogation (imaging) and therapy, via structural stabilisation or via their destruction.⁴

The evidence for G4 structures in vivo is compelling and has accrued over the past two to three decades from diverse methods including chemical mapping, DNA and RNA stalling and Direct imaging^{5–11} Chromatin immunoprecipitation (ChIP) assays with sequencing, (ChIP-Seq) using a G4 structure-specific antibody were performed by Hansel *et al.* that indicated that there are over 10,000 in the human genome that form G4 DNA structures under physiological conditions.^{12,13} G4s have been found to be particularly prevalent in nucleosome-depleted regulatory regions close to transcription start sites of genes that undergo enhanced transcription. They have also been shown to play a role in cancer, and prevalence is increased in cells showing higher proliferative capacity/immortalization. G4 structures have been demonstrated to play a role in genome instability and are highly prevalent in human oncogenes.¹⁴ Thus they have emerged as important therapeutic targets.¹⁵ There is also compelling experimental evidence that G4s exist within the chromatin, where they have the capacity to fold from multiple regions of the genome with intrinsic links to genome expression and genome stability.^{16–18} G4s in RNA are less

studied than DNA but computational models and biophysical studies in vitro have provided strong evidence for their existence and indeed, in solution, RNA quadruplex has been reported to be more thermodynamically stable than DNA analogues.¹⁹ There is currently less, but growing experimental evidence in-cellulo to suggest RNA G4 structures also occur in-vivo.^{20,21}

Development of small molecule G4 ligands for therapeutic applications has focused primarily on polycyclic planar ligands that can stack on tetrads designed to act as stabilising agents, such as Phen-DC3, Braco-19 and telomestatin.^{22,23} Stabilising small molecules can inhibit telomerase activity and induce a DNA damage response (DDR) often providing anticancer activity.^{24–29} Further development of G4 ligands has resulted in additional therapeutic functionalities such as alkylating, oxidising and metalating agents.^{9,22,30–33} However, there has been limited development of disruptive G4 ligands, that could be used to alleviate G4-mediated cellular process or manipulate G4s within cells.³⁴ Only a small number of G4 ligands have been reported to disrupt G4 structures and evidence can be challenging due to the complexity of assessing such mechanisms of action.^{35–37} In addition, disruption can be dependent on G4 type, experimental conditions and techniques. Nonetheless, G4 disruptive small molecules have significant therapeutic potential, as highlighted recently by Monchaud *et al.*, across diseases with their origins in helicase impairment, including in a number of genetic and neurological disorders.³⁸ Further, the implications of G4 stabilisation causing senescence and apoptosis could make G4 disruptors potential therapeutics.³⁹ Design of small molecules that can both probe and disrupt G4 structures offer an elusive but desirable therapeutic prospect.⁴⁰ Indeed, one of the most compelling ways to confirm G4 presence in vivo is by direct visualisation, e.g. through fluorescence imaging. Earliest imaging-based evidence for in-cellulo G4 originated from fixed cell studies using labelled anti-bodies toward G4.^{11,21} G4s have been recently visualised in live cells using fluorescence lifetime imaging.^{6,41} The

small G4 destabilising ligand PhpC described in chapter 1 has been shown to modulate the G4 landscape of RNA G4s in human cells.⁴² This was achieved through a combination of *in vitro* and cell-based assays. This was established in cells where a reduction in PhpC pre-treated cells resulted in a 30-68% reduction in N-TASQ foci caused through G4 binding. Due to the significant difference in apparent dissociation constant values of N-TASQ (apparent $K_d = 0.51 \mu\text{M}$) vs PhpC, (apparent $K_d > 100 \mu\text{M}$) it is argued that the diminishment in G4 cellular signal is related to the destabilisation of their structure in cells and not caused by competitive binding between N-TASQ and PhpC. There has been a number of other small disrupting ligands reported including a Ru(II) organo cation salt, though it has not been studied as thoroughly.⁴³

Inorganic probes are an attractive option for in-cellulo detection of G4s as a number of luminescent coordination compounds are well-established probes of DNA and have been characterised in detail in this regard.^{44–52} Complexes of Ru(II) and Ir(III) have been widely studied as DNA binders with capacity to report on structure and also to induce photodamage both in solution and more recently in cellulo.^{53–59} From the imaging perspective, Ru(II) and Os(II) complexes maintain a number of photophysical benefits over organic alternatives including their large Stokes shifts, environmentally sensitive emission and long-lived phosphorescent lifetimes. Combined, these properties make such probes suitable for multi-modal imaging making them an exciting prospect to explore G4 visualisation in live cells.^{60–66} While a number of G4 targeting metal complexes have been shown in solution to exhibit quadruplex binding, their high affinity for duplex DNA is likely to lead to poor discrimination from G4s *in vivo* so tuning the structures to improve selectivity is required.^{67,68}

In this chapter, the synthesis and characterisation of a supramolecular Ru(II) theranostic probe, Ru-PDC3, is described. The complex is rationally designed for G4 detection and imaging. Ru-PDC3 exploits two key moieties: 1,10-phenanthroline-2,9-

diylbis(carbonylimino)]bis[1-methylquinolinium] (Phen-DC3) one of the family of bisquinolinium dicarboxamides G4 ligands with high affinity and selectivity for quadruplex^{69,70} and, tetrapyrido[3,2-*a*:2',3'-*c*:3'',2''-*h*:2''',3'''-]]phenazine (tpphz) ligand, one of the family of phenazine containing ligands that instigate the characteristic water light switch effect when coordinated to Ru(II) complexes on DNA binding.^{45,71,72} We demonstrate that the Phen-DC3 affinity and light switch behaviour of tpphz are preserved in Ru-PDC3 on G4 binding. We compare the affinity of this complex for different G4 and other DNA structures using luminescence spectroscopy, Time correlated single photon counting (TCSPC), circular dichroism, Raman spectroscopy and experimental data is supported by TD-DFT calculations. The photophysical data confirms high affinity and specificity Ru-PDC3 for G4 structures compared to helical DNA and that clear discrimination between DNA and G4 binding can be made on the basis of emission lifetime data. Furthermore, photophysical data, for the first time, can clearly distinguish Ru-PDC3 with different G4 topologies. Marked changes in lifetime and emission intensity with different topologies were observed. In contrast to other reported Ru(II) based G4 ligands and indeed to Phen-DC3 itself, we have shown using CD titrations that Ru-PDC3 disrupts a variety of G4 structures.⁶⁷

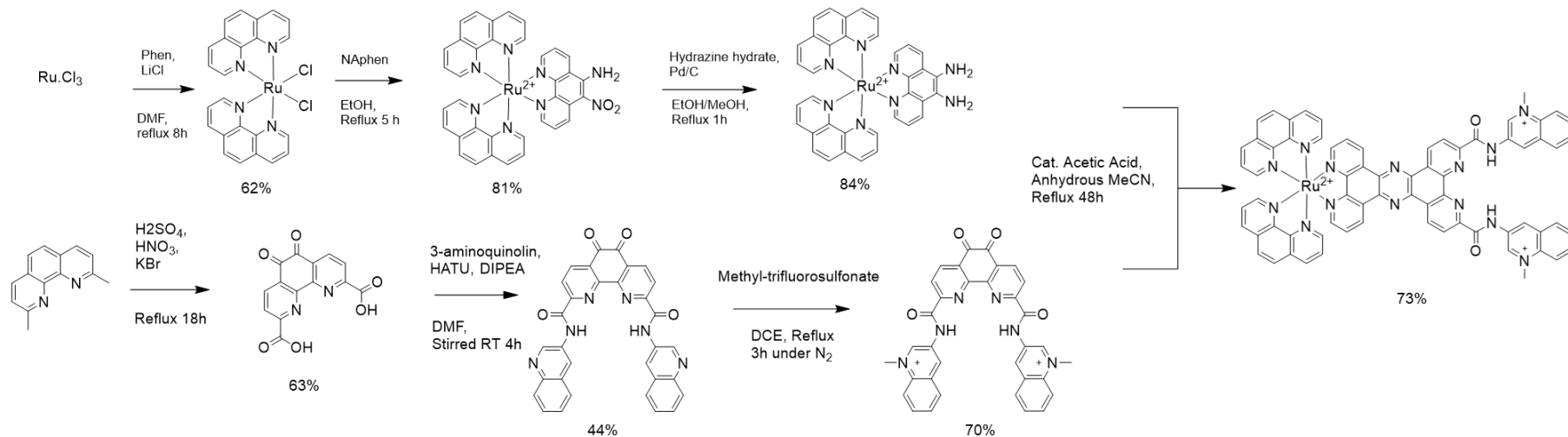
2.3 Synthesis and Characterisation

The primary aim of this chapter was to design and synthesise a novel ruthenium(II) polypyridyl light switch complex that incorporates a highly selective G4 binding ligand in order to achieve live cell uptake and explore its efficacy as a G targeted probe. There are some important considerations for choosing a G4 ligand. It is important that the G4 ligand can be functionalised to allow for incorporation with the Ru(II) complex. Some examples include the amino modified phen-DC3 to allow for ligand conjugation, or the azide functionalised pyridostatin capable of click chemistry in live cells.^{73,74} Furthermore, it was important that the ligand maintained high selectivity and biocompatibility. To this end, modification of Phen-DC3 was pursued, firstly, due to its well documented selectivity towards G4s. Secondly, the increased cationic charge and lipophilicity of this ligand was thought likely to promote cell uptake, where the comparable complexes $[\text{Ru}(\text{phen})_2(\text{tpphz})]^{2+}$ and $[\text{Ru}(\text{phen})_2(\text{dppz})]^{2+}$ proved impermeable. The complex Ru-PDC3 was designed, containing two auxiliary phenanthroline ligands and an extended dppz ligand containing Phen-DC3. Importantly, the complex was designed to include dppz as a bridging ligand, exploiting its light switch properties that occur when complexed to ruthenium(II). A switch was a desirable feature in order to reduce background when imaging and increase resolution. To obtain this complex, the novel phen-DC3 derivative, phendione-DC3, was synthesised.

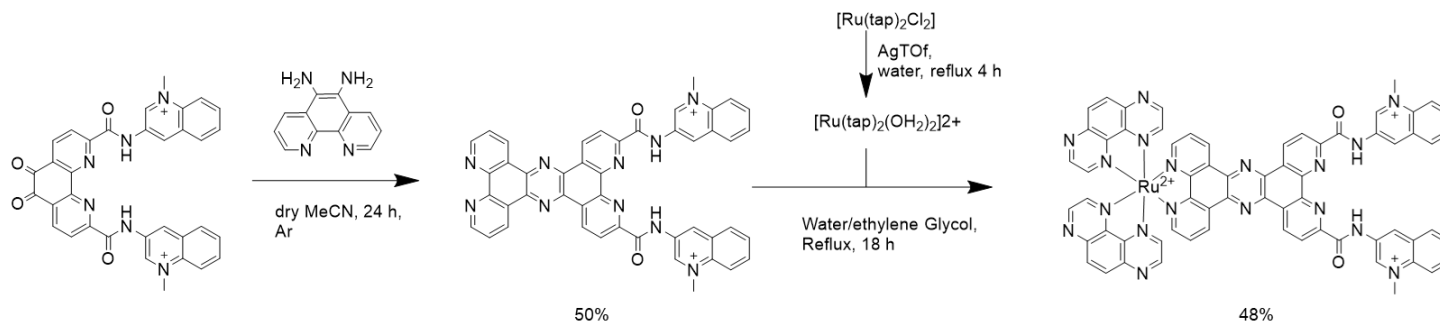
Synthesis of Ru-PDC3 was accomplished as outlined in Scheme 2.1 (A). By incorporating phendione functionality into the phen-DC3, it allows for Schiff-base condensation directly onto the complex without the need of a flexible linker. The synthetic route in this chapter was chosen to exploit the solubility of both ligand and complexes. In the case of phendione-DC3, no columns were required, and purification could be achieved by simply precipitating, filtering and washing the synthetic product. Further, by complexing 5,6' nitro-amino-phenanthroline directly followed by a reduction on complex, it allowed for simple

purification on a silica column to yield $[\text{Ru}(\text{phen})_2(5,6' \text{ diamino-phenanthroline})]^{2+}$. It was advantageous to have 5,6'diamino-phenanthroline complexed to ruthenium prior to the Schiff-base condensation because $[\text{Ru}(\text{phen})_2(5,6' \text{ diamino-phenanthroline})]^{2+}$ has better solubility in MeCN compared to 5,6'diamino-phenanthroline. It was considered that better in MeCN solubility would likely increase the yield of Schiff-base condensation. Another possible route is explored in chapter three, where the Schiff-base condensation is completed off complex to yield tpphz-PDC3, with subsequent complexation of the ligand. This was required due to difficulties arising during column chromatography that will be discussed in greater detail in chapter three. The exploration of this route did also highlight that the complexation of tpphz-PDC3 was significantly less efficient when compared to 5,6' nitro-amino-phenanthroline. This is unsurprising considering the much greater size of tpphz-PDC3 and reduced solubility.

A)



B)



Scheme 2.1 (A) Synthetic route to obtain Ru-PDC3. (B) Synthetic route to obtain Ru-TAP-PDC3.

Synthesis of $[\text{Ru}(\text{phen})_2\text{Cl}_2]$ was achieved through the widely reported route by refluxing RuCl_3 with 2 eq of 1,10'-phenanthroline in DMF with LiCl. Complexation of 5'-nitro,6'-amino-phenanthroline to $[\text{Ru}(\text{phen})_2\text{Cl}_2]$ by refluxing the solution in ethanol for 5 h. The complex was purified using a silica column with the mobile phase 80/20/1 (MeCN/ H_2O /20% w/v KNO_3). $[\text{Ru}(\text{phen})_2(5'\text{-nitro},6'\text{-amino-phenanthroline})]^{2+}$ was reduced using hydrazine hydrate on Pd/C refluxing in an ethanol/methanol mix, yielding $[\text{Ru}(\text{phen})_2(5,6'\text{-diamino-phenanthroline})]^{2+}$. Typically, pure complex could be obtained without the need of column chromatography. This was achieved by simply hot filtering the solution to remove Pd/C, reducing the solution under vacuum, and dissolving the complex in minimal acetone before precipitation as a PF_6 salt. Achieving purity of >95% based on HPLC chromatography analysis.

In parallel, phendione-DC3 was synthesised in an analogous manner to that previously reported for G4 targeting phen-DC3, however the starting material was 1,10-phenanthroline-2,9-dicarboxylic acid-5,6-dione, in place of 1,10-phenanthroline-2,9-dicarboxylic acid (Fig 2.1 (A)). Conjugation of aminoquinoline to the 1,10-phenanthroline-2,9-dicarboxylic acid-5,6-dione was completed via HATU coupling in DMF. The coupled intermediate precipitated from the reaction mixture as an orange solid and was isolated and purified by a simple filtration and wash. Subsequent methylation was achieved by reflux in dichloroethane with excess methyl trifluoromethanesulfonate. Similarly, the methylated product formed as a pale-yellow precipitate and was isolated by filtration and purified by washes with water and diethyl ether.

To synthesise $[\text{Ru}(\text{phen})_2(\text{tpphz-PDC3})]^{4+}$, a Schiff-base condensation between phendione-DC3 and $[\text{Ru}(\text{phen})_2(\text{diamino-phen})]^{2+}$ was performed in MeCN with a catalytic amount of glacial acetic acid for a 73% yield.

The final product was purified using silica column chromatography and isolated as a PF_6^- salt. Counterion exchange was performed using tertbutyl ammonium chloride to generate a water-soluble chloride salt. The excellent water solubility of the complex is an advantage as throughout the spectroscopic and cell-based studies reported herein, there was no requirement for use of mixed solvent systems to promote solubility.

^1H NMR was performed to characterise the structure and indicates that a successful condensation reaction occurred and that Ru-PDC3 was isolated (Fig 2.2). Gradient HPLC was performed to confirm purity by comparing $[\text{Ru}(\text{phen})_2(\text{diamino-phen})]^{2+}$ and Ru-PDC3 chromatograms by measuring absorbance at 440 nm. HPLC analysis of Ru-PDC3 indicated high purity with no residual $[\text{Ru}(\text{phen})_2(\text{diamino-phen})]^{2+}$ observed in the final product. Mass spectrometry was performed yielding a m/z $[\text{M}]^{3+} = 405.0975$ compared with calculated 405.0964 further confirmed the successful synthesis of Ru-PDC3.

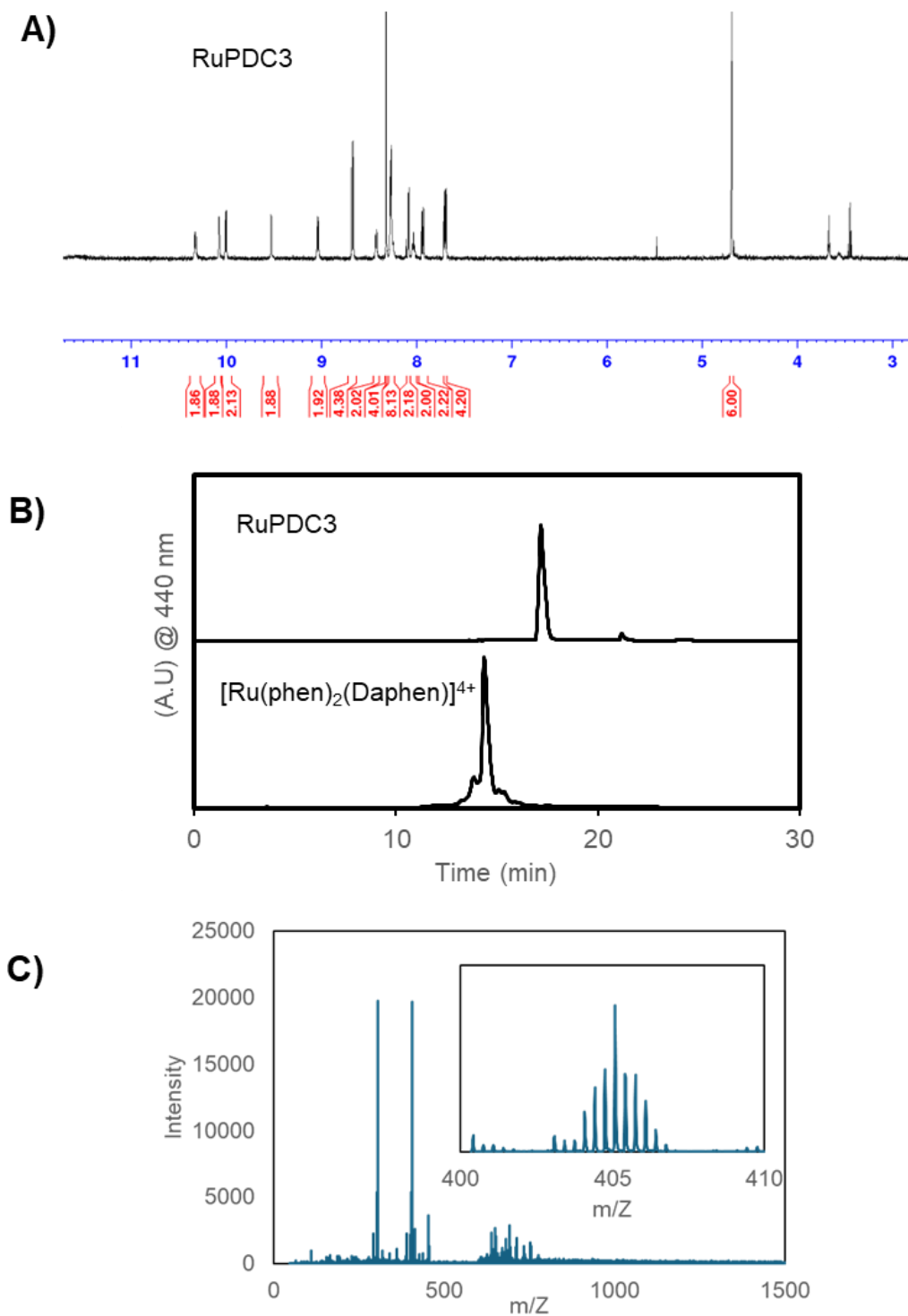


Figure 2.2 (A) ^1H NMR spectra of RuPDC3 in CD_3CN . (B) HPLC chromatogram of RuPDC3 (top) and $[\text{Ru}(\text{phen})_2(\text{Daphen})]^{2+}$ (bottom) using a C18 silica column with a gradient of 0.1% TFA in $\text{H}_2\text{O}/\text{MeCN}$ (C) Mass Spectra of RuPDC3 with inset indicating the 3+ m/z region.

2.4 Optical and Photophysical Properties of Ru-PDC3

Representative absorption and emission spectra of Ru-PDC3 are shown in Fig. 2.3 (A) and 2.3 B. The absorption maximum is centred around 450 nm in water and is red shifted and bandwidth decreases slightly in acetonitrile. To gain insight into the absorbance features TD-DFT calculations on Ru-PDC3 were carried out. The vertical excitation energies and oscillator strengths for the lowest 50 energy states were calculated using the hybrid functional UB3LYP and the LanL2DZ basis set. It was determined that the absorbance maximum, a $^1\text{MLCT}$, contains contributions from two CT transitions originating from the Ru(II) centre to the HOMO phen ligand and to the terminal methylquinolinium on PDC3. The tail of the absorbance contains a lower energy MLCT transition, originating from Ru(II) to the tetrapyrrophenazine of the PDC3 ligand. Additional absorption features at 393 nm and 373 nm are associated with MLCT and ILCT transition extended between the phenanthroline and the methylquinolinium pendants of the heteroligand.

Fig. 2.3 (B) shows that Ru-PDC3 exhibits light switch properties consistent with behaviour reported previously for related tpphz and dppz coordinated Ru(II) complexes. In aqueous media, the emission is essentially extinguished. In aprotic environments, in contrast, as shown here in acetonitrile, intense emission from the $^3\text{MLCT}$ state is observed with a maximum at 645 nm which shows some vibrational structure with a shoulder evident around 700 nm, extending to 850 nm. Exciting at 470 nm, the decay conforms to biexponential kinetics: with τ_1 of 223 ± 2 ns (intensity amplitude of 15%) and τ_2 82 ± 1 ns (intensity amplitude of 75%) under aerated conditions. On deaeration, we obtain τ_1 of 351 ± 7 ns (intensity amplitude of 9%) and τ_2 of 95 ± 3 ns (intensity amplitude of 91%) i.e, only the long-lived component shows significant oxygen sensitivity, but the amplitude of the short-lived component increases. Impurity can be excluded, as beyond the confirmation of purity from HPLC and NMR, as there is no batch-to-batch variation in the amplitude of the decays across different synthesis. The biexponential nature of the decay for Ru(II)

polypyridyl complexes, is concentration independent, so not attributable to aggregation, and may be due to genuine dual emission, as TD-DFT calculations indicate that there are orbitals on the dppz ligand with energies close to the LUMO that extend over the PDC ligand.

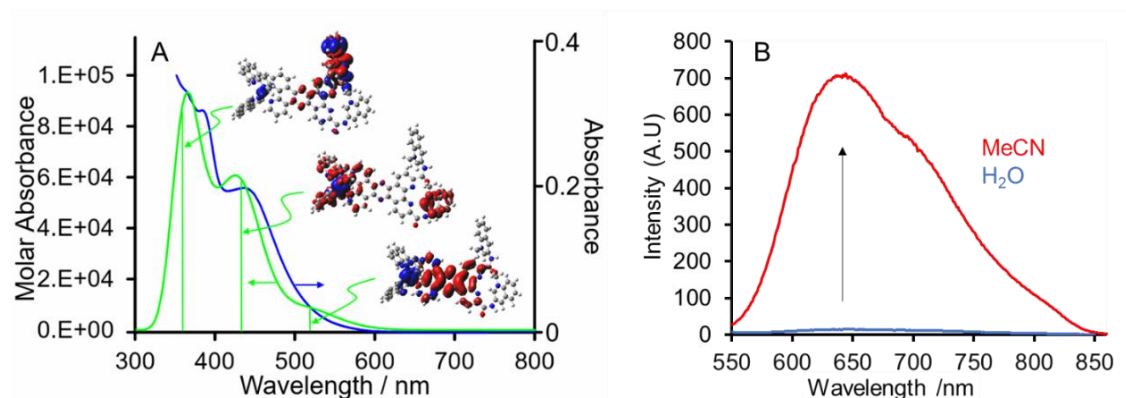


Figure 2.3 A) Experimental (blue) absorption of 10 μ M solution of Ru-PDC3 in water and theoretical (green) absorption Spectrum along with the Electron Density difference for the highest the oscillator strength transitions modelled at the UB3LYP/LanL2DZ for the excited state. Red volumes are region of the complex where the electron density is greater in the excited state compared to the ground state, and blue volumes are regions where the electron density is depleted in the excited state compared to the ground state. (B) Emission spectra of a 10 μ M Ru-PDC3 (λ =450 nm) in D.I H₂O (Blue) and MeCN (red).

We then compared the Raman spectrum, Fig. 2.4, of Ru-PDC3 obtained under 785 nm laser excitation with the resonantly excited Raman spectrum (λ = 473 nm) to gain deeper understanding of the origin of the optical transitions. To aid interpretation, we compared the Raman spectrum under non-resonance (785 nm) excitation conditions with the theoretically predicted Raman spectrum. These spectra are shown in Appendix A. There is excellent agreement between the experimental and predicted Raman spectrum of Ru-PDC3. Based on this interpretation, the vibrational modes are associated primarily with three ligand moieties of the complex, the phen ligands, the tpphz ligand and the terminal methylquinolinium termini are identified and, for simplicity, are indicated as pink, blue and

red in the colour coded scheme in Fig. 2.4 (A) and marked on the resonance Raman spectra shown in Fig. 2.4 (B-D).

Resonance Raman (RR) spectroscopy was then carried out under 473 nm excitation, which is close to the λ_{max} of the complex in water. Under such conditions the Raman active vibrational modes associated with the chromophores participating in the electronic transition undergo a dramatic enhancement in their vibrationally induced polarizability and thus in their Raman scattering cross section. This effect leads usually to between 5 and 7 orders of magnitude enhancement in Raman scattering from vibrational modes associated with the Frank Condon relaxation and so can provide clear insight into the origin of the optical transition.

The Raman spectrum of Ru-PDC3 at 473 nm excitation, shows notable complexity for a resonantly enhanced spectrum. Indeed, all of the vibrational modes observed under non-resonant excitation are also observed in the resonantly enhanced spectrum, albeit with a markedly different intensity profile. Vibrations associated with all three moieties: phen, tpphz and the more remote methylquinolinium unit (CC stretching mode at 1630 cm^{-1}) are evident in the RR spectrum. This observation is consistent with the computed assignment of the electronic spectrum which indicates that the lowest two energy optical transitions, both of which are expected to contribute at 473 nm, are complex MLCT transitions that extend across all three ligand moieties.

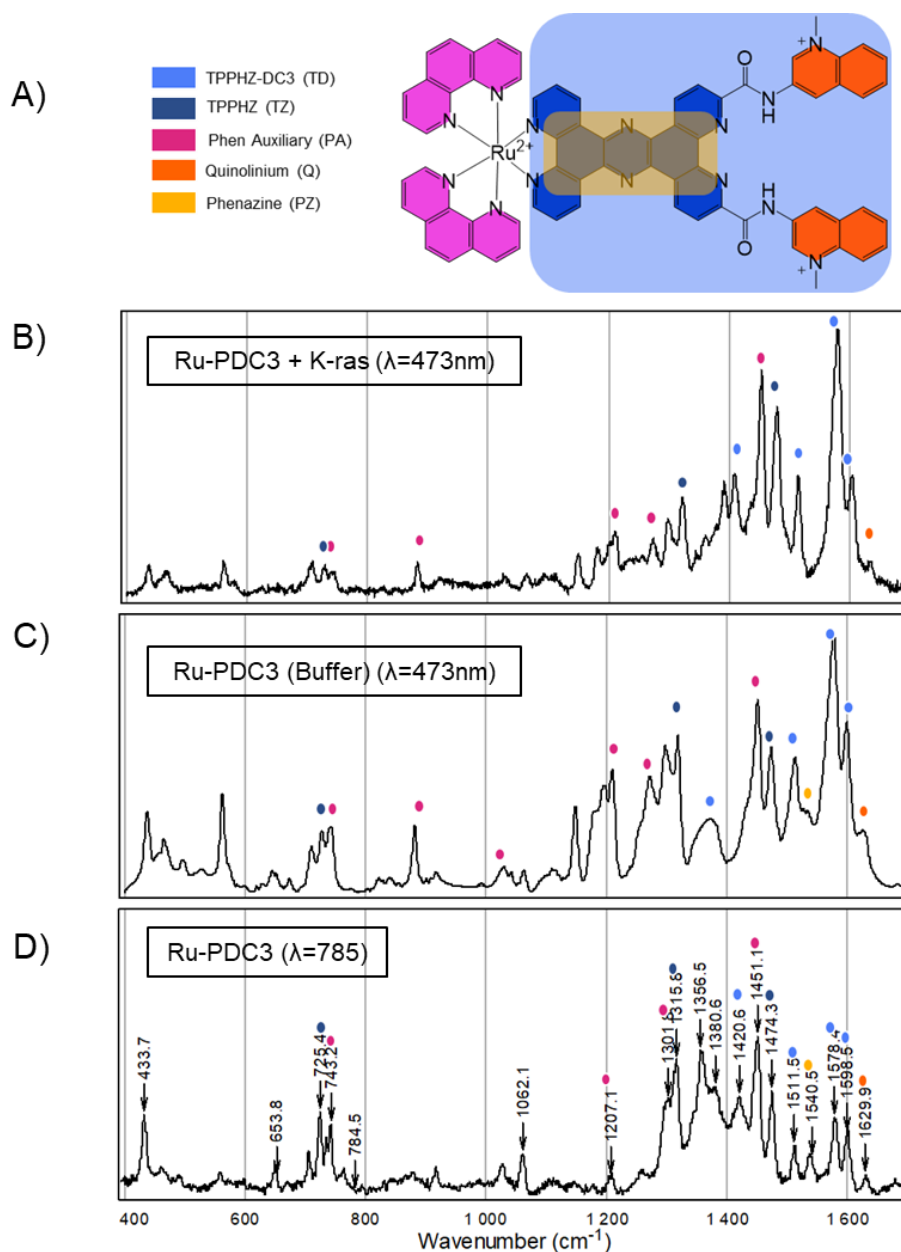


Figure 2.4 (A) Ru-PDC3 structural assignment for Raman Spectroscopy. (B) Raman spectra of Ru-PDC3 (100 μM) ($\lambda = 785 \text{ nm}$) (C) Resonance Raman spectra of Ru-PDC3 (100 μM) bound to K-ras (300 μM) ($\lambda = 473 \text{ nm}$). (D) Resonance Raman spectra of Ru-PDC3 (100 μM) in kpi buffer (10 mM potassium phosphate, 100 mM KCl at pH 7) ($\lambda = 473 \text{ nm}$). (D) Raman spectra of solid-state Ru-PDC3 ($\lambda = 785 \text{ nm}$).

2.5 DNA Binding

2.5.1 Absorption and Luminescent Titrations

To investigate the affinity and selectivity of Ru-PDC3 for G-quadruplex DNA, its interaction with duplex, calf thymus (ct) DNA, single stranded (ss) DNA and a range of guanine quadruplex forming oligonucleotides of representative topology were evaluated and compared using emission intensity, lifetime studies and circular dichroism. The panel of oligonucleotides tested in this chapter are detailed in Table 2.1. DNA G4s were annealed in buffer (10 mM potassium phosphate, 100 mM KCl) at 95 °C for 5 minutes before cooling slowly to room temperature at a rate of 1 °C/min. After cooling, stock solutions were kept at 4 °C overnight. This procedure was used for the preparation of all G4s, with the only exceptions occurring where use of a different buffer solution was required. Oligonucleotides were purchased directly from Eurofins genomics where they were purified by HPLC. For all DNA experiments, it is assumed that all oligonucleotides have formed guanine quadruplexes and that no stacking interactions have occurred between G4s that could result in the formation of multimeric guanine quadruplexes.

Table 2.1 Panel of oligonucleotides selected for testing.

Name	Secondary structure	Sequence 5'→3'	Ref.
22AG	Mixed hybrid	AGGGTTAGGGTTAGGGTTAGGG	75
KRAS	Parallel	AGGGCGGTGTGGGATAGGGAA	76
TBA	Anti-parallel	GGTTGGTGTGGTTGG	77
Pu24T	Parallel	TGAGGGTGGTGAAGGTGGGAAGG	75
INTER	Intermolecular	TAGGGTTA	78
ds17 A	Double Stranded	CCAGTTCGTAGTAACCC	79
ds17 B	Double Stranded	GGGTTACTACGAAGTGG	79
ctDNA	Double stranded		
ss17	Single Stranded	CCAGTTCGTAGTAACCC	79
A13	Single Stranded	AAAAAAAAAAAAA	
T13	Single Stranded	TTTTTTTTTTTTT	

DNA binding studies were performed using a procedure adapted from a literature protocol.⁵¹ Aliquots of DNA from a 1 mM stock solution were titrated into a 10 µM solution of Ru-PDC3 in KPi buffer. After addition, the solution was mixed with and incubated before luminescence measurements. Each titration was repeated at least 3 times. Addition of DNA was continued until saturation was evident. Sufficient incubation times were between the addition of DNA and measurement based on changes in absorption of the MLCT region, this period was typically 5 minutes. Average values for K_b and n were obtained by fitting results to the model described by Carter *et al.* with modifications by Poulsen *et al.*^{80,81} The model is outlined in the equations below and is derived from the Scatchard equation.⁸² MS Solver was used to calculate the best fit while minimising the sum of square residuals. Calculated binding affinities are tabulated in Table 2.2.

$$I_{app} = \frac{b - (b^2 - \frac{4K_b^2 C_t C_{dna}}{r})^{0.5}}{2K_b C_t} \cdot (I_b - I_f) + I_f$$

Equation 2.1

$$b = 1 + K_b C_t + \frac{K_b C_{bp}}{n}$$

Equation 2.2

Where: I_{app} is the apparent intensity of a given binding ratio, $r = [DNA]/[Ru]$; I_b is intensity at saturation; K_b is the binding constant; C_t is the ruthenium concentration; C_{DNA} is the total concentration of DNA by G4 strand equivalent, base pair or base for quadruplexes, dsDNA and ssDNA respectively; n is binding site size.

The emissive response of RuPDC3 was dependent on sequence that the complex was titrated against, as illustrated in Fig. 2.5. In all cases, DNA titration resulted in an increased emission intensity. Fig. 2.5 illustrates the greater emissivity of Ru-PDC3 when bound to the INTER quadruplex in comparison to other G4s indicating effective shielding

of Ru-PDC3 suggesting different binding modes. Interestingly, there does not appear to be a correlation between emission intensity and binding affinity between G4s. The binding mode to G4s is likely dependant on the sequence and folding of the individual sequences, with past studies on ruthenium polypyridyl complexes binding to G4s suggesting binding through loops, bulges and intercalation.^{83–85}

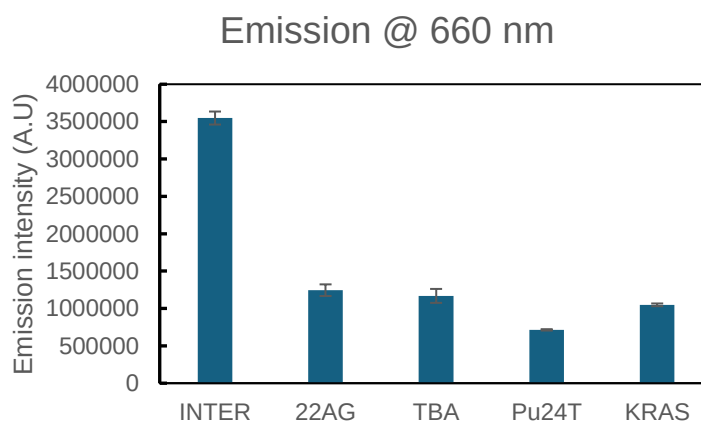


Figure 2.5 Emission intensity of Ru-PDC3 (10 μ M) when bound to G4s at a ratio of [3]:[1], [G4]:[Ru] in KPi buffer. Excitation wavelength = 450 nm. (n = 3)

Representative emission titrations for ds17 and ss17 are illustrated in Fig. 2.6, with their respective binding models depicted as insets. Interestingly, binding affinities for dsDNA and ssDNA were comparable and similar to values previously reported Ru(II)-dppz complexes in buffers of high salt concentration (>50 mM NaCl). Affinity for ds17 and ctDNA were determined as $1.9 \pm 0.3 \times 10^6 \text{ M}^{-1}$ and $2.3 \pm 0.6 \times 10^6 \text{ M}^{-1}$ respectively. For single stranded DNA binding affinities for ss17, T13 and A13 were determined as $1.3 \pm 0.4 \times 10^6 \text{ M}^{-1}$, $1.4 \pm 0.5 \times 10^6 \text{ M}^{-1}$ and $2.2 \pm 1.3 \times 10^6 \text{ M}^{-1}$ respectively, these values are markedly high, and comparable to affinity with ds DNA, and may be attributed to 4+ positive charge on the complex.^{86,87}

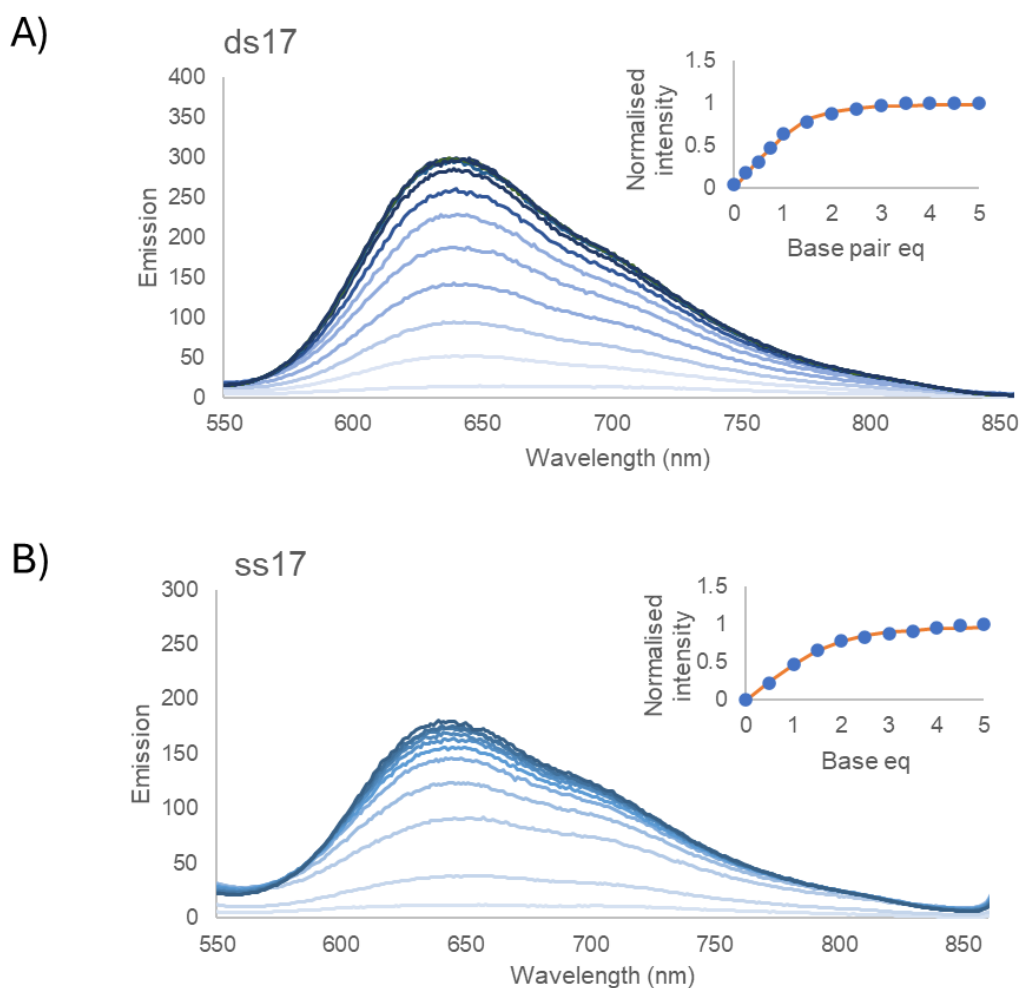


Figure 2.6 Representative emission titration curves for ds17 and ss17. The inset for each graph indicates their respective binding curve and binding model applied to extract binding affinities (Equation 2.1). Excitation wavelength = 450 nm.

For G4 sequences, the quadruplexes were annealed as a concentrated stock in Kpi buffer (10 mM potassium phosphate, 100 mM KCl, pH 7). A panel, of five G4 sequences were selected to represent different possible G4 topologies including parallel, anti-parallel, mixed hybrid and intermolecular. These G4s include, KRAS and Pu24T which are parallel G4s, TBA is an anti-parallel G4, 22AG forms a mixed-hybrid structure when annealed in potassium buffer and INTER a four stranded intermolecular G4. Ru-PDC3 binding affinity for KRAS, INTER and 22AG was at least an order of magnitude greater than recorded for

ds or ss DNA. The affinities were determined as $1.4 \pm 0.1 \times 10^7 \text{ M}^{-1}$, $1.4 \pm 0.2 \times 10^7 \text{ M}^{-1}$ and $2.2 \pm 0.7 \times 10^7 \text{ M}^{-1}$ respectively for KRAS, INTER and 22AG quadruplexes (Fig. 2.7).

The significant variance in binding affinities of Ru-PDC3 for different G4 is interesting and may be explained by considering that binding sites for G4s will be different depending on topology and loop structure. The difference in binding affinity between Pu24T and KRAS is interesting, as they both (and confirmed from CD spectroscopy, vide infra) are expected to have parallel structures. However, while their strands are oriented in the same direction and both contain three quartets, their loop structures are distinctive. KRAS exhibits three regular propeller loops but Pu24T, one of seven nuclease-hypersensitive elements (NHEs), NHE III1 c-myc gene, has unusual fold-back and reversal loop that bridge across the tetrad layers, that are likely the origin of the difference in binding affinity. Differences in affinity between organic ligands between parallel structures has been noted previously.^{88–90} The increased affinity observed for Ru-PDC3 when compared to other Ru(II) analogues may, in part, be associated with the additional electrostatic interactions as a result of the positively charged quaternary nitrogen's. Compared to reports by Su *et al.* Δ - and Λ [Ru(phen)₂(dppz)]²⁺ were reported to bind to 22AG with an affinity of $4.6 \times 10^6 \text{ M}^{-1}$ and $2.7 \times 10^6 \text{ M}^{-1}$.⁶⁷ Further reports by Ji *et al.* indicated a binding affinity of [Ru(bpy)₂(dppz)]²⁺ to 22AG to be $1.4 \times 10^6 \text{ M}^{-1}$.⁹¹ While the additional charge of Ru-PDC3 may increase the binding affinity with G4s, we hypothesise that binding to other DNA structures is less specific as a result of steric hinderance from the bis-quinolinium functional groups. The difference observed in fluorescence intensity/quantum yield on saturation binding indicates differences in binding mode resulting in different degrees of phenazine exposure to the aqueous environment.

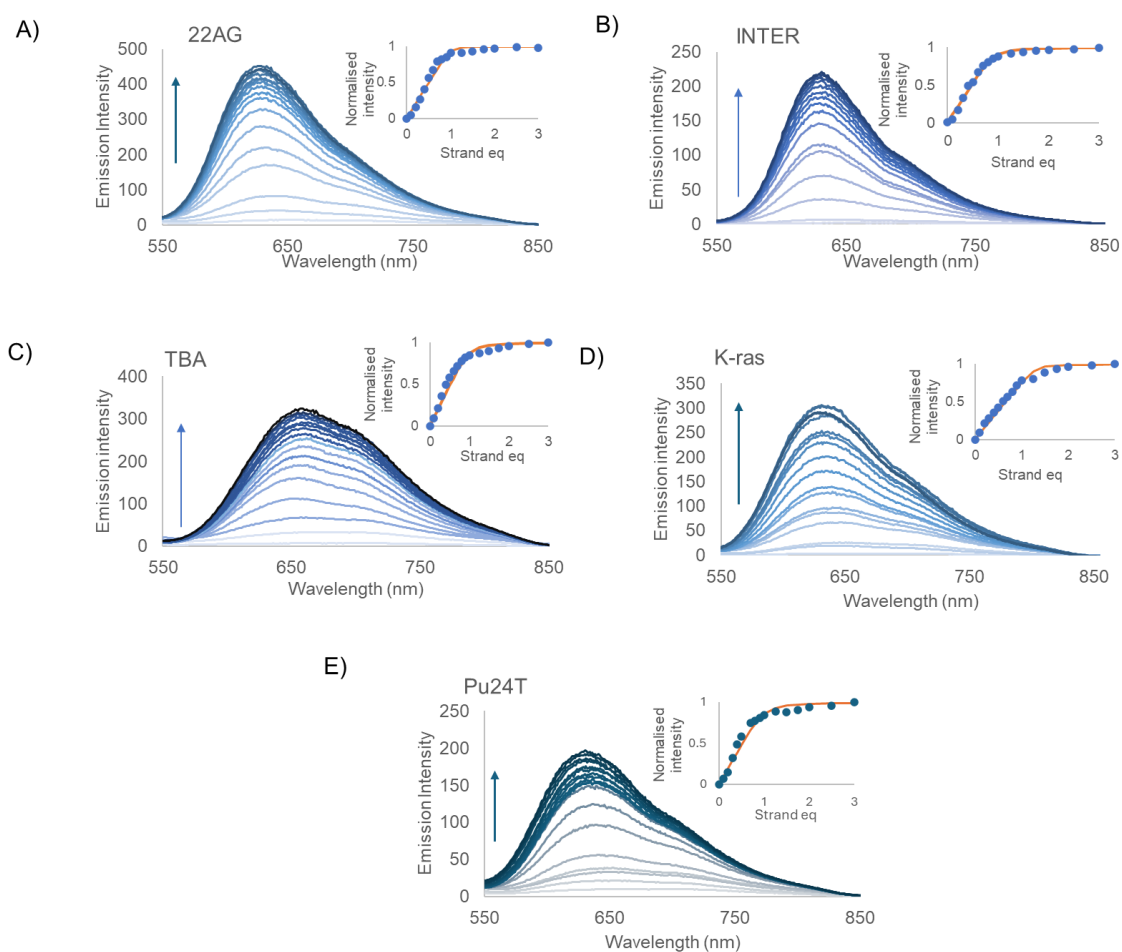


Figure 2.7 Representative emission titration curves for the G4s (A) 22AG, (B) INTER, (C)TBA, (D) KRAS and (E) Pu24T. The inset for each graph indicates their respective binding curve and binding applied to extract binding affinities. Excitation was performed at 450 nm.

The relative emissivity of the bound complex is reflected in the intensity of the graphed plots in Fig. 2.8 (A), as the excitation conditions are identical in each case and followed the following pattern ss<ds<G4. With a notably dramatic increase in emissivity on binding to INTER where emission intensity was roughly an order of magnitude higher than for other G4s and weak emission enhancement, comparable to ss and ds DNA for Pu24T. The bar chart in Fig. 2.8 (B) shows clear discrimination in affinity of Ru-PDC3 for G4 structures over dsDNA and ssDNA. Notably, Ru-PDC3 shows highest affinity for the mixed hybrid quadruplex 22AG with notably lower affinity toward TBA and particularly Pu24T

quadruplexes, where affinity was $5.6 \pm 1.7 \times 10^6 \text{ M}^{-1}$ and $2.6 \pm 0.1 \times 10^6 \text{ M}^{-1}$, though still greater than duplex DNA.

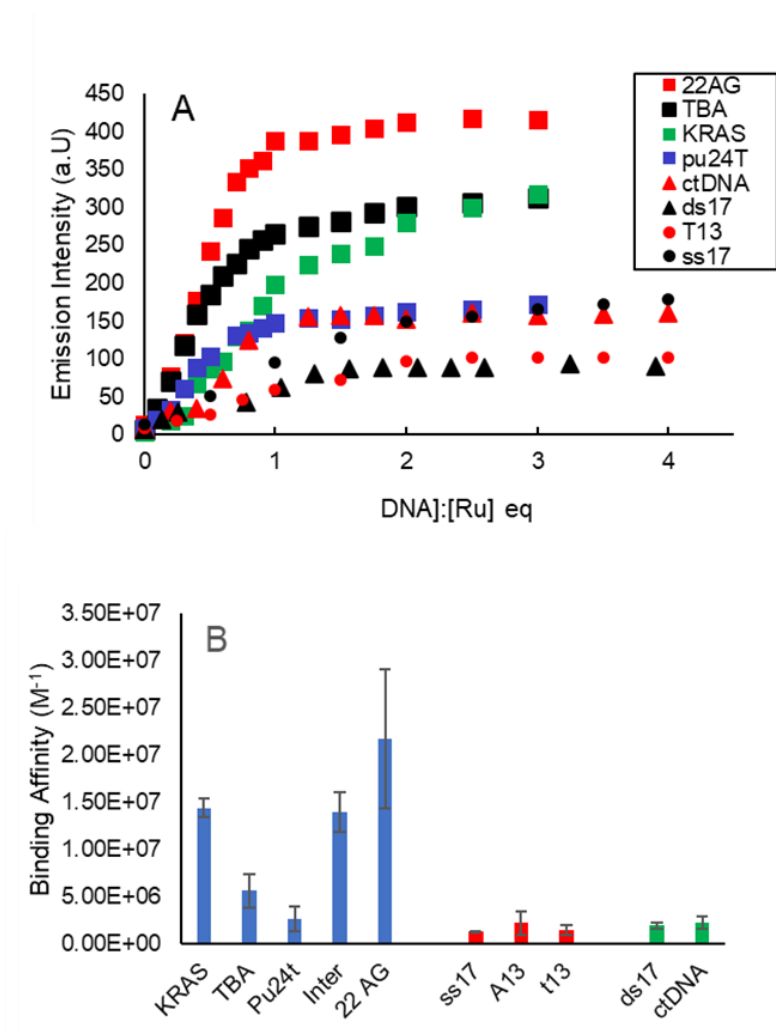


Figure 2.8 (A) Representative titration binding curves for Ru-PDC3 against a series DNA structures (ds17, T13, 22AG, TBA, and K-ras) Concentration is calculated as strand equivalent for G4s, Base pair equivalents for dsDNA Base equivalents for ssDNA. (B) Calculated binding affinities based on binding curves for G4s (blue) ssDNA (red) and dsDNA (green).

Table 2.2 DNA sequences tested and their respective binding affinity

Sequence	Secondary	PDB	K_b (M^{-1})	Site Size	SSR
22 AG	Mixed hybrid	1kf1	$2.2 \pm 0.7 \times 10^7$	0.5	3.6%
KRAS	Parallel	512v	$1.4 \pm 0.1 \times 10^7$	0.5	3.8%
Pu24t	Parallel	2MGN	$2.6 \pm 0.1 \times 10^6$	0.5	3.4%
Inter	Intermolecular		$1.4 \pm 0.2 \times 10^7$	0.5	3.2%
TBA	Anti-parallel	148D	$5.6 \pm 1.7 \times 10^6$	0.5	4.9%
ctDNA	Double stranded		$2.3 \pm 0.6 \times 10^6$	1	3.7%
ss17	Single stranded		$1.3 \pm 0.4 \times 10^6$	1	1.4%
ds17	Double stranded		$1.9 \pm 0.3 \times 10^6$	1	2.2%
A13	Single stranded		$2.3 \pm 1.2 \times 10^6$	1	1.2%
t13	Single stranded		$1.4 \pm 0.6 \times 10^6$	1	1.9%

2.5.2 Influence of DNA Binding on the Lifetime Decay Ru-PDC3

Although there is clearly discrimination in terms of emissivity and affinity of Ru-PDC3 between the DNA structures and individual quadruplexes, as binding stimulates the light switch effect across all nucleic acid structures explored, structural discrimination will not be possible by conventional fluorescence imaging. Fluorescence lifetime imaging (FLIM) however, can provide discrimination if there is sufficient distinction between the lifetimes of the complex as it associates with different structures. Villar *et al.* for example have

reported recent success in discriminating the response of organic dyes against G4s and dsDNA in FLIM.^{6,41,92} To investigate the prospect of discriminating DNA secondary structures on the basis of lifetime, TCSPC was carried out on the Ru-PDC3 with each of the DNA structures. Measurements were performed under binding conditions with [DNA]:[Ru] ratio of [3]:[1] for quadruplexes, [5]:[1] for dsDNA and [5]:[1] for ssDNA. All DNA measurements were performed in a buffer of 10 mM potassium phosphate, 100 mM KCl at pH 7 under aerated conditions.

Table 2.3 presents the resulting lifetime data. As described, the complex emits with biexponential decay kinetics in MeCN while emission is extinguished in aqueous media. In the presence of nucleic acid, emission switches on and decays according to triexponential decay kinetics. τ_1 is > 150 ns and $\tau_2 > 30$ ns. With the exception of A13 and T13 ssDNA the behaviour is comparable to those observed for the parent complex in acetonitrile. In addition, a short-lived component, τ_3 , is observed with values of < 40 ns and less than 10 ns in the case of A13 and T13 ssDNA. This component was not observed in MeCN and is tentatively attributed to emission from complex, bound to DNA but in an aqueous exposed environment. Comparable kinetics have been noted previously for related dppz complexes of Ru(II) on association with DNA structures including quadruplex.⁸³ The long-lived components of the decay, τ_1 and τ_2 are attributed to the dual exponential decay observed in acetonitrile, originating from the complex in a binding mode that offers best protection from water. τ_3 is tentatively attributed to the complex in binding configuration that leaves the complex more exposed to aqueous solution as on the basis of the electrochemistry and optical properties of the complex we do not expect direct electron transfer to the DNA structures. Notably, there is clear discrimination in the lifetime on binding to dsDNA and ssDNA structures and the quadruplexes. This is presumably attributed to different modes between e.g. duplex and quadruplex. Ruthenium complexes have been reported to intercalate between guanine tetrads in intermolecular

G4 structures in addition to terminal capping reported for mononuclear and binuclear complexes.^{49,85} τ_1 in particular is sensitive to quadruplex binding with decays in the range of 389-430 ns, which is roughly 100 ns longer in most cases compared to τ_1 when bound to ds or ssDNA. Indeed, the lifetime of this component exceeds the lifetime observed in deaerated acetonitrile. Indicating, in all cases, that at least one mode of quadruplex binding offers significant protection to Ru-PDC3 from the aqueous environment. However, notably the amplitude of τ_1 varies significantly across nucleic acids.

Consistent with the steady state fluorescence, binding to 22AG yields one of the longest, τ_1 emission decays from the ruthenium centre at 426 (± 8) ns. Indicating that at least one mode of binding to this G4 provides significant protection of the phenazine centre. Although overall, τ_1 is fairly consistent, across all of the quadruplex topologies, ranging from approximately 390 to 430 ns, there is no correlation between affinity for the G4s and τ_1 . For example, τ_1 on TBA association is comparable to 22AG at 426 (± 8) ns even though the affinity was dramatically lower for this polymorph. Rather, it is taken to reflect the most phenazine protected binding mode for a given topology. The amplitudes of the decay components τ_1 and τ_3 are taken to reflect the relative predominance of at least two binding modes. For example, for Ru-PDC3 associated with Inter G4, over 40% of the intensity amplitude of the decay comes from the long-lived component (418 ns) indicating a binding mode that protects the phenazine is more prevalent in this complex, compared for example to K-ras or TBA where 4 and 8% of amplitude of decay is derived from the long-lived component. Indeed, in all G4s, τ_3 dominates the decay, at nearly 50% of amplitude. τ_2 is relatively stable both in value and in amplitude across G4s, with the exception of INTER G4 where it is roughly 50% of the decay amplitude. Interestingly dsDNA, although showing a low τ_1 compared to G4 (and as expected similar to ctDNA), has a significant amplitude of >30 % for this component of the decay, consistent with intercalation. The notable variation of lifetime and relative amplitudes of the contributing components may

provide the opportunities to distinguish between different G4 in a cellular environment. Although the complexity of the decays may make it difficult to analyse in practice, where average lifetimes are easier to collect in imaging. Indeed, discrimination remains clear when, as shown Fig. 2.9, the averaged lifetimes are compared as the shorter-lived components contribute greater amplitude in the lower affinity polymorphs. 22AG and INTER show clear discrimination from the other polymorphs from both intensity and amplitude averaged lifetimes. Representative decays of INTER, ss17 and ds17 are illustrated in Fig.2.10. Overall, the lifetime data shows promise, from the perspective of FLIM, that DNA forms may be discriminated on the basis of lifetime, an increase in intensity weighted lifetimes across all quadruplex environments is clear compared to dsDNA and ssDNA environments.

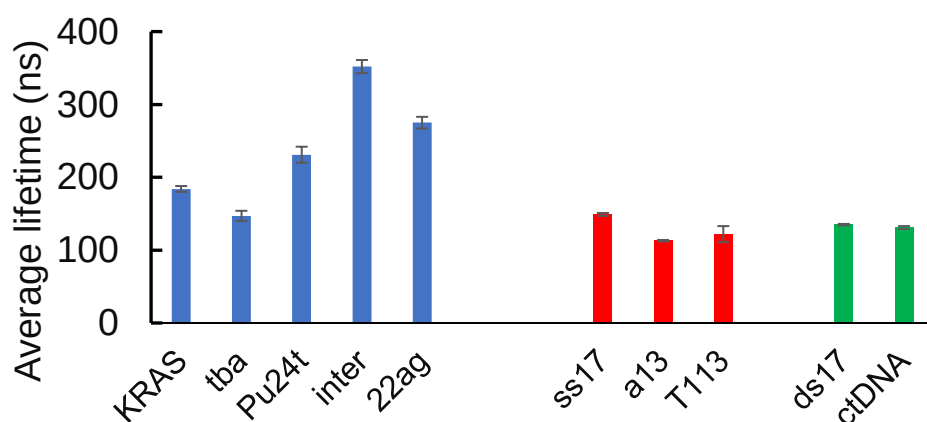


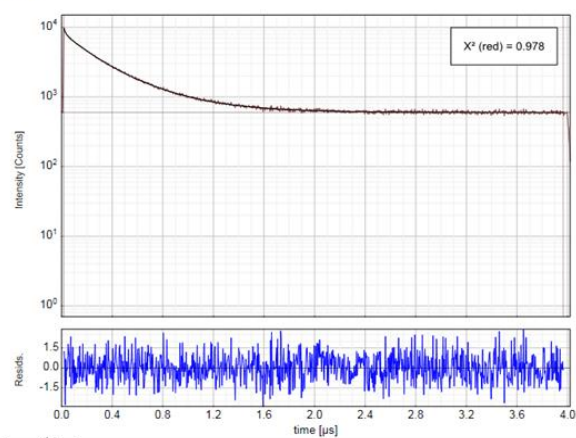
Figure 2.9 Intensity weighted average lifetimes (ns) of Ru-PDC3 upon saturated binding to a panel of DNA structures. Average lifetimes are an average of 3 repeats and obtained using the PicoQuant Fluofit software.

Table 2.3 Lifetime and amplitude of each component obtained from TCSPC measurements ($\lambda = 450$ nm). Ru-PDC3 (10 μ M) in the presence of G4s (30 μ M) dsDNA (50 μ M), and ssDNA (50 μ M). Values are the average of three repeats. Triexponential fit was applied using the PicoQuant Fluofit tailfit analysis software

	τ_1 (ns)	α_1 %	τ_2 (ns)	α_2 %	τ_3 (ns)	α_3 %	$\tau_{av.1}$ (ns) intensity
Aerated (MeCN)	223 (± 2)	14.3	82 (± 1)	75.7	-	-	130 (± 4)
Deaerated (MeCN)	351 (± 7)	8.9	95 (± 3)	91.1	-	-	163 (± 3)
K-ras	389 (± 6)	8.5	93 (± 5)	44.0	24 (± 6)	47.5	184 (± 4)
22AG	426 (± 8)	14.5	110 (± 5)	39.2	19 (± 3)	46.4	275 (± 8)
Pu24t	430 (± 30)	8.7	101 (± 10)	37.3	19 (± 4)	54.0	231(± 11)
Inter G4	418 (± 13)	43.6	197 (± 9)	40.4	13 (± 2)	16.0	352 (± 9)
TBA	395 (± 17)	4.5	90 (± 8)	44.7	37 (± 4)	50.8	147 (± 7)
ds17	284 (± 5)	31.7	74 (± 1)	57.0	16 (± 1)	11.3	135 (± 1)
ctDNA	291 (± 4)	6.5	62 (± 1)	53.4	12 (± 1)	40.1	131 (± 2)
ss17	329 (± 12)	6.6	75 (± 1)	43.4	17 (± 1)	50.0	149 (± 2)
A13	152 (± 4)	8.4	40 (± 2)	52.4	4 (± 1)	39.2	113 (± 1)
T13	205 (± 26)	9.8	46 (± 5)	51.3	8 (± 2)	37.8	122 (± 11)

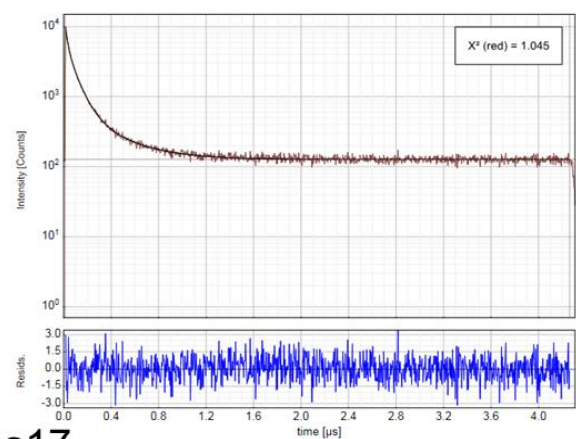
Inter

Main Plot



ds17

Main Plot



ss17

Main Plot

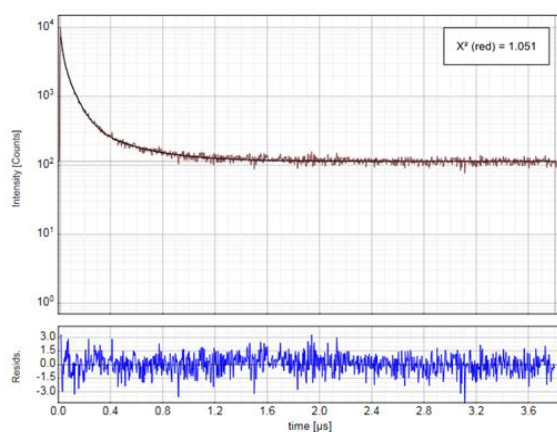


Figure 2.10 Lifetime decays of Ru-PDC (10 μM) with INTER (30 μM)., ds17 (50 μM). and ss17 (50 μM). Excitation = 450 nm.

2.5.3 The Influence of Ru-PDC3 on DNA Structure Through CD Spectroscopy

As G-quadruplex DNA is optically active, circular dichroism (CD) spectroscopy is a useful method to interrogate structural changes to G4s induced by their interaction with Ru-PDC3. The G4 topologies for each sequence used here can be readily distinguished by CD spectroscopy. The metal complex used herein is a racemic mixture so as expected does not show optical activity in absence of DNA. In all cases, the CD of the G4s in Kpi buffer was consistent with the expected topography as reported previously.^{75–79} Peak maxima and minima were observed at approximately 264 and 245 nm respectively for K-ras and Pu24T, consistent with their parallel topology, at 295 and 260 nm respectively for TBA consistent with anti-parallel structure and at 295 max, $\approx$ 260 max, and 245 nm respectively for 22AG which has a mixed hybrid structure.⁹³ Peak maxima for INTER were observed at 215 and 260 nm consistent with a four stranded intermolecular quadruplex. The peak maxima or minima shifts, changes of amplitude or loss of these features on G4 interaction with small molecules can reflect formation or disruption of the G4 structure.

In these experiments, Ru-PDC3 was titrated into a solution where G4 DNA was at a constant concentration to follow changes to the G4 topology. Recently, such an assay has been established to investigate known stabilisers and disruptors, where it was shown that a reduction in CD peak intensity at a characteristic quadruplex wavelength can relate to disruption of G4s.³⁸

Remarkably, as illustrated in Fig. 2.11, titration of Ru-PDC3 against the above described G4s resulted in exceptional disruption. This was evident as significant loss of ellipticity occurred on addition of the metal complex. Such a disruptive response is relatively unusual upon small ligand binding, particularly so for ruthenium polypyridyl complex binding, where mainly stabilisation of quadruplexes has been reported to date. One

exception was a mononuclear complex of $[\text{Ru}(\text{phen})_2(\text{PHEHAT})]^{2+}$ where similar reductions in ellipticity of Tel22 G4 at high ratios ($> 5:1$ ratio of Ru:G4) were evident.⁸⁴

When titrated against the antiparallel TBA G4, the signal at 295 nm was reduced by 82% at 5 equivalents of Ru. Such dramatic, near total loss of ellipticity is exceptional for a small molecule, particularly under such low [Ru]:[G4] ratio and under high salt conditions (100 mM KCl).

Notably across several G4 topologies, induced CD (ICD) was observed in features associated with the metal complex. ICD can occur when an achiral molecule displays CD within their absorption bands after association with a chiral molecule. Following titration of 3 equivalents of Ru(II) against TBA, ICD is observed at 400 nm and 260 nm that grow upon further titration. Such a response may indicate selective affinity of one enantiomer, Δ or Λ , for the G4 structure. Similarly, the CD signal of the two parallel complexes Pu24T and K-ras, was greatly reduced when titrated against Ru-PDC3 both G4s exhibited remarkably similar destabilisation with a total reduction in ellipticity at 265 nm of 71% and 73% for Pu24T and K-ras, respectively. As the destabilisation is similar for both parallel G4s, it is possible that the similarity relates to the influence of the sequence direction and loop structure. Unlike TBA, ICD signals did emerge to a significant extent during the titration. Titration of Ru-PDC3 against the INTER G4 resulted in the most extensive disruption of the quadruplex signal and these changes were also accompanied by an intense ICD. Ellipticity decreases by 95% at a 10:1 [Ru]:[G4] ratio at 210 nm. The intense ICD of Ru-PDC3 occurring 248 and 264 nm obscures potential changes in ellipticity arising from G4 topology, to overcome this analysis was performed at multiple wavelengths. Interestingly, Ru-PDC3 did not induce complete disruption of the mixed hybrid quadruplex 22AG. The decrease in ellipticity at 265 nm and positive ellipticity at 295 and 245 nm indicates a conformational change to an anti-parallel quadruplex in which guanines are stacked in alternating orientations. This ligand-induced change is

remarkably similar to the previously reported interaction between Phen-DC3 and a human telomeric G4 analogous to 22AG, where a K^+ ion was ejected by Phen-DC3 followed by the stabilisation of a two quartet anti-parallel quadruplex.^{94,95}

The promising disruptive activity of Ru-PC3 was unexpected and, we believe, currently unparalleled in small molecule G-quadruplex association across the literature and wasn't repeatable in later chapters with analogous Ru(II) complexes. As a comparison, TMPy4, one of the best known G4 small molecule ligands shows results in less disruption across a narrower range of G4s. As reported by Mitteaux *et al.*, when TMPy4 is titrated against 22AG in K^+ buffer at a ratio of [10]:[1] ligand to G4, a reduction in CD ellipticity of 68.7% was observed when monitored at 293 nm.³⁸ Based on computational studies it has been suggested that unfolding occurs after an initial binding of TMPy4 to the top face of a G4 disrupting H-bonding in the tetrad, the ligand then intercalates causing consecutive disruption of the remaining tetrads.⁹⁶ A Schiff-Stilbene ligand has also been reported to disrupt G4s through groove binding and intercalation.⁹⁷

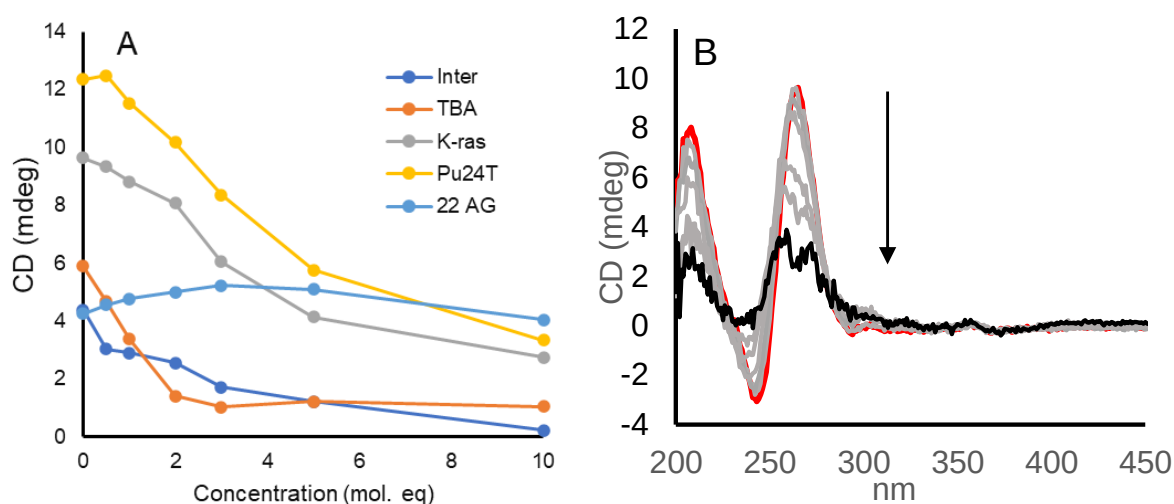


Figure 2.11 A) Intensity of peak CD signal from different G4s as a function of of Ru-concentration. Ru-PDC3 concentration ranges from 0-10 mol. equivalent to [Ru]:[G4].

Quadruplex concentration is maintained at 5 μM in KPi buffer. CD signal intensity was monitored a peak maxima, that depended on the topology which were 265 nm for Pu24T and K-ras, 295 nm for 22AG and TBA, and 210 nm for INTER. (B) Representative CD spectra of K-ras (5 μM) when titrated against increasing equivalents of Ru-PDC3 (0-10 mol. Eq [Ru]:[G4]).

2.5.4 Resonance Raman Spectroscopy of G4 bound complex.

The resonance Raman spectroscopy of the Ru-PDC3 was examined under 473 nm excitation following its incubation with each of the nucleic acids studied. Under resonance conditions only the Raman features of Ru-PDC3 are evident in the spectrum. Association with the nucleic acids exerted subtle but significant influence on the resonance Raman spectrum. There were small shifts in the key Raman features on nucleic acid association and these were greater for G4 than DNA, but in particular there are clear changes in relative intensities of the phen based vibrational modes between 1400 and 1500 cm^{-1} that increase in relative intensity compared to the tpshz features that dominate above 1500 cm^{-1} . Similar behaviour has been noted previously in dppz based Ru(II) complexes on DNA binding. In addition, most notably, the vibrational mode at 1534 cm^{-1} isolated on the phenazine is lost exclusively on quadruplex binding (Fig. 2.12).⁹⁸ The feature although reduced in intensity on duplex binding remains evident in all other nucleic acid materials, especially single stranded structures. This is a potentially important finding that may permit discrimination of G4 structures in resonance Raman in cells.

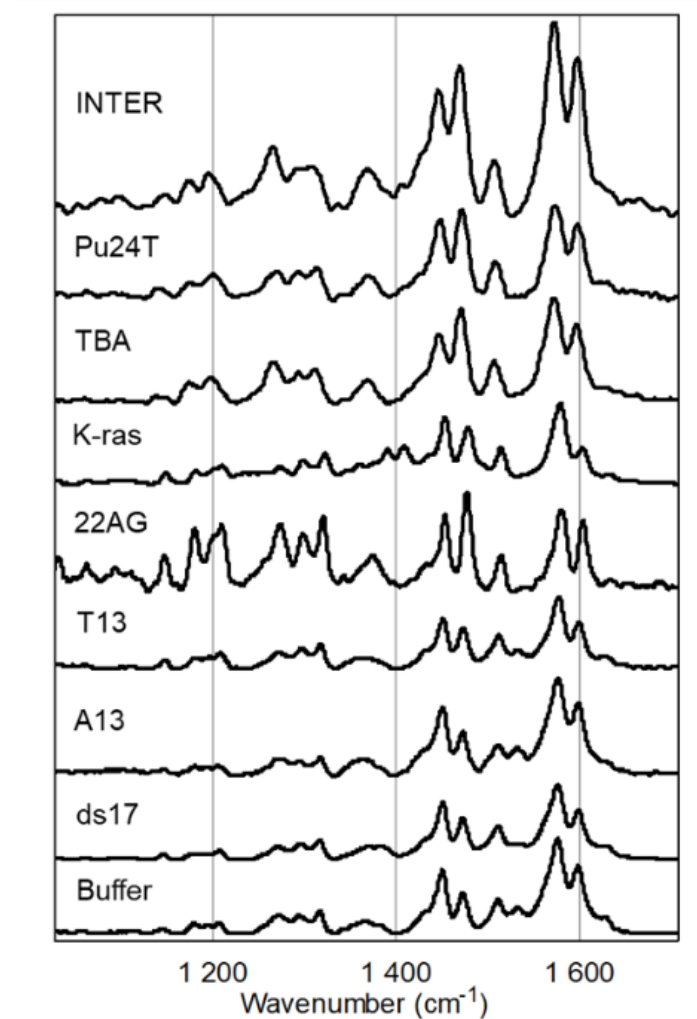


Figure 2.12 Resonance Raman spectra of Ru-PDC3 (100 μM) bound to G4 DNA, ssDNA, and double stranded DNA (300 μM , 500 μM and 500 μM , respectively) in kpi buffer ($\lambda = 473 \text{ nm}$). Spectra are normalised to the vibrational band at 1508 cm^{-1} .

2.6 Cell Studies

2.6.1 Cellular Uptake

Ultimately for application of these complexes as imaging probes or in therapy, cell permeability and targeting is important. Given the high cationic charge of Ru-PDC3 and its water solubility, we explored whether the complex, without further modification, is

permeable to live cells. We examined uptake by A549 lung carcinoma cells using confocal fluorescence microscopy.

Fig. 2.13 shows representative cell imaging following incubation of Ru-PDC₃ at 50 μ M in A549, lung cancer cells for 24 h. Due to the light-switch properties of the complex in the aqueous environment, monitoring its uptake and true distribution within the cell is challenging as it emits from only aqueous exclusive environments. Nonetheless, following uptake, the complex exhibits bright punctuate emission from within the cytoplasm, although it appears to be largely nuclear excluding.

To evaluate, then if the punctate distribution of the probe is due to endosomal entrapment we evaluated colocalization of the complex with Rab7a-GFP, a commercial dye used to stain late endosomes.⁹⁹ Representative co-localisation studies are shown in Fig. 2.13 (E) where Ru-PDC₃ (shown in red) was found to colocalise to an extent with Rab7a-GFP, (in yellow), evident by the overlap regions (in orange) and the Pearson's coefficient value of 0.73, though it is clear that Ru-PDC₃ is not solely confined to the endosomes. Late endosomes serve as a sorting station for internalised extracellular molecules and can fuse with lysosomes as part of the endocytic pathway.¹⁰⁰ We thus evaluated co-localisation of emission of Ru-PDC₃ with LysoTracker Green, a lysosomal staining dye representative and obtained a Pearson's coefficient value of 0.51 ± 0.03 suggesting partial localization of the complex in lysosomes under these conditions.

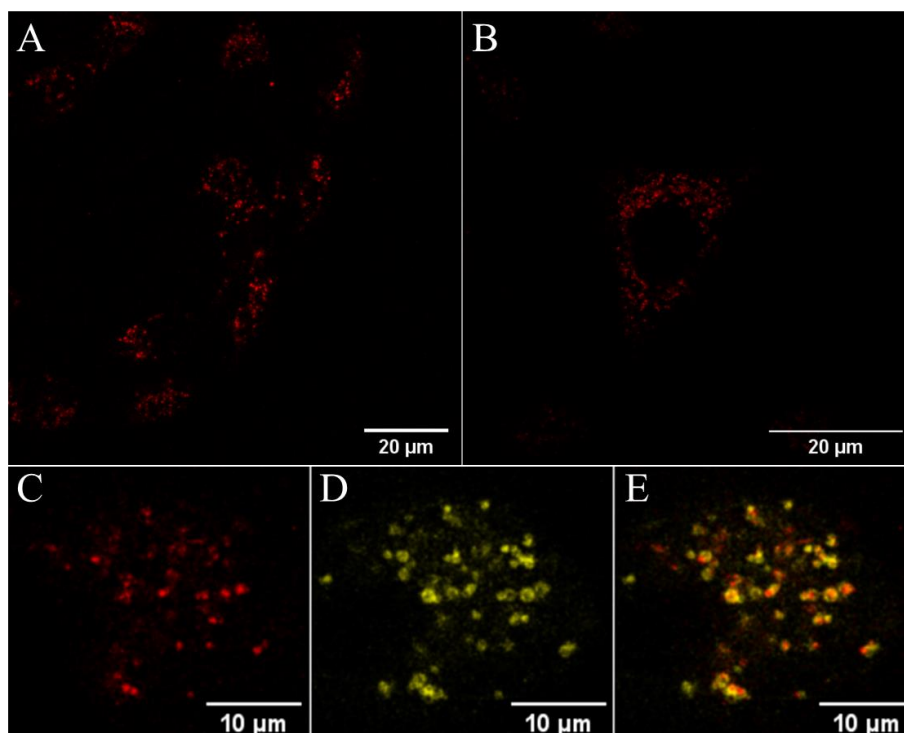


Figure 2.13 Confocal images showing the uptake of (A-B) Ru-PDC3 in live A549 cells at 50 μ M following 24 h incubation in the absence of light. A 475 nm white light laser was used to excite the ruthenium complex and emission was collected between 550 and 800 nm. In colocalization studies cells were incubated with (C) 50 μ M Ru-PDC3 for 24 h and co-stained with (D) Rab7a-GFP that indicated confinement in some late endosomes evident by the overlap of the two channels in the (E) overlay image (Pearson's coefficient, $r = 0.73 \pm 0.03$). Rab7a-GFP was excited at 488 nm and emission was collected between 490 nm – 540 nm.

To investigate if cellular localisation may be cell line dependent, additional studies were performed in HeLa cells. Interestingly, a significant difference in localisation was evident with nuclear and perinuclear uptake occurring after 2.5 h at 70 μ M (Fig. 2.14). To confirm nuclear uptake, co-staining with Hoechst was performed and indicated that punctate emission arising from RuPDC3 emission occurred within the nucleus. This is a significantly quick incubation time to achieve nuclear localisation for a metal complex and a promising outcome for therapeutic avenues. Although nuclear localisation is evident, further studies were completed to determine if the emission was the result of nucleic acid binding.

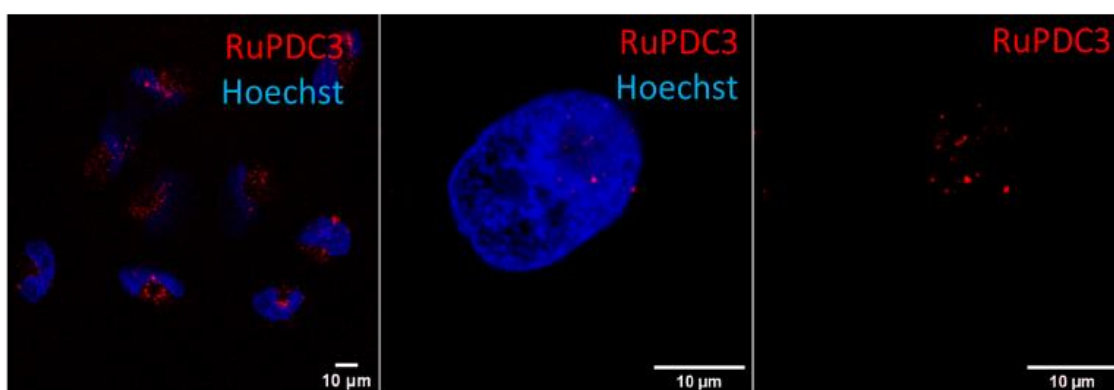


Figure 2.14 Confocal images of cells incubated with Ru-PDC3 (70 μ M/ 2.5 h) and co-stained with Hoechst nuclear staining dye; scale bar: 10 μ m.

To confirm emission was a result of DNA binding, immunofluorescence studies with the anti-G4 antibody BG4 were employed in combination with RNase A and DNase (Fig. 2.15). When HeLa cells were treated with RNase A, the staining pattern remained largely unchanged, indicating that RNA binding was not a significant contributor to the emission of RuPDC3. Whereas, after treatment from DNase, a significant reduction in signal arising from the nucleus is evident, further confirming the emission occurs as a result of DNA binding.

Significantly, overlap of RuPDC3 and BG4 foci was evident and a Pearson's Co-efficient of 0.55 was observed indicating successful G4 binding. This is further visualised in Fig. 2.15 (C), where emission intensity mapping in the nucleus is compared between BG4 and RuPDC3. It is evident that good correlation between BG4 and RuPDC3 foci is observed.

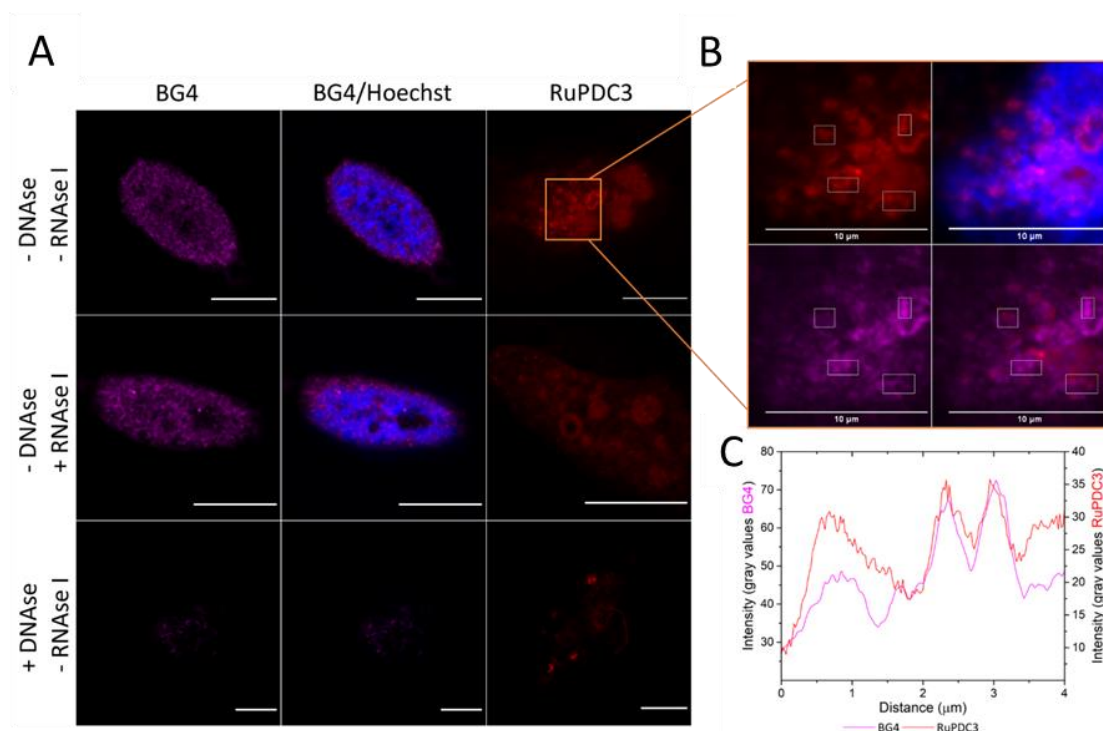


Figure 2.15 (A) Detection of G-quadruplexes in HeLa cells and enzymatic treatments with DNase and RNase I. Control cells were prepared by labelling with anti-G quadruplex recombinant antibody BG4 and co-staining with DAPI. Test cells were prepared by treating cells with Ru-PDC3 while live and then performing BG4 immunofluorescence following fixation. (B) Enlarged image region within the cell nucleus showing Ru-PDC3 staining, Ru-PDC3/Hoechst overlap, BG4 staining and Ru-PDC3/BG4 overlap (Pearsons's coefficient, $r = 0.55 \pm 0.02$); scale bar: 10 μm . (C) Representative intensity line profile for BG4 and RuPDC3 (ROIs shown in SI).

Since the choice of cell line clearly impacts the uptake of RuPDC3, an additional cell type, MCF 7, investigated. Again, interestingly, a different localisation was observed. Under the same incubation conditions as HeLa, RuPDC3 localises exclusively in the cytoplasm with no signal arising from the nucleus. When compared to A549 which also results in cytoplasmic uptake, localisation is observably different. In the case of MCF 7, RuPDC3 is less punctate and resembles mitochondrial staining. To confirm, colocalisation studies with biotracker 488 green were performed and image analysis resulted in a strong Pearson's coefficient of 0.73 (Fig. 2.16). Further emphasising the importance of testing multiple cell

lines. Additionally, it highlights the benefits of theranostics for their built-in capacity for visualisation, in this case fluorescence microscopy.

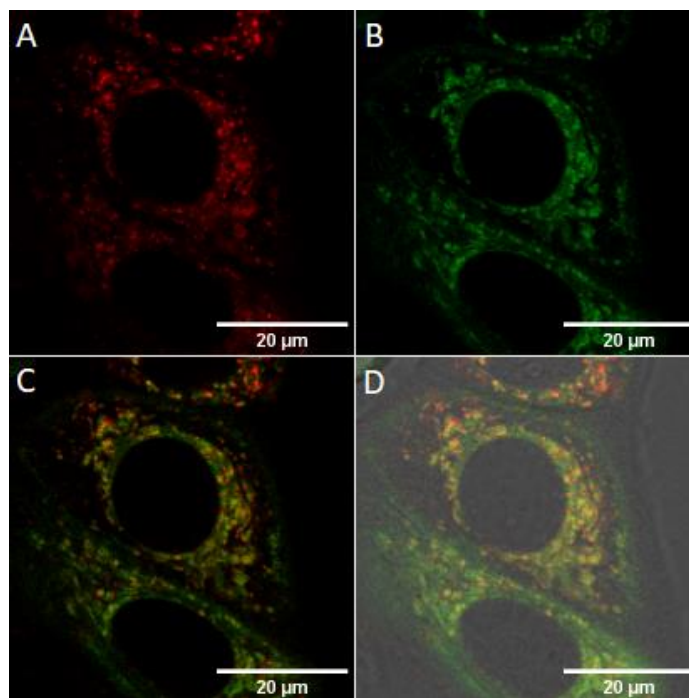
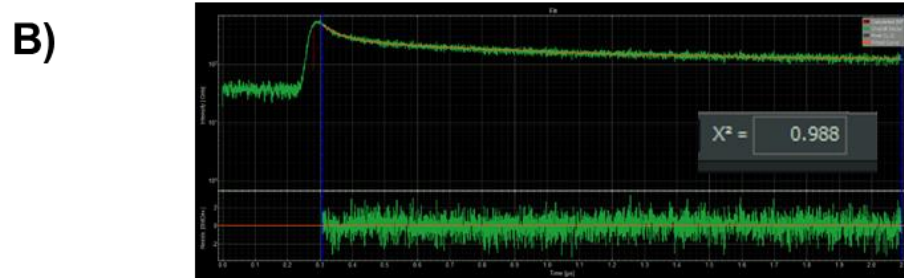
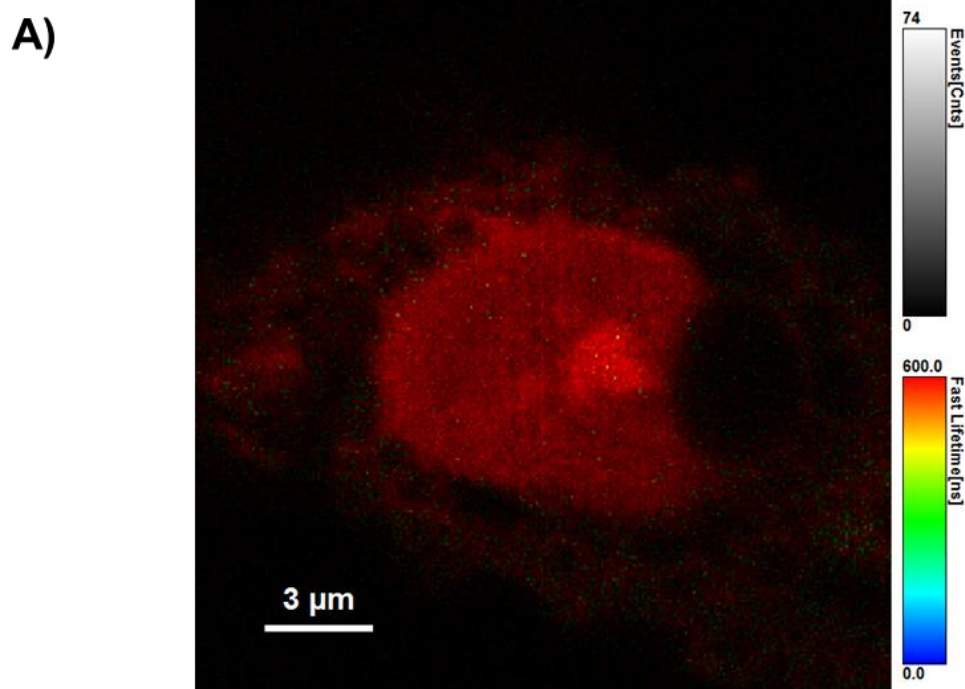


Figure 2.16 Confocal images of MCF7 cells incubated with Ru-PDC3 at the same incubation conditions applied in HeLa studies (70 μ M/ 2.5 h) and co-stained with BioTracker 488 Green (100 nM; 25 min) staining dye. Shown are the A) RuPDC3 , and B) BioTracker 488 Green emission channels and overlay C) of the two channels and with D) brightfield. Pearson's coefficient, $r = 0.73 \pm 0.02$.

2.6.2 PLIM

As RuPDC3 is bound to nuclear DNA, PLIM was undertaken to study the nature of RuPDC3s decay in a live cell environment. The complex was imaged after a 2.5 h incubation period at 70 μ M (Fig. 2.17). Since this decay is arising from RuPDC3 bound to DNA, comparisons to solution studies can be analysed in order to assess binding environments. The complex showed a biexponential decay of 600 ns and 50 ns in the nucleus of the cell, which is notably longer than RuPDC3 in A549 cells. In HeLa cells, the

intensity weighted average was 400 ns, and the amplitude weighted lifetime was 200 ns. In comparison, RuPDC3 recorded a long decay of approximately 400 to 450 ns in when bound to G4 DNA in solution. Crucially, the long decay when bound to duplex DNA or ssDNA was less than 300 ns. Comparatively, it is evident that the decays in a live cell environment are similar to decays observed upon G4 binding from solution studies. It is important to highlight that cellular conditions and buffer solutions are not identical, due to this variations in decays will occur. This variation in decay has been noted in previous PLIM and FLIM studies. Over the previous decade, there has been an increase in papers using these techniques to identify G4 formation in cells. The Vilar group have used the dye DOATA-M2 with some success and have more recently have followed this with additional cellular studies. It should be noted that the weighted average lifetimes recorded in solution were similar to solution studies of G4 and ss/dsRNA, studies incubating cells with the G4 binder pyridostatin resulted in a reduced average lifetime that the authors link to a reduction in G4 binding. The same group recently reported RNA G4 imaging with the novel dye, TOR-G4, although it should be noted that average lifetimes observed in U2OS cells were between 2.2 and 3.5 ns and in solution the reported average lifetimes for RNA G4s were >4.9 ns. There is clearly a need for exploration of decays recorded in solution and comparisons to those recorded in cell environments. The picture becomes further complicated when considering Ru(II) polypyridyl complexes that contain two isomers. Lincoln *et al.* have explored the how different isomers can result in different decays as a result of a difference in DNA binding between isomers. This is important when considering the RuPDC3 PLIM studies where differences in the solutions studies could be a result of the cellular environment or specific enantiomer binding, uptake or localisation. Nonetheless, the long decay of RuPDC3 would suggest direct G4 binding.



C)

Model Parameters: n 2

Parameter	Value	Fit
t_0 [ns]	$306.176 \pm \text{-----}$	
$A_{[1]}$ [kCnts] Limits	0.1482 ± 0.0014	✓
$A_{[2]}$ [kCnts] Limits	0.2157 ± 0.0021	✓
$\tau_{[1]}$ [ns] Limits	600.0 ± 7.3	✓
$\tau_{[2]}$ [ns] Limits	52.0 ± 1.2	✓
Bkgr Deco [Cnts] Limits	117.68 ± 0.45	✓
$I_{[1]}$ [kCnts]	173.8 ± 1.7	
$I_{[2]}$ [kCnts]	21.91 ± 0.51	
I_{Sum} [kCnts]	196 ± 2	
A_{Sum} [kCnts]	0.3639 ± 0.0032	
$\tau_{\text{Av Int}}$ [ns]	539.0 ± 6.6	
$\tau_{\text{Av Amp}}$ [ns]	275.3 ± 4.3	

Figure 2.17 PLIM mapping of RuPDC3 in nuclear region of a single live cell; scale bar: 3 μm . Cells were incubated with 70 μM RuPDC3 for 2.5 h prior to imaging.

2.6.3 Resonance Raman Mapping

Multi-modality in probes can be enormously beneficial when investigating biophysical phenomena, particularly for validating observations. An advantage of Ru(II) polypyridyl probes is their large Stokes shift that can facilitate resonance Raman mapping. To this end, resonance Raman mapping of live HeLa cells was performed after treatment with RuPDC3. Cells were treated with 70 μM for 2.5 h prior to mapping experiments. As evident from Fig. 2.18, resonance Raman spectra was successfully collected from the nucleus of HeLa cells further confirming nuclear accumulation of RuPDC3. Interestingly, the vibrational mode observed at 1544 cm^{-1} does not appear suggesting G4 binding. Loss of the same band was evident in in solution studies of RuPDC3 bound to a series of G4s as seen in Fig. 2.12.

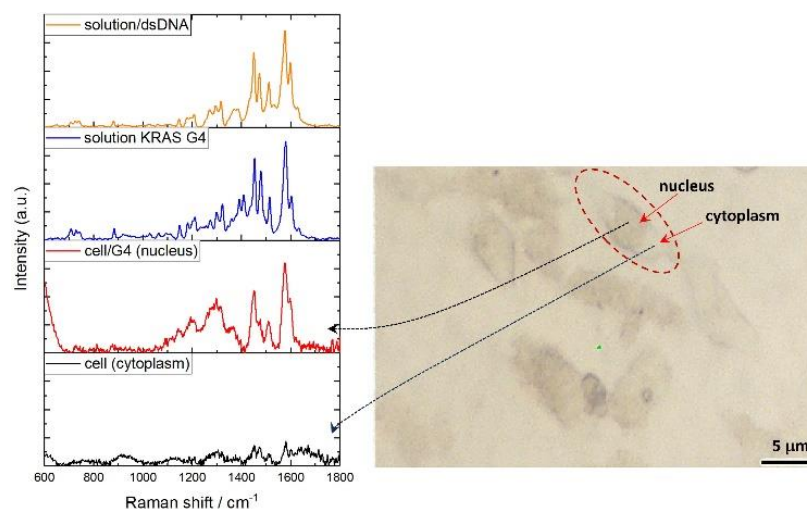


Figure 2.18 Resonance Raman mapping in live HeLa cells pre-treated with Ru-PDC3 (70 μM / 2.5 h). The Raman spectrum of Ru-PDC3 in the cell nucleus was compared to the cytoplasm spectrum and to Ru-PDC3 in PBS solution upon addition of dsDNA or KRAS.

2.6.4 Toxicity Studies

Ultimately as these complexes are intended as therapeutics in combination with diagnostics, studies were carried to assess cytotoxicity and phototoxicity (Table 2.4). Cytotoxicity studies using the Alamar Blue assay showed that the Ru-PDC3 complex was mildly toxic toward the A549 cell line with an IC_{50} of $85 \pm 10 \mu M$. Although extensive distribution of the complex within cells was observed, the low cytotoxicity is tentatively attributed to significant endosomal entrapment of the complex under these conditions. By comparison, a series of ligand (PDC)-based derivatives in A549 cells showed moderate toxicity over a 72 h incubation with IC_{50} values ranging from $16 \mu M$ to above $100 \mu M$.¹⁰¹ Due to uptake occurring in the mitochondria and nucleus, dark toxicity and phototoxicity was evaluate for MCF7 and HeLa cells. Dark toxicity was low with an IC_{50} greater than $100 \mu M$ but when irradiated at 470 nm, the complex was significantly more toxic with an IC_{50} of $3.51 \mu M$. This is unsurprising considering the essential function of mitochondria for cell function by the generation of ATP. Targeting of mitochondria with PDT agents is very affective according to the literature. Groups have had great success targeting the mitochondria with ROS generating probes, particularly with Iridium due to their proclivity towards mitochondrial accumulation, though one drawback is their dark toxicity typically around $2 \mu M$. This reduced dark toxicity of RuPDC3 in comparison is an attractive property. The complex recorded a photoirradiation factor of >28 making it a viable phototherapeutic for MCF7. Further investigating the result of localisation on toxicity, cytotoxicity of RuPDC3 was tested in HeLa cells. RuPDC3 had a dark IC_{50} of $70 \mu M$ and a light IC_{50} of 1.35 resulting in a PIF of 37.84. The high phototoxicity of RuPDC3 when accumulated in the nucleus can be attributed to the generation of ROS, damaging DNA causing cell death. The well-studied G4 ligand, pyridostatin has reported an IC_{50} of $81.6 \mu M$ in MCF-7, a similar toxicity to RuPDC3, in HeLa cells under dark conditions.¹⁰²

Table 2.4 PIF and IC_{50} values of RuPDC3 on MCF7 and HeLa adherent cells.

Cell Line	IC ₅₀ (μM) ^[a]	IC ₅₀ (μM) ^[b]	PIF ^[c]
MCF7 adherent	> 100	3.51 ± 1.39	> 28
HeLa adherent	70 ± 3.21	1.85 ± 1.69	37.84
A549	85 ± 10 μM	N.D	N.D

2.7 Conclusions

The light switch light switch capability of the well know Ruthenium tetrapyrido[3,2-*a*:2',3'-*c*:3'',2''-*h*:2''',3'''-]]phenazine (tpphz) complex was combined with a high affinity G4 ligand (Phen-DC3) to create a high affinity reporter for G-quadruplex DNA that could be applied to live cell imaging. The complex shows complex absorption and emission behaviour. DFT computation indicates overlapping contributions from three charge transfer transitions originating from the Ru(II) centre to the phen ligand, the terminal methylquinolinium and to tetrapyridophenazine of the PDC3 ligand. This is supported by resonance Raman data which shows that under 473 nm excitation, all of the ligand systems are enhanced. The complex shows the light switch effect, emission is extinguished in water and switched on in acetonitrile or on association with nucleic acid. From emission titrations, the complex shows particularly high affinity for G4 quadruplex DNA, although the affinity was G4 dependent with K_a over an order of magnitude greater for K-ras and 22AG compared with ctDNA. The emission intensity and average emission lifetime are also greatly enhanced on binding to these two G4 structures. Interestingly, the resonance Raman spectroscopy shows a small but important change on quadruplex binding; the vibrational mode at 1534 cm⁻¹ isolated on the phenazine is lost on G4 binding but not on association with other nucleic acids.

Critically, and unexpectedly the complex was found to be a potent G4 disrupter. Circular dichroism revealed that across all G4s studied a dramatic loss of G4 ellipticity occurred,

which was particularly prevalent for KRAS and TBA, both of which showed significant induced CD at the metal complex absorbance. Furthermore, interestingly the disruption does not correlate with the binding affinity or enhancement in emission intensity.

The complex, as a chloride salt is water soluble and was found to be readily cell permeable through an activated uptake mechanism. The complex was found in A549 cells to be localised to a significant degree, although not exclusively, to endosomes and lysosomes in live cells, consistent with an endocytotic uptake mechanism. It showed strong emission from within the cells and based on emission lifetime imaging the emission switch is observed due to association with organelle membrane rather than nucleic acid materials.

When studies were expanded to include additional cell lines, nuclear uptake was observed in HeLa cells and mitochondrial uptake was observed in MCF7. DNA binding in HeLa cells was confirmed through DNase and RNase experiments. G4 binding was explored through a combination of immunofluorescence, PLIM and Raman mapping. Colocalisation with BG4 indicated successful G4 binding in nucleus. PLIM studies recorded a long lifetime that was representative of G4 binding in solutions of approximately 500 ns. Similar to solution studies when RuPDC3 binds to G4s, rRaman mapping of RuPDC3 in the nucleus showed depletion of the 1544 cm^{-1} vibrational band.

Toxicity experiments were performed in A459, HeLa and MCF7 showing a dark toxicity of 85 μM , 70 μM and >100 μM respectively. Light induced IC_{50} of RuPDC3 was 3.51 and 1.89 μM in MCF7 and HeLa respectively, showing highly phototoxicity and significant PIFs of >28 and 31.

2.8 Materials and Methods

Synthetic procedures

[Ru(phen)₂(diamino-phen)]²⁺

[Ru(phen)₂(5,6'-nitro-amino-phen)]²⁺ (50 mg) and Pd/C (25 mg) were added to a 50:50 MeOH: EtOH solution and brought to reflux. Hydrazine hydrate (0.2 ml) was added, and the solution was refluxed for 1 h before vacuum filtration. The filtrate was concentrated before purification on a silica gel column. Purified fractions were collected and reduced under pressure, redissolved in minimal MeCN before precipitation in a NH₄PF₆ solution to yield a red solid. (40 mg, 84% yield) ¹H NMR (600 MHz, CD₃CN): 8.60 (m, 4 H) 8.48 (dd, 2 H), 8.25 (t, 4 H), 8.03 (t, 2 H), 8.01 (t, 2 H), 7.78 (dd, 2 H), 7.60-7.66 (m, 4 H), 7.51 (dd, 2 H), 4.75 (s, 4 H).

1,10-phenanthroline-2,9-dicarboxylic acid-5,6-dione (1)

1) Was synthesised as previously reported by Gislason *et al.*¹¹ Neocuproine (0.51 g) and KBr (0.42 g) were added to a RBF. Fuming sulfuric acid (5 ml) was added in portions to the round bottom flask followed by the dropwise addition of conc. nitric acid (2.5 ml). The solution was refluxed for 6 hours. After refluxing the solution was allowed to cool, poured over ice water (25 ml). The resulting yellow ppt. was filtered and washed with distilled water, diethyl ether. ¹H NMR (600 MHz) DMSO-d₆: 8.58 (d, J = 8.0 Hz 2H), 8.26 (d, J = 7.9 Hz, 2H).

5,6-dioxo-N2,N9-di(quinolin-3-yl)-5,6-dihydro-1,10-phenanthroline-2,9-dicarboxamide (2)

1,10-phenanthroline-2,9-dicarboxylic acid-5,6-dione (100 mg) was dissolved with anhydrous DMF in a N₂ purged RBF. DIPEA (0.34 ml) was added followed by HATU (0.38 g) previously dissolved in DMF and allowed to stir for 30 minutes before addition of 3-

aminoquinoline (0.97 g) previously dissolved in anhydrous DMF. The solution was stirred overnight. The orange precipitate was filtered and washed with minimal DMF, deionised water and diethyl ether. (82 mg, 44% yield) ^1H NMR (600 MHz): 11.69 (s, 2 H), 9.62 (d, $J = 2.9$ Hz, 2 H), 9.08 (d, $J = 2.3$ Hz, 2 H), 8.76 (d, $J = 7.7$ Hz, 2 H), 8.52 (d, $J = 7.8$ Hz, 2 H) 8.07-8.11 (m, 4 H), 7.76 (t, $J = 8.0$ Hz, 2 H), 7.67 (t, $J = 8.0$ Hz, 2 H). m/z calculated = 551.1473, Found 551.1464.

Phendione-DC3 (3)

2) (47mg) was dissolved in 30 ml of anhydrous dichloroethane and brought to reflux under N_2 . Methyl trifluorosulfonate (0.3 ml) was added and the solution refluxed for 18 hrs. The yellow ppt. was filtered and washed with water and diethyl ether. (52 mg, 70% yield) ^1H NMR (600 MHz) 11.98 (s, 2 H), 10.20 (d, $J = 2.3$ Hz, 2 H), 9.80 (d, $J = 1.6$ Hz, 2 H), 8.83 (d, $J = 8$ Hz, 2 H) 8.61 (d, $J = 8.1$ Hz, 2 H), 8.54 (d, $J = 8.8$ Hz, 2H), 8.49 (d, $J = 7.92$ Hz, 2 H), 8.26 (t, $J = 8.2$ Hz, 2 H), 8.06 (t, $J = 7.1$ Hz, 2 H), 4.69 (s, 6 H) Meas. m/z calculated = 729.1385, Found 729.1384.

Ru-PDC3

$[\text{Ru}(\text{phen})_2(\text{DAphen})](\text{PF}_6)_2$ (30 mg) and Phendione-DC3 (36 mg) were dissolved in dry MeCN. A catalytic amount of glacial acetic acid was added, and the solution was refluxed for 72 hrs under nitrogen. The solution was concentrated under vacuum and purified on silica column using a 80/20/1 (MeCN/ H_2O /20% KNO_3) mobile phase. Product fractions were collected and combined. Sat. Ammonium hexafluorophosphate was added and the solution was concentrated under vacuum. The red ppt. was filtered and redissolved in minimal MeCN. The product was further precipitated in a sat. ammonium hexafluorophosphate solution. The orange product was filtered and washed with deionised water and diethyl ether. Yield = orange solid (41 mg, 73% yield). ^1H NMR (600 MHz, CD_3CN): 10.35 (d, $J=7.8$ Hz, 2 H), 10.14 (s, 2 H) 10.02 (d, $J=7.8$ Hz, 2 H) 9.51 (s, 2

H), 9.03 (d, J=8.0 Hz, 2 H), 8.67 (dd, J= 8.4, 1.1 Hz, 4 H), 8.43 (d, J= 9.0 Hz, 2 H), 8.20-8.34 (m, 12 H) 8.08 (d, J=5.0 Hz, 2 H) 8.04 (t, J= 7.8 Hz, 2 H) 7.92 (m, 2H) 7.69 (m, 4 H) 4.73 (s, 6 H). Meas. m/z $[M]^{3+}$ calculated = 405.0964, Found 405.0975.

Absorbance measurements were performed using a Jasco V670 spectrophotometer, data was manipulated using Jasco Spectra Manager software and MS Excel. Emission titration spectra was obtained using a Varian Cary Eclipse Fluorimeter. Slit widths were set to 20 nm for all DNA, with the exception of emission studies using the G4, INTER, where 10 nm was used due to greater emissivity. Lifetime measurements were performed on a PicoQuant FluoTime 100 Compact FLS TCSPC system using a 450 nm pulsed laser from PicoQuant PDL800-B source and an external Thurlby Thandar Instruments TGP110 10 MHz pulse generator to enable acquisition of long lifetime data. Data was collected up to 10,000 counts and decay curves were analysed using PicoQuant Fluofit software and tail-fit statistical modelling. De-aeration was performed by bubbling samples with argon for 15 minutes before measurement. Circular dichromism measurements were performed on a Chirascan Series and data manipulation was performed using the Global 3 Analysis Software and MS Excel. Comparative emission measurements of Ru-PDC3 bound to G4s were recorded on a Clariostar Plus plate reader in triplicate. High resolution Mass Spectrometry (HR-MS) was performed by the HR-MS facility, Trinity College Dublin. Analytical HPLC was performed on a Varian 940-LC Liquid Chromatograph using an Agilent Zorbax Pursuit XRs C18 column (5 μ m, 4.6 x 250 mm). Samples were prepared in distilled water, gradient elution was performed with a flowrate of 1 ml/min starting with a 0.1% TFA in H₂O/MeCN mobile phase starting with 95/5 increasing linearly to 50/50 over 20 mins. Peak detection was performed using PDAD at 440 nm.

Stock concentrations of Ru-PDC3 were stored as PF₆⁻ salts in MeCN or as Cl⁻ salts in deionized water at a concentration of 5 mM.

Oligonucleotides were purchased from Eurofins Genomics and stored at -20°C. Preparation of G4s were performed similarly to previous reports.¹⁰³ A KPi buffer (10 mM potassium phosphate, 100 mM KCl) was used to anneal the quadruplex for all measurements. Quadruplexes were annealed at 95 °C for 5 mins before cooling slowly to room temperature. Fluorescent and absorbance titration experiments were performed in tandem using a stock concentration of 1mM quadruplex strand concentration.

Chemicals were purchased either from Sigma-Aldrich (Merck) or Fluorochem and used without further purification. ¹H NMR spectra were recorded on a 600 MHz Bruker spectrometer. ¹H NMR spectra were processed using the Bruker Topspin software. The complexes [Ru(phen)₂(nitroamino-phen)]²⁺ and [Ru(phen)₂(diamino-phen)]² were synthesised as described by Gillard *et al.*¹⁰⁴

Raman studies

Resonance Raman spectroscopy was carried out on Ru-PDC3 (100 µM) in KPi. Instrument calibration was performed prior to measurement. Ru-PDC3 was measured in the presence of DNA with a 473 nm laser with 20 accumulations per measurement. Measurements were repeated at least three times. Spectra in this section are representative spectra. Baseline correction and peak picking were completed using NGS LabSpec5 software.

Circular dichroism

Circular Dichroism was performed using a G4 DNA solution annealed at 5 µM in KPi buffer. Titrations were repeated at least three times, each measurement presented is the average of two accumulations. Spectra were recorded over a window of from 200 nm to 550 nm. Ru-PDC3 was titrated (2.5-50 µM) in each sample and measured after at least 5 min to allow binding to complete following prior confirmation that this length of time was sufficient to see no additional changes.

2.8.1 Cell culture

The $[\text{Ru}(\text{phen})_2(\text{tpphz-PDC3})]^{4+}$ parent complex was studied in a lung carcinoma cancer cell line (A549). The cells were subcultured using Dulbecco's modified Eagle's medium supplemented with 10% FBS and 1% penicillin–streptomycin and were grown at 37 °C with 5% CO₂.

Uptake studies of $[\text{Ru}(\text{phen})_2(\text{tpphz-PDC3})]^{4+}$

A549 cells were seeded at 8×10^4 cells in 35 mm glass bottom culture dishes (Ibidi, Germany) and were allowed to grow for 48 h at 37 °C with 5% CO₂. Cells were treated with $[\text{Ru}(\text{phen})_2(\text{tpphz-PDC3})]^{4+}$ at the desired concentration using a dye stock solution of 1 mM in PBS. Following the desired incubation time, at 37 °C at 5% CO₂, the cells were washed twice with supplemented PBS (1.1 mM MgCl₂ and 0.9 mM CaCl₂). Treated cells were imaged directly using a Leica TCS DMI8 confocal microscope (63 X oil immersion objective lens) with a heated stage at 37 °C. A 475 nm white light laser was used to excite $[\text{Ru}(\text{phen})_2(\text{tpphz-PDC3})]^{4+}$ and the emission range was set to between 550 and 800 nm. In the metabolic inhibitor study, the cells were pre-treated with 5 μM oligomycin and 50 mM 2-deoxy-D-glucose for 40 minutes and the contact solution was replaced with $[\text{Ru}(\text{phen})_2(\text{tpphz-PDC3})]^{4+}$ at 50 μM / 24 h in fresh media.

Rab7a-GFP dye, used to stain late endosomes, was added to the cells, and incubated overnight at 37 °C prior to addition of the ruthenium complex. Rab7a-GFP was excited at 488 nm and emission was collected between 490 nm – 540 nm. LysoTracker Green DND 26, used for staining lysosomes, was added at 60 nM and incubated for 30 min prior to imaging (λ_{exc} 504 nm, λ_{em} range: 510 – 515 nm). BioTracker 488 Green (100 nM; 25 min), used to stain mitochondria, was excited at 488 nm and emission was collected between

510 – 520 nm. A nuclear staining dye was added (DRAQ7; 3 μM) during uptake studies to distinguish intact live cells from permeabilized/dead cells. The 633 nm laser was used to excite DRAQ 7 and emission was collected between 650–750 nm.

Cytotoxicity studies

The Alamar blue assay (Promocell GmbH) was used to assess the cell viability of A549 cells treated with the $[\text{Ru}(\text{phen})_2(\text{tpphz-PDC3})]^{4+}$ complex. The cells were seeded in flat bottomed- culture treated 96-well plates at 10^4 cells in 100 μl per well for 24 h at 37°C with 5 % CO_2 . The complex was added at concentrations: 100, 75, 50, 25, 10 and 5 μM ($n = 3$) and incubated for 24 h prior to the addition of the Resazurin reagent (10 % v/v) for 6 h at 37°C in the absence of light. Absorbance readings were carried out at 570 nm and 600 nm (corrected for background subtraction) using a CLARIOstar (plus) (v 5.70) plate reader. The viability assay was performed in triplicate.

Phosphorescence Lifetime Imaging Microscopy (PLIM)

Live A549 cells were prepared and treated with with $[\text{Ru}(\text{phen})_2(\text{tpphz-PDC3})]^{4+}$. Luminescence lifetime imaging was carried out using a PicoQuant 100 system attached to Leica TCS inverted (DMI8) confocal microscope using a 63X oil immersion objective. Each sample was acquired for 120 s with a 512 x 512 resolution using a 405 nm laser line. Data was analysed using PicoQuant SymphoTime software.

Mammalian Cell Culture

RuPDC3 was tested in two cancerous adherent cell lines; a cervical cancer cell line (HeLa) and a breast cancer cell line (MCF7) and tumour spheroids prepared from HeLa cells.

Adherent cell monolayers

HeLa or MCF7 cells were subcultured using Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 1% penicillin–streptomycin and were grown at 37 °C with 5% CO₂.

Three-dimensional tumour spheroids

HeLa tumour spheroids were prepared in HPAC media supplemented with 5% FBS by seeding at 2×10^4 cells/well in clear U-bottom 96-well TC BIOFLOAT plates (faCellitate) and grown for 96 hours at 37 °C with 5% CO₂ with partial media changes at 48 hours.

MTT Cell Proliferation assay

HeLa or MCF 7 cells were seeded at 10^4 cells per well in 96-well plates and incubated for 24 h at 37 °C. Cells were treated with RuPDC3 between 0 – 150 µM. After 2.5 h incubation (uptake confirmed by confocal imaging), the compound/media mixture was removed and replaced with phenol-red free media. A 470 nm blue light LED array was used to irradiate the cells (*irradiated plate*) at 4.94 mW/cm² irradiance for 10 minutes (irradiation dose 2.96 ± 0.02 J/cm²). The control (non-irradiated) plate was placed in the incubator. The irradiated and non-irradiated cells were further cultured for 24 h before adding thiazolyl blue tetrazolium bromide (MTT) reagent (at 25 µL per well) and incubated for 3 h at 37 °C. Next, the medium was removed, and the formed formazan was solubilized in DMSO (100 µL/well) and incubated for 5 minutes. Absorbance readings at 550 nm and 620 nm (background) were performed following a pre-mixing step using a BMG Labtech ClarioStar (plus) plate reader. Percentage cell viability (corrected for background) was determined relative to untreated control cells. The assay was performed by including six replicates per testing condition and repeated twice (N-2). Initial control studies were performed to ensure irradiation dosage did not compromise viability of non-treated cells.

CellTiter-Glo 3D spheroid viability assay

Spheroids were prepared as described above and treated with RuPDC3 between 0 – 75 µM for 24 h. An irradiated (LED 470 nm; dosage 5.26 ± 0.03 J/cm²) and non-irradiated (in the dark) 96-well

plate were prepared. Next, the spheroids were cultured for 24 h and the CellTiter-Glo 3D assay was performed to determine cell viability as per the manufacturer's protocol. Percentage cell viability was determined relative to untreated control spheroids. For fluorescence gain adjustment, automatic gain adjustment at 90% was performed on the control well. The assay was performed by including four replicates per testing condition and repeated three times (N-3). Initial control studies were carried out to ensure irradiation dosage did not compromise viability of non-treated spheroids.

Confocal Microscopy

HeLa or MCF 7 cells were seeded at 2×10^4 cells in a μ -slide 8 well (Ibidi) and incubated overnight at 37 °C with 5% v/v CO₂. RuPDC3 was added to the cells at 70 μ M in phenol red-free media and incubated for 2.5 h at 37 °C at 5% CO₂ in the absence of light. Following incubation, the media/compound solution was removed, the cells were washed twice with supplemented PBS (1.1 mM MgCl₂ and 0.9 mM CaCl₂) and replacing media was added. Cells were imaged directly using a Leica TSP DMI8 confocal microscope (63 X oil immersion objective lens) with a heated stage at 37 °C. A 476 nm white light laser was used to excite RuPDC3 emission was collected between 560 and 795 nm. In colocalization studies, the cells were co-stained with commercial dyes Hoechst 33342 (1 μ g/mL) or BioTracker 488 Green (100 nM; 25 min) to stain the nucleus or mitochondria respectively. Hoescht 33342 dye was excited at 405 nm and emission was collected between 422 – 550 nm and BioTracker 488 was excited at 488 nm and emission was collected between 510 – 520 nm. For ROS detection in spheroids, spheroids were incubated with a ROS indicator dye, H₂DCFDA, at 15 μ M in PBS for 30 min at 37 °C. The cells were imaged by collecting the emission from the ROS dye between 518 – 525 nm following excitation at 495 nm.

Raman Imaging

HeLa cells were seeded at 2×10^4 cells in a μ -slide 8 well (Ibidi) and incubated overnight at 37 °C with 5% v/v CO₂. RuPDC3 was added to the cells at 70 μ M in phenol red-free media and incubated for 2.5 h at 37 °C at 5% CO₂ in the dark. Following incubation, the media/compound solution was removed, the cells were washed twice with supplemented PBS (1.1 mM MgCl₂ and 0.9 mM CaCl₂) and replacing media was added. Cells were imaged directly and Raman spectra from the cytoplasmic and nucleus of cells were collected (70 μ M/ 2.5 h).

Immunofluorescence studies

HeLa cells were seeded at 2×10^4 cells/well on poly-D-Lysine coated coverslips in 24-well plates and incubated for 24 h at 37 °C with 5% v/v CO₂. Cells were incubated with RuPDC3 at 70 µM while live (and prior fixation). Immunofluorescence labelling: HeLa cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature. The cells were rinsed with 1X PBS twice and permeabilized with 0.1 % Triton X-100 for 10 minutes at room temperature and rinsed with 1X PBS twice. The following incubations were carried out at 37 °C with 5% v/v CO₂. For enzymatic treatments, cells were pre-treated either with RNase A (10 mg/mL) or DNase I (60 U) and incubated for 1 h before proceeding to immunofluorescence staining. Blocking was performed by incubation with 0.5 % goat serum in 0.1 % Tween20 in PBS for 1 h. Cells were incubated with anti-DNA G-quadruplex BG4 antibody (1:100, Merck #MABE917), Rabbit anti-DYKDDDDK Tag (1:800, Cell Signalling Technology, #2368) and secondary anti-rabbit Alexa Fluor 647 (1:600, Invitrogen) for 1 h each with 5-minute washes using PBS/0.1% Tween20 between each incubation. For comparison, additional samples were prepared by incubating fixed cells with RuPDC3 before BG4 and after BG4 labelling. The cells were co-stained with DAPI (0.5 µg/mL), washed with PBS and mounted with Prolong Gold Antifade mounting medium. Biological replicates of N-2 were carried out. Alexa Fluor 647 was excited at 647 nm and emission was collected between 678 – 727 nm. DAPI was excited at 405 nm and emission was collected between 410 – 459 nm. A 476 nm white light laser was used to excite RuPDC3 emission was collected between 560 and 795 nm.

2.9 References

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Chapter 3 Targeting Mitochondrial Guanine Quadruplexes for Photoactivatable Chemotherapy in Normoxic and Hypoxic Environments

Contributions: TD-DFT calculation were performed by Prof. Conor Long. All cellular studies were performed by Rhianne Curley. Gel electrophoresis studies were performed by Dr. Giuseppe Avella.

3.1 Abstract

Chapter 3 modifies the Ru-PDC3 complex presented in chapter 2, developing a type-III Ru(II) therapeutic designed to target and directly photodamage G4 DNA. Type-III photosensitisers (PSs) are capable of directly interacting with and damaging their biomolecular targets. This is an exciting prospect for a phototherapeutic, as it avoids the requirement for photosensitiser interaction with co-reagents, for example, the requirement of molecular oxygen to generate singlet oxygen in type-II PDT. The novel complex, Ru-TAP-PDC3, is capable of undergoing photoinduced electron transfer from guanine monophosphate, where a combination of static and dynamic luminescent quenching was observed. Similarly, Ru-TAP-PDC3 was observed to undergo significant quenching when bound to mitochondrial G4 sequences. Strong binding to these parallel G4 structures was demonstrated using a well-established fluorescent intercalator displacement assay. Importantly, the complex showed a stronger fluorescent intercalator displacement for mitochondrial G4s over duplex DNA. The complex showed uptake into live cells where it localised in the mitochondria. Upon irradiation, the complex depleted mtDNA resulting in mitochondrial dysfunction and apoptosis. Crucially this was achieved in both normoxic and hypoxic conditions.

3.2 Introduction

Phototherapy comprises several modalities, including Photodynamic therapy (PDT) and photoactivatable chemotherapeutics (PACT). It is an evolving treatment for cancer and infection that can offer high specificity and reduced off-site toxicity that can be a feature of conventional chemotherapy. In clinical PDT, a photosensitiser (PS) is used to harness visible or Near-infrared (NIR) light and generate cytotoxic reactive oxygen species (ROS). To date most clinically approved PSs are based on porphyrin chlorins, and phthalocyanines.¹ For instance, Photofrin a clinically approved hematoporphyrin derivative is used to treat oesophageal, endobronchial and lung cancer.^{2,3} The majority of clinically approved PSs operate through a Type-II mechanism where cytotoxic singlet oxygen ($^1\text{O}_2$) is generated as result of Dexter energy transfer between a triplet state of the PS and triplet oxygen ($^3\text{O}_2$) (Fig. 3.1). A limitation of the Type-II mechanism is its dependence on ambient oxygen within the tissue environment since hypoxia is a feature common to 90% of solid tumours.^{4,5} Hypoxia contributes to cancer proliferation, migration, and resistance to treatment.⁶ Type-I processes can also accompany Type-II where the excited PS undergoes electron transfer either via substrate oxidation or to molecular oxygen forming superoxide ($\text{O}_2^{\cdot-}$) leading to subsequent formation of other ROS catalysed by substrates in the cell environment including peroxide and hydroxyl radical ($\cdot\text{OH}$).⁷ An alternative approach is PACT where photoactivation is oxygen independent, typically the PACT agent functions as a prodrug where upon light activation it dissociates into an activated compound capable of inducing cell death. Another type of PACT agent can be classified as type-III photosensitisers.^{8,9} This class interact directly with biological substrates causing damage without the need of molecular oxygen or ROS.^{10,11}

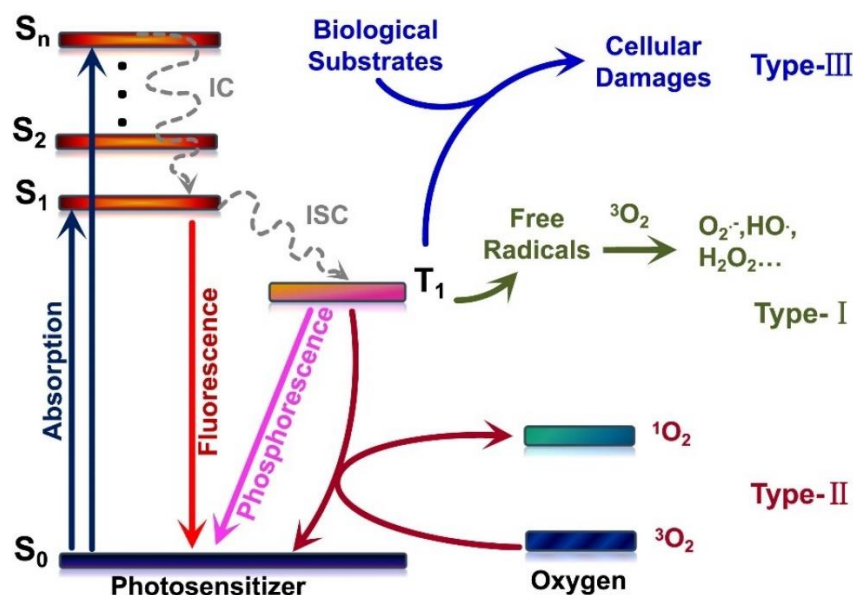


Figure.3.1 Illustration of the different types of mechanisms involved in PDT. (Adapted from Ref. 8).

Typically, Ru(II) polypyridyl complexes have been shown to function as Type II PSs for photodynamic therapy.¹² They are attractive PSs due to their chemical stability, photostability, and their long-lived ^3MLCT rendering them suitable triplet PSs for both $^1O_2$ and ROS generation.¹³ Some modifications can be made to Ru(II) complexes in order to achieve a greater $^1O_2$ quantum yield, thus generating greater quantities of singlet oxygen. For example, through expanding the bite angle of ligands towards the ruthenium metal centre, promoting ligand field splitting and reducing singlet-triplet state mixing, enhancing luminescent quantum yields and extending lifetime decays.^{14,15} Other approaches have been to target Ru(II) complexes towards organelles to increase the cytotoxic effect of $^1O_2$. This has been achieving through altering ligand lipophilicity or through bioconjugations.¹⁶ The drawback of the conventional PDT/singlet oxygen route to phototherapy is the requirement for molecular oxygen as a co-reagent. The reactions work well in normoxic environments but suffer a significant reduction in efficacy when applied in hypoxia, which is a characteristic of many solid tumours.³

Ruthenium(II) polypyridyl complexes have been developed as PACT agents, through careful tuning of s/p ligand donor character, photoreactivity can be built into the complex facilitating photoinduced ligand dissociation, where both ligands and parent complex possess anticancer activity.^{9,17–19} For example, Glazer *et al.* have developed probes that can eject a phototoxic ligand when irradiated while also generating cytotoxic Ru(II) aquo species.^{20,21}

As discussed in chapter 1, Type III Ru(II) PDT complexes can be achieved through complexation of Ru(II) to the electron deficient ligand, tetraazaphenanthrene (TAP). The high photooxidation potential of these complexes means they have sufficient thermodynamic driving force to directly photo-oxidise guanine bases through what is believed to be a proton coupled electron transfer (PCET).^{22–24} PCET has been shown to effectively cleave DNA and TAP containing complexes may also produce covalent photoadducts that are detrimental to cell health. Our group has previously reported a strategy to drive complexes of this type to the nucleus through conjugation of the NLS targeting peptide (Fig. 3.2). They exhibited phototoxicity with a low light dosage in live cells but low dark cytotoxicity making them excellent Type-III PDT candidates.²⁵ Cloonan *et al.* reported a $[\text{Ru}(\text{tap})_2(\text{pdppz})]^{2+}$ complex capable of targeting mitochondria for type III PDT.²⁶ The complex showed good light induced toxicity however conditions under hypoxia were not explored.

Ruzza *et al.* have explored their $[\{\text{Ru}(\text{tap})_2\}_2(\text{tpphz})]^{4+}$ complex in 3D multi-cellular tumour spheroids (MCTS) which is significantly more representative of a tumour cancer environment with proliferative, quiescent, senescent and hypoxic or necrotic regions making them an ideal choice of 3D cell mode.^{27,28} Importantly these MCTS contain a hypoxic necrotic core which provided the environment to explore the efficacy of Ru(II)-tap complexes in hypoxia. It should be noted that the paper does not provide IC_{50} values for spheroids and that the concentration used in irradiation experiments was very high at 100

μM meaning results towards the impact of light induced toxicity were not quantitative. Elias *et al.* have also explored the cellular uptake of their $[\text{Ru}(\text{tap})_2(\text{cpip})]^{2+}$ series of G4 targeted complexes in U2OS, though these results were largely preliminary as no IC_{50} was determined in light or dark conditions.²⁹ Our group has previously been successful in delivering a Ru(II)-TAP bearing peptide conjugate to the nucleus of HeLa cells where the complex induced phototoxicity in normoxic conditions. To date, only a small number of Ru(II)-TAP therapeutics have been reported and their potential phototoxicity has yet to be fully exploited, particularly under hypoxic conditions, as summarised by Table 3.1.

Table 3.1 Phototoxic and cytotoxic values of Ru(II)-tap complexes, with the corresponding cell line and light dosage.

Complex	Cell line	IC_{50} Dark (μM)	IC_{50} Light (μM)	Fluence	Ref
Ru-Tap-NLS	HeLa	83.4	51.8	1.5 J cm^{-2}	²⁵
$[\text{Ru}(\text{tap})_2(\text{pdppz})]^{2+}$	HeLa	70 ± 6.3	8.8 ± 2.9	18 J cm^{-2}	²⁶
$[\text{Ru}(\text{tap})_2(\text{mpdppz})]^{2+}$	HeLa	60 ± 15	4.8 ± 0.5	18 J cm^{-2}	³⁰
$[\text{Ru}(\text{tap})_2(\text{dtp})]^{2+}$	HeLa	63 ± 8	4.0 ± 1.5	18 J cm^{-2}	³⁰
$[\{\text{Ru}(\text{TAP}_2)\}_2(\text{tpphz})]^{4+}$	C8161	>200	40 (approx.)	18 J cm^{-2}	²⁷

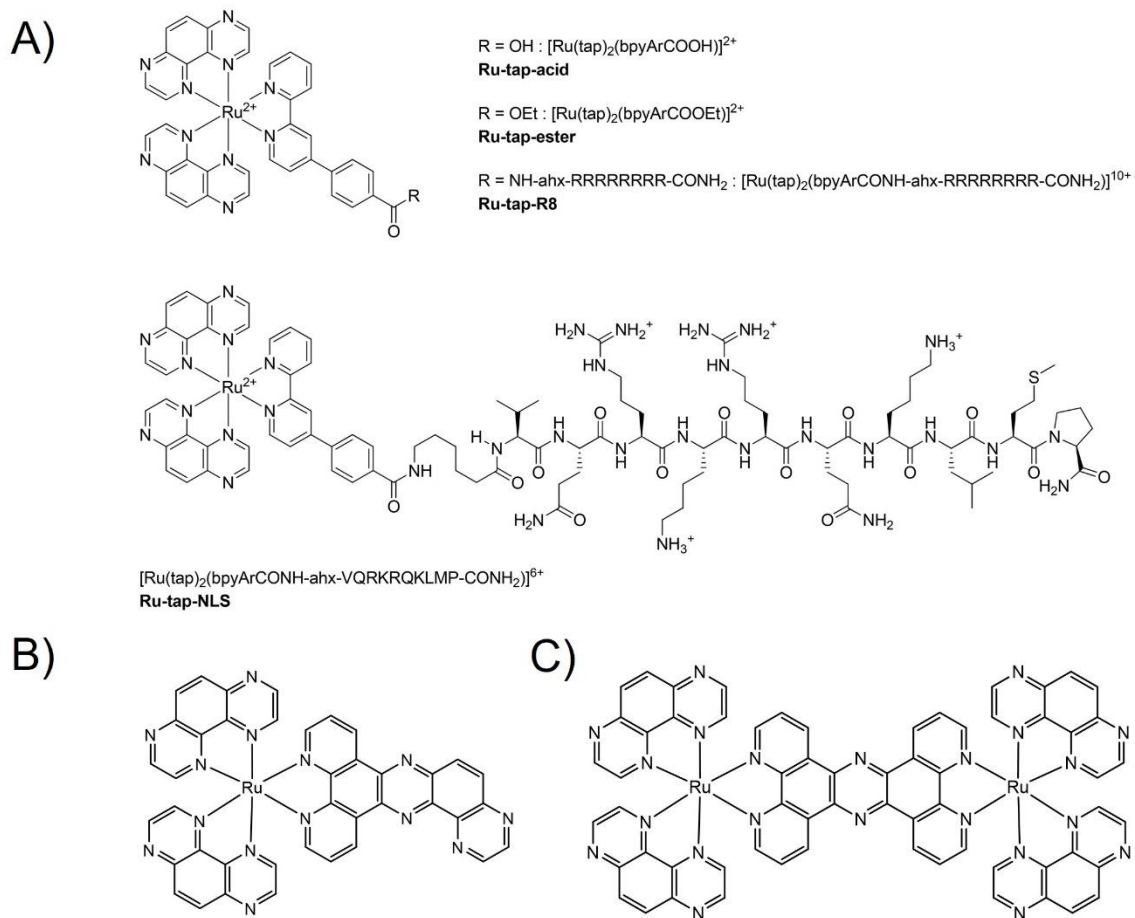


Figure 3.2 Structures of Ru(II)-tap complexes that have been targeted towards live cells. (A) Ru(II)-tap peptide conjugates (adapted from ref. 108). (B) Structure of $[Ru(tap)_2(mpdppz)]^{2+}$ (C) Structure of $[\{Ru(TAP_2)\}_2(tpphz)]^{4+}$.

Mitochondria are an attractive target for PSs largely due to their essential role in oxidative phosphorylation, where they generate ATP for cellular function and play a role in cell death signalling and in maintaining redox homeostasis.^{31,32} Mitochondrial G4 structures (mitoG4s) are less frequently investigated compared to their nuclear counterparts and their presence and impact on cellular functions is not yet fully resolved. Nonetheless, mitochondria have been a rational target for therapeutics due to reasons outlined above. Mitochondria possess their own genome, approximately 16.5 kb in length with circular double stranded nucleoids encoding for 37 genes involved in oxidative phosphorylation in

most mammalian cells. Furthermore, the Mitochondria present favourable K^+ rich conditions for G4 formation (150-180 mM). G-rich sequences are prevalent in mammalian mtDNA with roughly 170 potential quadruplex forming sequences identified in the heavy strand of a mitochondrial genome. In comparison to nuclear DNA, mtDNA lacks protective histones and has reduced repair machinery resulting in high vulnerability to mtDNA targeting drugs. Therapeutic drugs designed to target and irreparably damage mtDNA can cause loss of mitochondrial function, caspase 9 activation and apoptosis. There is currently a lack of probes designed as diagnostic or therapeutics agents to facilitate study of mitoG4s.^{33,34}

As sites of contiguous guanine, G4 structures are potentially very particularly vulnerable to oxidative damage.³⁵ Further, when multiple guanines in a DNA sequence are in proximity, the oxidation potential of the 5' most guanine is further reduced, making them ideal therapeutic targets to under photo-redox processes.^{36; 37–39}

This chapter presents Ru(II) complex directed toward photodamage of G4s located in the mitochondrial genome. We evaluate the affinity of the Ru-TAP-PDC3 complex toward putative mitoG4 structures and compare it to duplex DNA. We also demonstrate that the complex localises within mitochondria of live HeLa cells where, upon irradiation at 405 nm, PicoGreen studies indicate mtDNA is depleted, mitochondrial potential is reduced, caspase activation occurs, and apoptosis is induced. The process is proposed to originate from photoinduced electron transfer induced damage to the mtDNA and occurs in normoxic and hypoxic conditions, generating a potent and selective light activated therapeutic that targets mitoG4s and mtDNA.

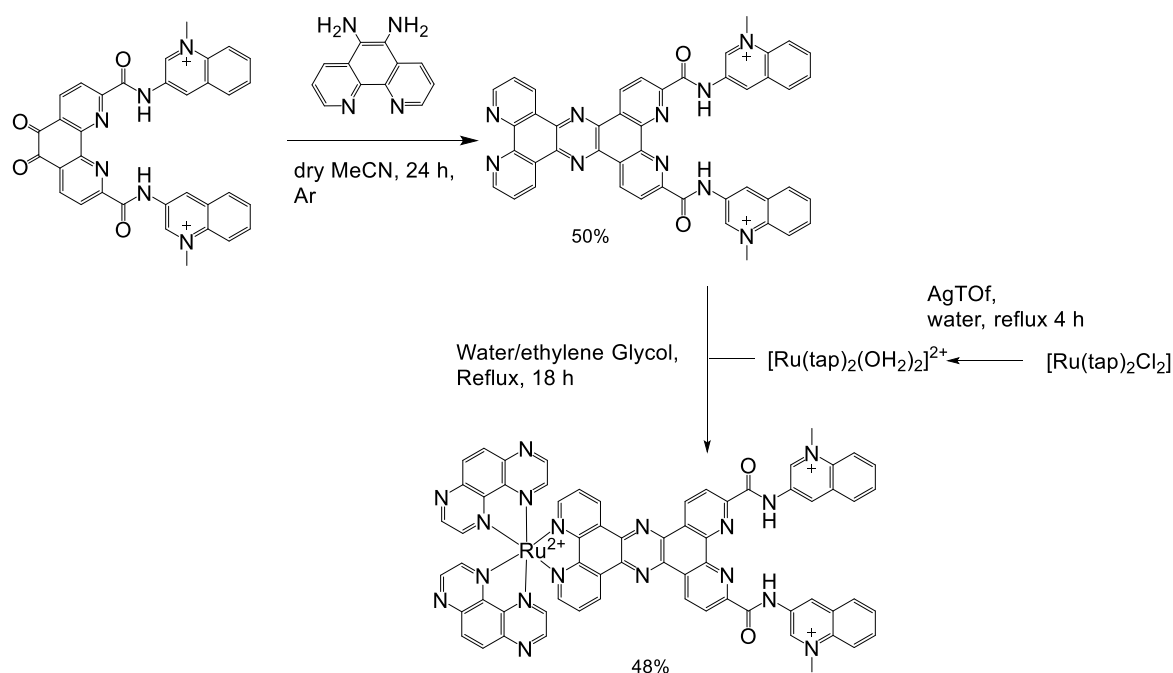
3.3 Results and Discussion

3.3.1 Design and Synthesis of Ru-TAP-PDC3

This chapter aims to further expand on work developed previously in chapter 2 which saw strong DNA binding and selectivity for G4s in addition to successful uptake across multiple cell lines. Crucially in this chapter, the aim was to develop a complex that has similar strong affinity and selectivity for G4s but is able to directly undergo PET when binding to DNA. To this end, the synthetic target Ru-Tap-PDC3 was selected.

The synthetic route to Ru-TAP-PDC3 is illustrated in Scheme 3.1. Although $[\text{Ru}(\text{phen})_2(\text{PDC3})]^{4+}$ and $[\text{Ru}(\text{tap})_2(\text{PDC3})]^{4+}$ are synthetic analogues, different synthetic routes were necessary to obtain each product. Attempts to repeat the synthesis outlined in chapter 2 with the tap analogue were unsuccessful. This was largely due to the instability of $[\text{Ru}(\text{tap})_2(5,6'\text{-diaminophenanthroline})]^{2+}$ and consequent difficulties in purifying it on silica columns. Attempts to purify $[\text{Ru}(\text{tap})_2(5,6'\text{-diaminophenanthroline})]^{2+}$ resulted in the complex sticking to the column when using the mobile phase 80/20/1 (MeCN/H₂O/ 20% w/v KNO₂). The complex was eluted from the column by adding dilute glacial acetic acid and was found to be degraded. To overcome this, an alternative route was undertaken in this chapter. Here, Schiff-base condensation was completed off-complex between phendione-DC3 and 5,6-diamino-phenanthroline, yielding tp-phz-PDC3 (Fig. 3.3). tp-phz-PDC3 was then directly complexed to the metal centre. 5'6-nitro, amino-phenanthroline was synthesised as previously described in chapter 2 and was confirmed through ¹H NMR analysis indicating an asymmetric ligand with 8 protons in the aromatic region. Proton shifts were compared to the literature, further confirming successful synthesis (Fig 3.3 (A)). The 5'6-nitro,amino-phenanthroline was then reduced by refluxing the ligand in a MeOH/EtOH solution with hydrazine hydrate and Pd/C. The solution was filtered to

remove Pd/C and the filtrate was evaporated to obtain pure 5'6-diamino-phenanthroline. This was confirmed through ^1H NMR analysis where the reduced ligand is now symmetrical and has three peaks in the aromatic region, each integrating for two protons and an amine peak integrating for 4 protons (Fig. 3.3 B).



Scheme 3.1 Scheme outlining the synthetic route to obtain Ru-TAP-PDC3.

This ligand was used without further purification in a Schiff-base condensation with phendione-DC3. Molar equivalents were refluxed under N_2 in dry MeCN with a catalytic quantity of acetic acid. The final tpphz-PDC3 ligand precipitated out of solution as an off-white solid. The product was filtered, washed and dried before complexation. ^1H NMR analysis indicated a successful condensation had occurred (Fig 3.3). The peak previously attributed to the two amine groups was no longer present and a new peak attributed to the two methyl groups was evident at 4.8 ppm and integrated for 6 protons.

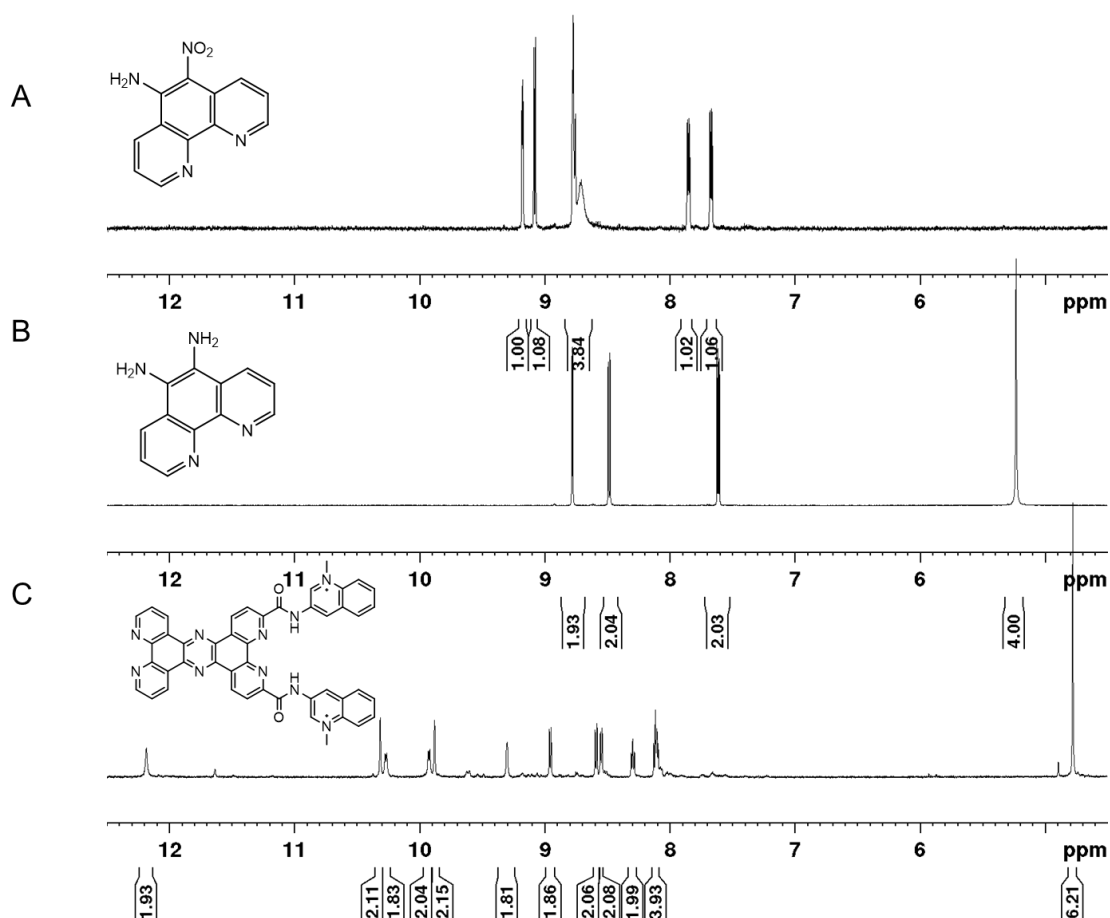


Figure 3.3 ^1H NMR spectra of (A) 5',6'-nitro, amino-phenanthroline (B) 5',6'-diamino-phenanthroline and (C) tp-phz-PDC3.

As $[\text{Ru}(\text{tap})_2\text{Cl}_2]$ is less reactive than comparable $\text{Ru}(\text{II})$ polypyridyl chloride complexes, $[\text{Ru}(\text{bpy})_2\text{Cl}_2]$ for example, the Ru-aquo intermediate ($[\text{Ru}(\text{tap})_2(\text{H}_2\text{O})_2]$) was first prepared prior to complexation. This was generated by the treatment of $[\text{Ru}(\text{tap})_2\text{Cl}_2]$ with silver triflate and refluxing in H_2O for three hours. tp-phz-PDC3 complexation was achieved by refluxing with $[\text{Ru}(\text{tap})_2(\text{H}_2\text{O})_2]$ in a 4/1 ethylene glycol/water mix for 24 h. The reaction was monitored by TLC and when complete the complex the complex was purified on C-18 preparation silica plates with an 80/20/1 mobile phase of comprised of $\text{MeCN}/\text{H}_2\text{O}/\text{KNO}_2$ (20% w/v). Characterisation was completed using ^1H NMR and high-resolution mass spec and purity was confirmed through HPLC (Figure 3.4).

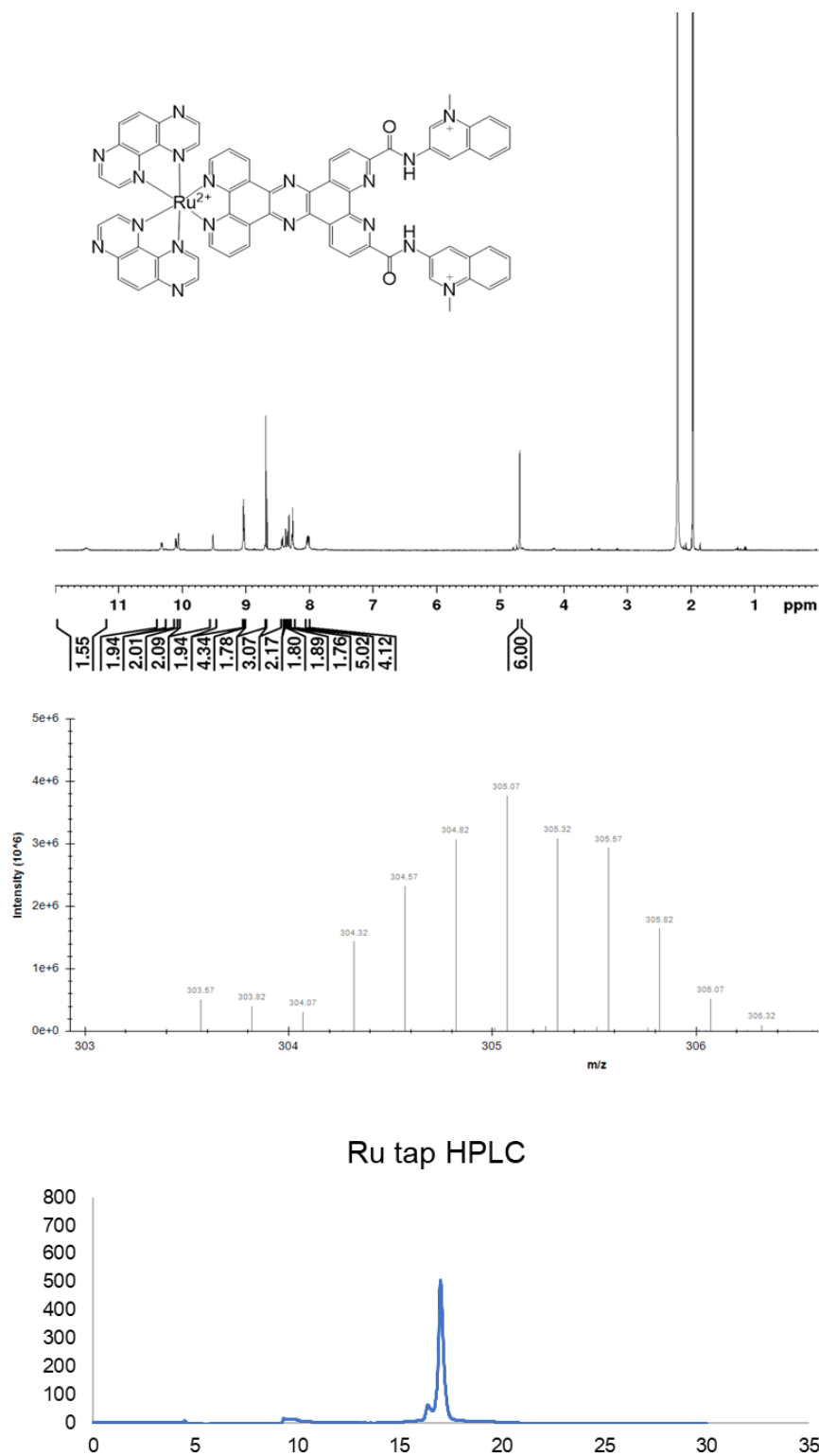


Figure 3.4 (A) ^1H NMR (600 MHz) of Ru-TAP-PDC3 in CD_3CN (B) Mass spec of Ru-TAP-PDC3 (C) HPLC trace of Ru-TAP-PDC3 recorded at 440 nm using C18 silica column with a gradient of 0.1% TFA in $\text{H}_2\text{O}/\text{MeCN}$.

3.3.2 Photophysical Characterisation

As shown in Figure 3.5 (B), the absorption spectra of Ru-TAP-PDC3 shows features at 375, 415 and 465 nm with a shoulder extending to 525 nm in water, buffer and MeCN. DFT calculations indicate that the visible absorbance originates from $^1\text{MLCT}$ transitions. Interestingly, as reflected in the electron density difference maps, the lowest energy transition contains contributions from both MLCT localised on the TAP ligands and a transition involving the tpphz bridge. Excitation into the MLCT absorbance (Fig. 3.5(B)) results in an intense emission with maxima at 628 nm (H_2O), 625 nm (buffer), and at 615 nm (MeCN). A hypochromic shift occurs when exciting the complex in buffer compared to DI water (ca. 15%) under absorbance matched conditions. The emission decays according to biexponential kinetics. In water the complex has lifetimes and fractional amplitudes of $\tau_1 = 795$ ns (81%) and $\tau_2 = 75$ ns (19%), in MeCN $\tau_1 = 634$ ns (90%) and $\tau_2 = 39$ ns (10%), and in phosphate buffer $\tau_1 = 616$ (70%) ns and $\tau_1 = 93$ ns (30%). Similar biexponential decay was observed from the previously reported phenanthroline derivative, RuPDC3.⁴⁰

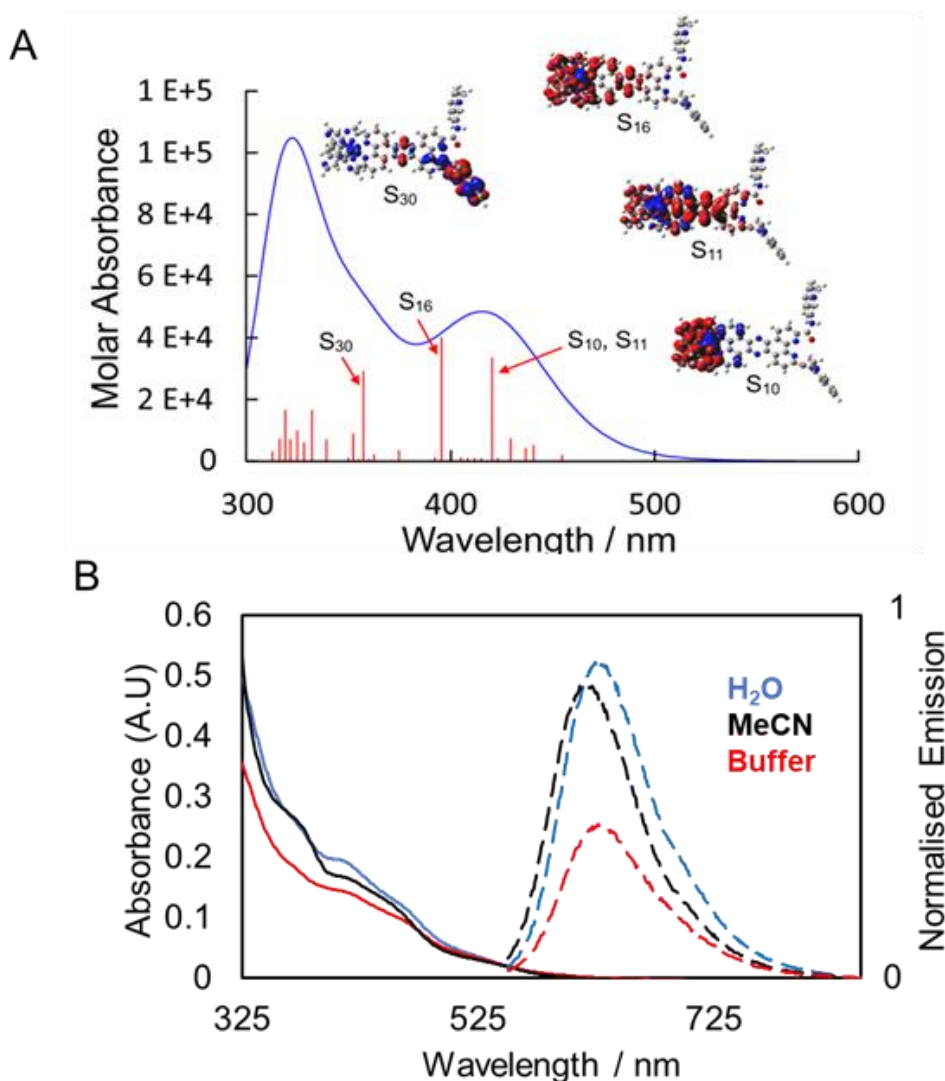


Figure 3.5 (A) Theoretical absorption spectrum modelled and electron density difference maps for highest oscillator strength transitions from DFT calculations in water. (B) Absorption and emission spectra of Ru-TAP-PDC3 (10 μ M) in DI H₂O, MeCN and KPi buffer (10 mM potassium phosphate, 100 mM Potassium chloride 7 pH), with excitation at 470 nm.

To investigate if the excited state of Ru-TAP-PDC3 is quenched by guanine, emission and lifetime studies were performed with increasing concentrations of GMP (guanosine monophosphate). A significant decrease in luminescence intensity was observed when GMP was titrated, consistent with quenching by photoinduced electron transfer. GMP was titrated up to a concentration of 1 mM into a solution of Ru-TAP-PDC3 (10 μ M), where a total decrease in intensity of approximately 85% was evident. In parallel, lifetime decay

measurements were made to understand the impact of GMP towards Ru-Tap-PDC3 when excited. These experiments saw a reduction in lifetime, where Ru-TAP-PDC3 has an average lifetime of 592 ns (intensity weighted) in buffer solution but when in a solution titrated with 1 mM GMP the average lifetime was reduced to 234 ns (intensity weighted). The decrease in in luminescence intensity and lifetime is consistent with PCET. To further quantify the relationship between the concentration of GMP and resulting excited state quenching, Stern-Volmer plots were constructed, as shown in the insets of Fig. 3.6. The Stern-Volmer plots reveal a notable difference in the slopes of data derived from luminescence lifetime and luminescence intensity (0.0015 and 0.0065 respectively), indicating that the quenching observed is a combination of static and dynamic quenching, the static component is attributed to ion pairing between the cationic complex and anionic GMP. The lifetime data can be used to estimate the quenching constant using the Stern Volmer equation outlined below.

$$\frac{I_0}{I} = 1 + K_{sv}[Q]$$

Equation 3.1

Where I_0 is the luminescent intensity in the absence of the quencher, I is the luminescent intensity in the presence of the quencher, K_{sv} is the Stern-Volmer constant and Q is the concentration of the quencher

The Stern-Volmer plot derived from quenching studies was used to calculate the quenching constant, $K_q=2.5 \times 10^9$ consistent with a diffusion-controlled process for the dynamic component of quenching attributed to photoinduced electron transfer. The PCET observed is expected and can be attributed to the estimated E^*_{red} value of approximately 1.3 eV.

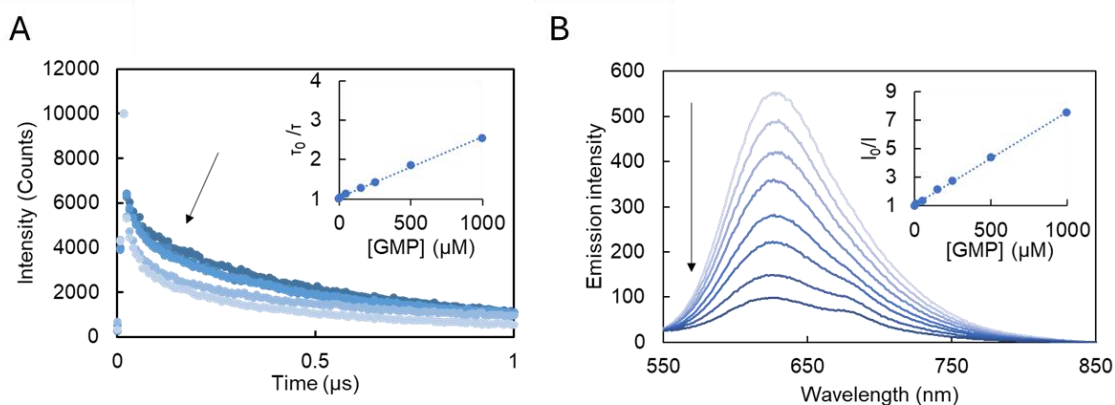


Figure 3.6 (A) Lifetime quenching of Ru-TAP-PDC3 (10 μM) lifetimes with increasing concentrations of GMP (0 mM- 1 mM) in kpi buffer, insets: Stern-Volmer plot of lifetimes and (B) Luminescent intensity quenching of Ru-TAP-PDC3 (10 μM) lifetimes with increasing concentrations of GMP (0 mM- 1 mM) in kpi buffer, insets: Stern-Volmer plot of luminescence intensity.

To investigate the quenching dynamic when Ru-TAP-PDC3 was bound to G4s, analogous studies were performed with three mitoG4 sequences PMPS, HRCC and KSS as illustrated by Stern-Volmer plots in Fig. 3.7. Here the luminescent lifetime and emission intensity of Ru-TAP-PDC3 (5 μM) is measured in the presence of increasing concentrations of G4 (0-50 μM). It is clear that the quenching of Ru-TAP-PDC3 is considerably more complex when binding to G4s than in comparison to GMP. This is unsurprising, as when the complex has fully bound to a G4, it can be expected that the interaction and subsequent quenching becomes primarily static in character. This is evident in in Fig. 3.7, as at increasing DNA concentrations (15- 50 μM) of G4, the slope of each Stern Volmer remains largely unchanged. This is indicative in of a static quenching mode.

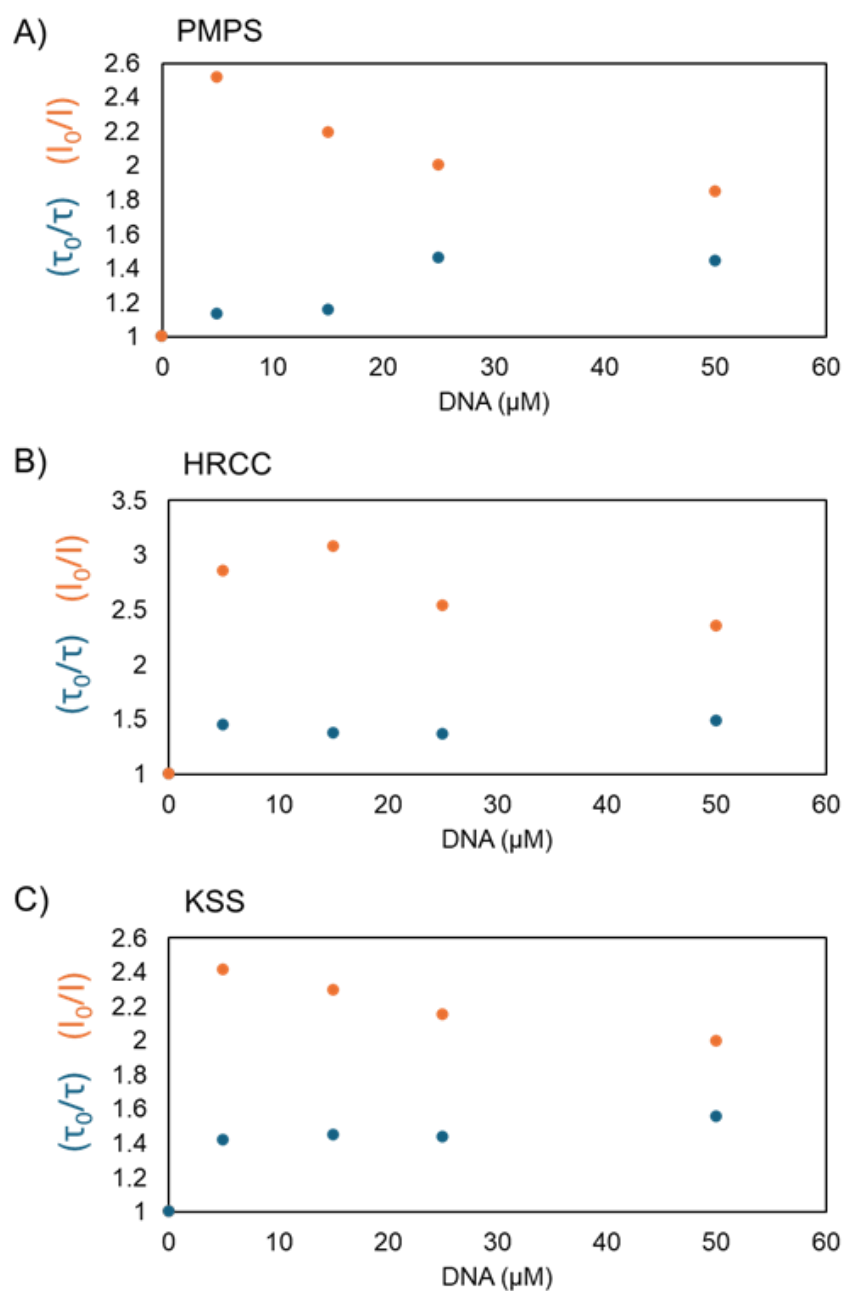


Figure 3.7 Stern-Volmer plots of steady state and emission lifetime quenching data for Ru-TAP-PDC3 (5 μM) with increasing concentrations of the mitoG4 (A) PMPS, (B) HRCC and (C) KSS from 0 to 50 μM . Luminescence intensity-based data is shown in orange. Emission lifetime data is presented in blue.

The E^*_{red} value of Ru-TAP-PDC3 can be estimated from the voltametric and 77 °K emission studies illustrated in Fig. 3.8. This was calculated to be approximately 1.3 eV which is sufficiently reducing enough to successfully cause the photooxidation evident in Fig. 3.8.⁴¹

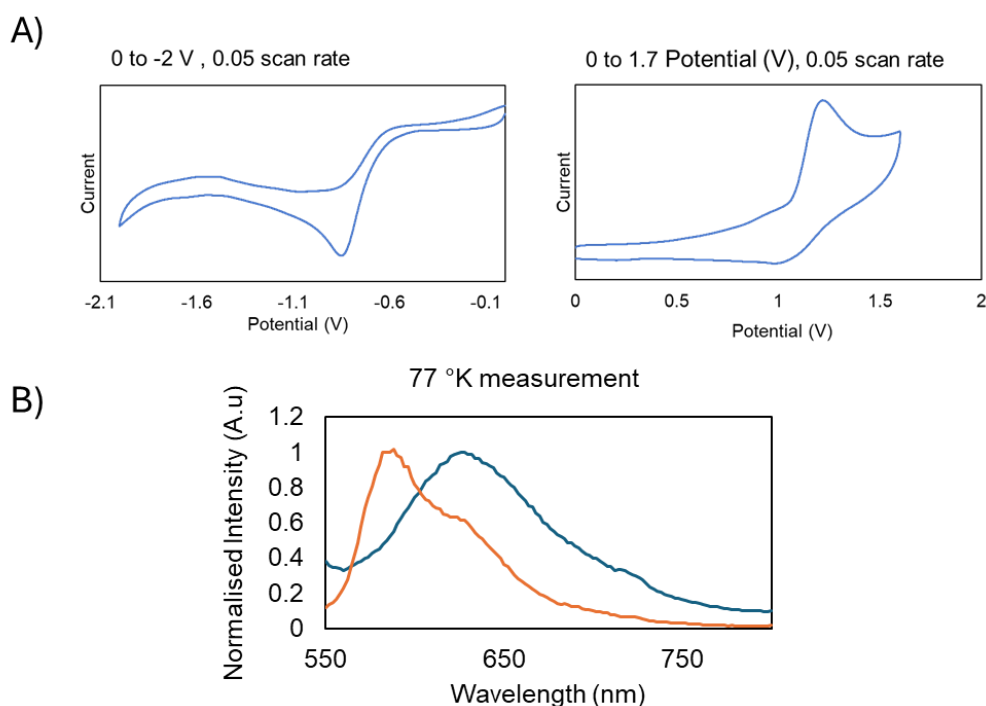


Figure 3.8 (A) Cyclic voltammetry of Ru-Tap-PDC3 in MeCN (1 mM), at a scan rate of 0.05. (B) Luminescent measurement of Ru-TAP-PDC3 in ethanol: methanol (4:1) taken at 77 °K (Blue) and 293 °K (Orange).

3.3.3 DNA Binding

To investigate the properties of Ru-TAP-PDC3 when interacting with DNA, a series of absorbance and luminescence titrations were performed. Oligonucleotides representative of nuclear G4, MitoG4s and dsDNA sequences were used. The selected oligonucleotides were: the human telomeric quadruplex; 22AG, the MitoG4s; KSS, PMPS, and HRCC and

the hairpin oligonucleotide; ds26. Oligonucleotide sequences are outlined in Table 3.2. Titrations were carried out in KPi buffer (10 mM potassium phosphate buffer with 100 mM KCl at 7 pH). Oligonucleotides were annealed at for 5 min at 95 °C before cooling slowly to room temperature at a rate of 1 °C/min. Oligonucleotides were kept at 4 °C overnight before use. 22AG forms a mixed hybrid structure when annealed in Kpi buffer, whereas the three mitoG4s all form parallel structures under the same conditions. Topological confirmation of G4 assembly was confirmed using circular dichroism. As mitoG4s typically form parallel structures, three of these were selected as a series in order to compare and contrast if alterations in loop length and loop bases impacted the binding affinity of Ru-Tap-PDC3. Titrations were carried out in KPi buffer (10 mM potassium phosphate buffer with 100 mM KCl at 7 pH). Oligonucleotides were annealed at for 5 min at 95 °C before cooling slowly to room temperature at a rate of 1 °C/min. Oligonucleotides were kept at 4 °C overnight before use.

Table 3.2 Panel of oligonucleotides selected for testing.

Name	Sequence (5'→3')	Reference
22AG	AGGGTTAGGGTTAGGGTTAGGG	42
HRCC	GGGGGTTGGGTATGGGGAGGGGGG	43
PMPS	GGGACGCGGGCGGGGGATATAGGG	43
KSS	GGGGAGGGGTGTTTAAGGGGTGGCTAGGG	43
ds26	CAATCGGATCGAATTCGATCCGATTG	44

For all titrations in this study, measurements were made after sufficient time was given to ensure equilibrium had been reached and any features observed were confirmed beyond time dependent spectral changes. This was achieved by monitoring any changes that occur in the absorbance of Ru-Tap-PDC3 after the addition of DNA, typically the binding

solution reached equilibrium within five minutes, though this was occasionally aided by mixing the solution with a pipette after an addition of DNA.

Absorbance titrations of the dsDNA hairpin oligonucleotide, ds26, are shown in Fig. 3.9. dsDNA induced a hypochromic response in Ru-TAP-PDC3 across the full absorbance range measured. Measuring the change in absorbance of the MLCT region is of particular interest because excitation into this region will be pursued for luminescence experiments and photoirradiation toxicity studies. The MLCT of the complex underwent a hypochromic response, particularly at 415 nm with increasing concentrations of ds26. The addition of ds26 DNA was completed stepwise and the response was found to be saturable. To compare, the response to ctDNA was also examined. A similar hypochromic response was observed upon binding to ctDNA and a similar response in saturation was also evident suggesting a similar binding mode to both types of duplex DNA. This type of response to DNA has been reported for Ru(II)-Tap complexes, especially when possessing a dppz ligand.⁴⁵ It is thought the hyperchromic response arises upon DNA binding and is often attributed to stacking of the dppz ligand within DNA base pairs. This $[\text{Ru}(\text{tap})_2(\text{dppz})]^{2+}$ study also indicated a hyperchromic shift at higher concentrations of DNA that were not evident for Ru-tap-PDC3.

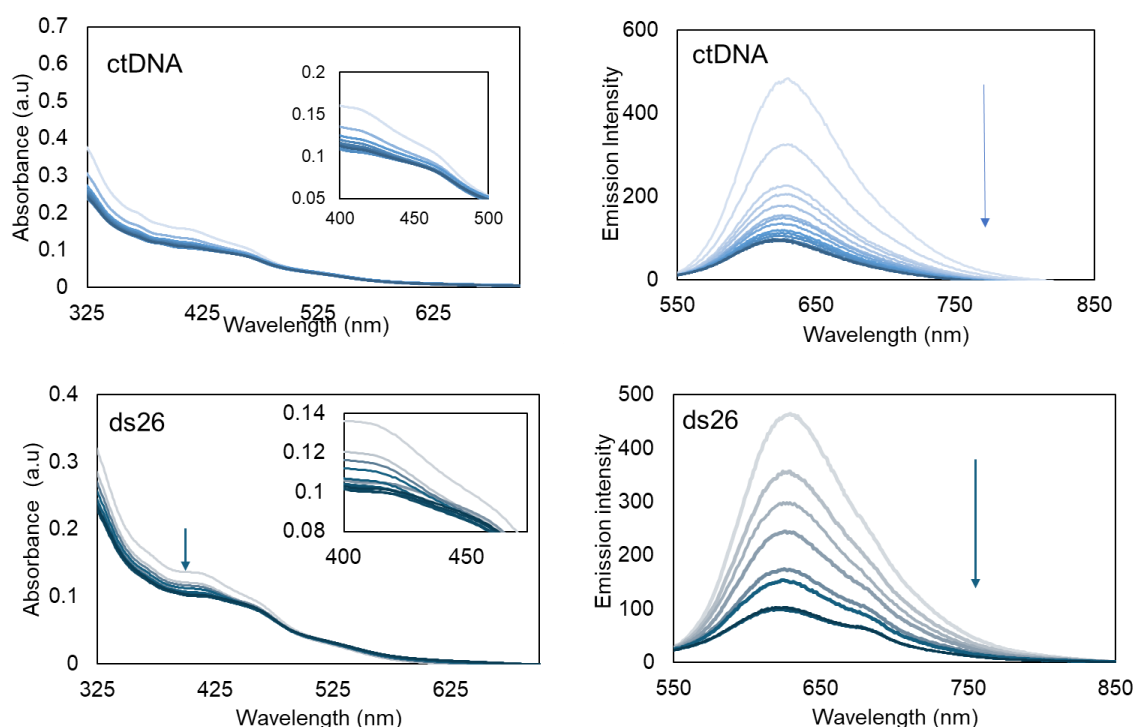


Figure 3.9 Representative UV-Vis absorption for Ru-TAP-PDC3 (10 μ M) when titrated against ctDNA and ds26 insets: Absorption spectra of titrations from 400 nm to 475 nm. Representative luminescence titration of Ru-TAP-PDC3 (10 μ M) when titrated with KSS, HRCC and PMPS (λ_{ex} = 465 nm)

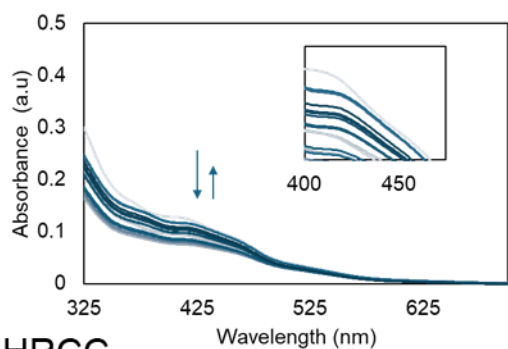
In contrast, for parallel mitoG4s, there is hypochromism on initial Ru-TAP-PDC3 binding with KSS, HRCC and PMPS (Fig. 3.10). However, this is followed by a hyperchromic shift at higher G4 concentrations. Interestingly, this response is similar for all mitoG4s and could indicate similar binding mode and affinity at low DNA concentrations.⁴⁶ Similar to dsDNA, this hypochromic response is evident in the MLCT at 415 and 450 nm. It is noted that the decrease in absorbance as a response to DNA is more drastic for mitoG4s in comparison to dsDNA, where a reduction of 42% was evident for KSS and 28% for ds26. The hyperchromic response that occurs at higher concentrations of DNA may originate from multiple binding modes e.g. Ru-TAP-PDC3 binding through end-stacking interactions with parallel G4s, within grooves of G4s or possibly between intercalation at the G4 interface. The drastic changes in absorbance introduce a level of complexity when

attempting to extract binding affinities. To gain a greater understanding of the interaction between Ru-Tap-PDC3, the luminescence response of the complex was explored.

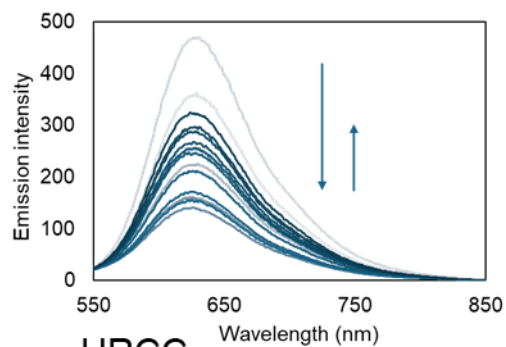
As no isosbestic point was clearly resolved after performing the series of absorbance titrations, 465 nm was selected for excitation directly into the MLCT band since it had the lowest extent of change to the absorbance cross section. Consistent with absorption titrations, the luminescent response of dsDNA titrations were saturable with a decrease in luminescence intensity of 80% observed for ds26 and 70% for ctDNA. The decrease in luminescent intensity is attributed to both the decrease in absorbance and the complex excited state undergoing PCET. Fig. 3.11 shows the luminescence response after correction for changes in the absorbance of the complex. Similarly, the decrease in luminescence remains further confirming the complex undergoes PCET with dsDNA.

When Ru-TAP-PDC3 is titrated with MitoG4s the luminescence is partially quenched. Given that G4s are comprised of continuous guanine, a short decrease in emission intensity is observed, indicating that PET occurs when Ru-TAP-PDC3 binds. Consistent with the absorption titration, following initial quenching, a modest increase in luminescence occurs at G4 concentration 2 μ M. When the luminescence is corrected to account for changes to absorbance cross section, the modest increase in luminescence remains. This again suggests multiple G4 binding modes are active that vary electron transfer efficiency, e.g. through distance the effects. Typically, the McGhee-von Hippel binding model can be applied to extract binding affinities, however, attempts to fit our data were unsuccessful in case of G4s, due to the complexity of the response and the fact that quenching is likely occurring across different rates corresponding to different binding modalities to the G4 structures.⁴⁷

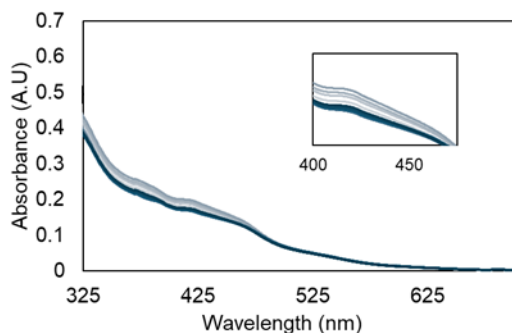
KSS



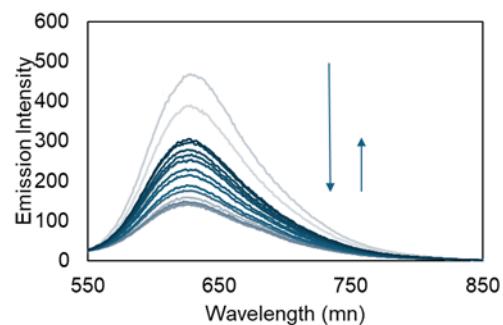
KSS



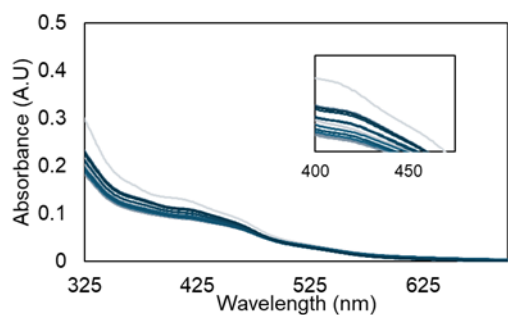
HRCC



HRCC



PMPS



PMPS

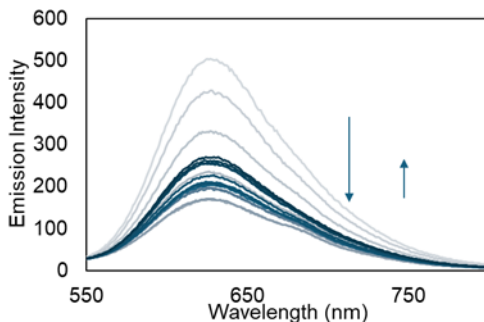


Figure 3.10 Representative UV-Vis absorption for Ru-TAP-PDC3 (10 μ M) when titrated against KSS, HRCC and PMPS (mitoG4s) insets: Absorption spectra of titrations from 400 nm to 475 nm. Representative luminescence titration of Ru-TAP-PDC3 (10 μ M) when titrated with KSS, HRCC and PMPS (λ_{exc} = 465 nm).

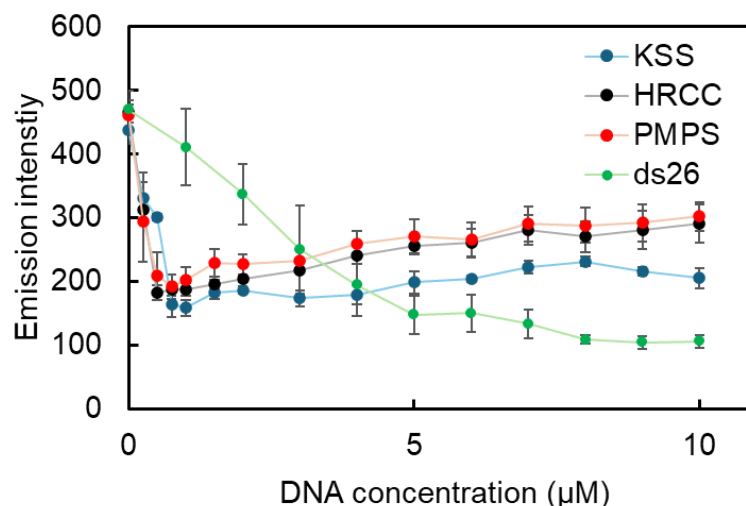


Figure 3.11 Luminescent quenching curves of Ru-TAP-PDC3 (10 μ M) against a series of DNA oligonucleotides (KSS, HRCC, ctDNA and PMPS). (E) G4-FID curves of Ru-TAP-PDC3 against a series of DNA oligonucleotides (HRCC, KSS, PMPS and ds26).

3.3.4 G4-FID Displacement Assay

Next, to estimate binding affinity a G4 fluorescent intercalator displacement (G4-FID) assay was carried out. This assay is widely applied to study binding affinity of novel DNA probes based on the competitive displacement of thiazole orange (TO) from G4s or dsDNA. TO displays strong fluorescence when bound to G4s and dsDNA, which is decreased when displaced by an analyte. Affinities are reported as concentrations required to displace 50% of TO (DC_{50}). Figure 3.12 outlines representative displacement graphs, where TO fully bound to each oligonucleotide without the presence of any displacing ligand is highlighted in red. The displacement assay was performed on a plate reader, exciting TO at 495 nm and gathering the emission spectra from 515 nm to 800 nm. This broad spectral range was measured to ensure that increasing concentration of Ru-TAP-PDC3 did not contribute to the decrease in emission intensity of TO after displacement. As Ru-TAP-PDC3 is not very emissive when excited at 495 nm and its emission is quenched when bound to DNA, contributions to emission spectra were not evident of the displacement assay was not evident. Upon addition of Ru-Tap-PDC3 a

decrease in emission intensity is evident across all oligonucleotides tested indicating TO was successfully displaced.

It is evident from Fig. 3.13 that Ru-TAP-PDC3 binds with higher affinity to G4s when compared to ds26. The complex particularly shows a preference for the parallel mitoG4s HRCC, PMPS and KSS with DC_{50} values 0.20, 0.53, 0.40 μ M respectively. These values indicate strong binding consistent with a high affinity ligand for each mitoG4. The mixed hybrid, 22AG, had a DC_{50} value 0.90 μ M, and can be defined as a median affinity ligand, likewise, dsDNA had a DC_{50} of 0.91 μ M and is a medium affinity ligand. In comparison to literature reported of Phen-DC3 G4-FID experiments, Ru-TAP-PDC3 shows a similarly strong displacement values. Phen-DC3 has a significant DC_{50} 0.31 μ M when binding to 22AG μ M and 0.25 μ M binding to c-kit under the same conditions.⁴⁸ Comparing Ru-TAP-PDC3 to PhenC3, it is clear that Ru-TAP-PDC3 has a preference for parallel structures which is not evident for phenDC3. Nonetheless, it is clear that Ru-TAP-DC3 binds with high affinity.

While this assay did facilitate the extraction of binding constants there are some conditions that are necessary yield this result that should be considered. Firstly, in order to displace TO, a similar binding mode is required. As G4s have multiple potential binding sites, it is possible that a ligand can strongly bind a quadruplex without successfully displacing TO. Similarly, when considering interaction with dsDNA, TO can reportedly intercalate between base pairs as well as in grooves of DNA.⁴⁹

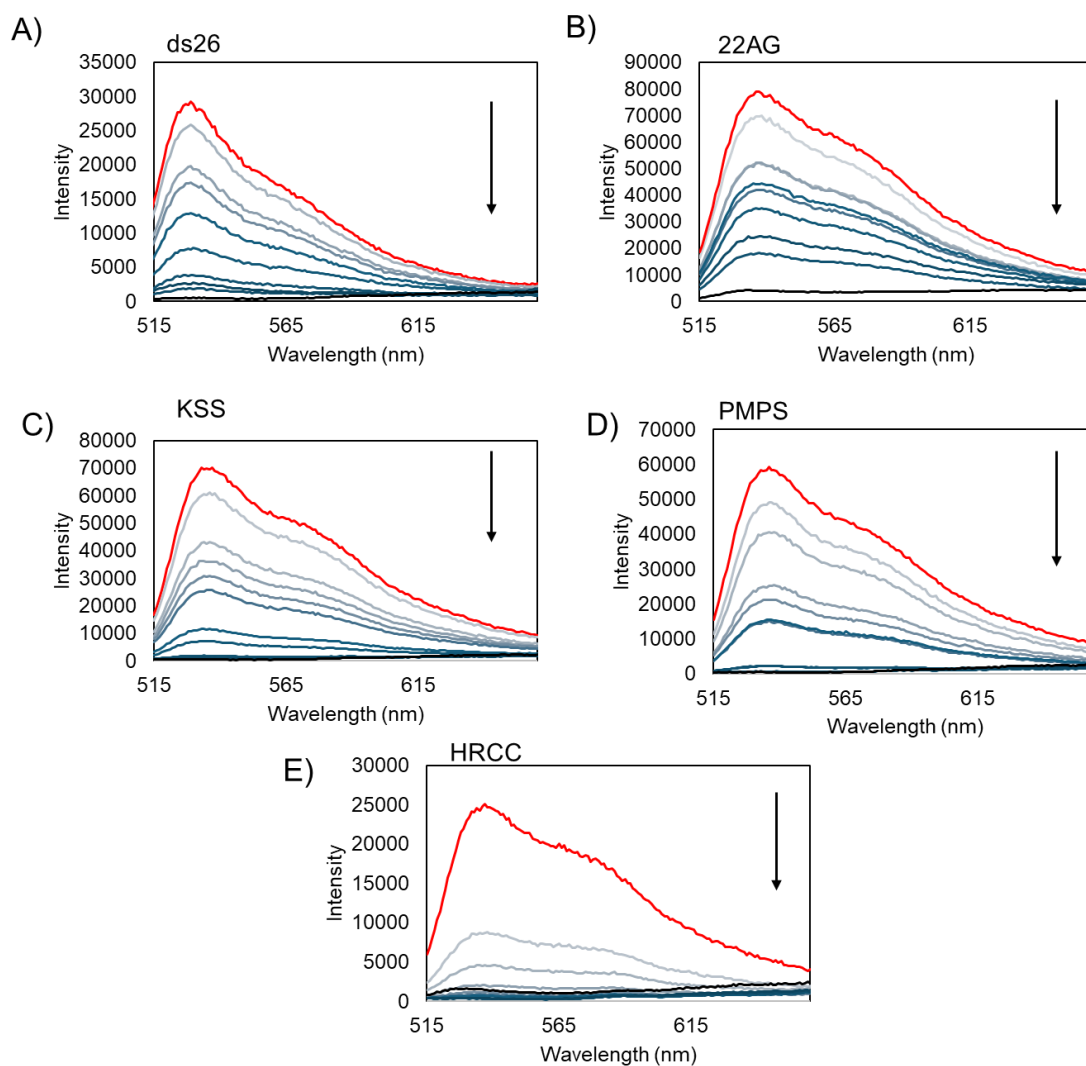


Figure 3.12 Representative emission spectra of thiazole orange displacement. The initial spectra of thiazole orange when bound is red and the final spectra of thiazole orange when displaced is black.

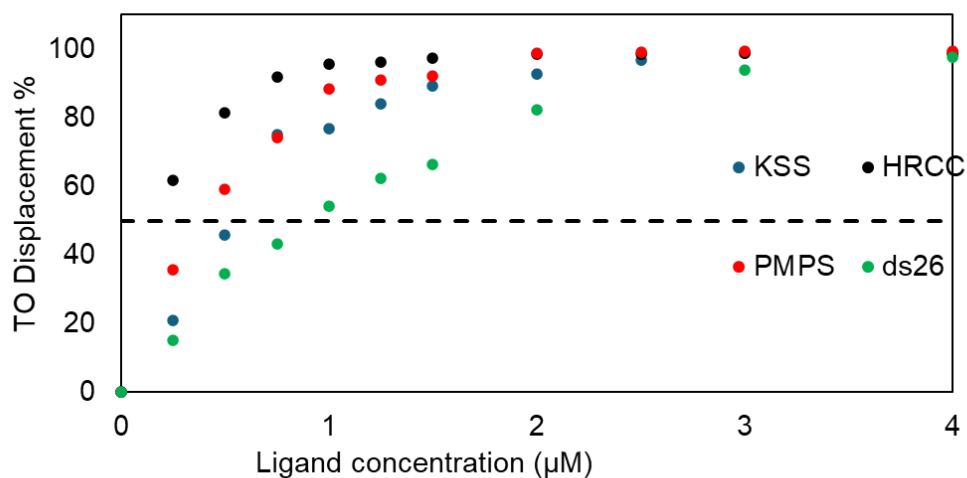


Figure 3.13: G4-FID graph illustrating the displacement of TO from the series of mitoG4s (KSS, HRCC and PMPS) and ds26.

3.3.5 Influence of Ru-TAP-PDC3 Towards G4 structure

Circular dichroism measurements were made to understand the impact of Ru-TAP-PDC3 on the different G4 structures. When Ru-TAP-PDC3 binds to the mitoG4s, no significant change in CD or ICD can be observed. These observations in combination with significant displacement of TO may indicate an end-capping as the binding mode for the parallel mitoG4s. However, when bound to 22AG at increasing concentrations ($[1]:[5]$, $[G4]:[Ru]$) a decrease at ca 270 nm is evident (Figure 3.14). The cause of this change in the CD spectra of human telomeric G4s has previously been reported for Phen-DC3, where authors suggest the ligand disrupts a tetrad. As the Phen-DC3 moiety is incorporated into Ru-TAP-PDC3, this binding mode should also be considered as a possibility to explain the difference in binding affinities. Interestingly the behaviour contrasts markedly with related complex Ru-phen-PDC3, where binding resulted in significant structural disruption G4s evident in chapter 2.⁴⁰

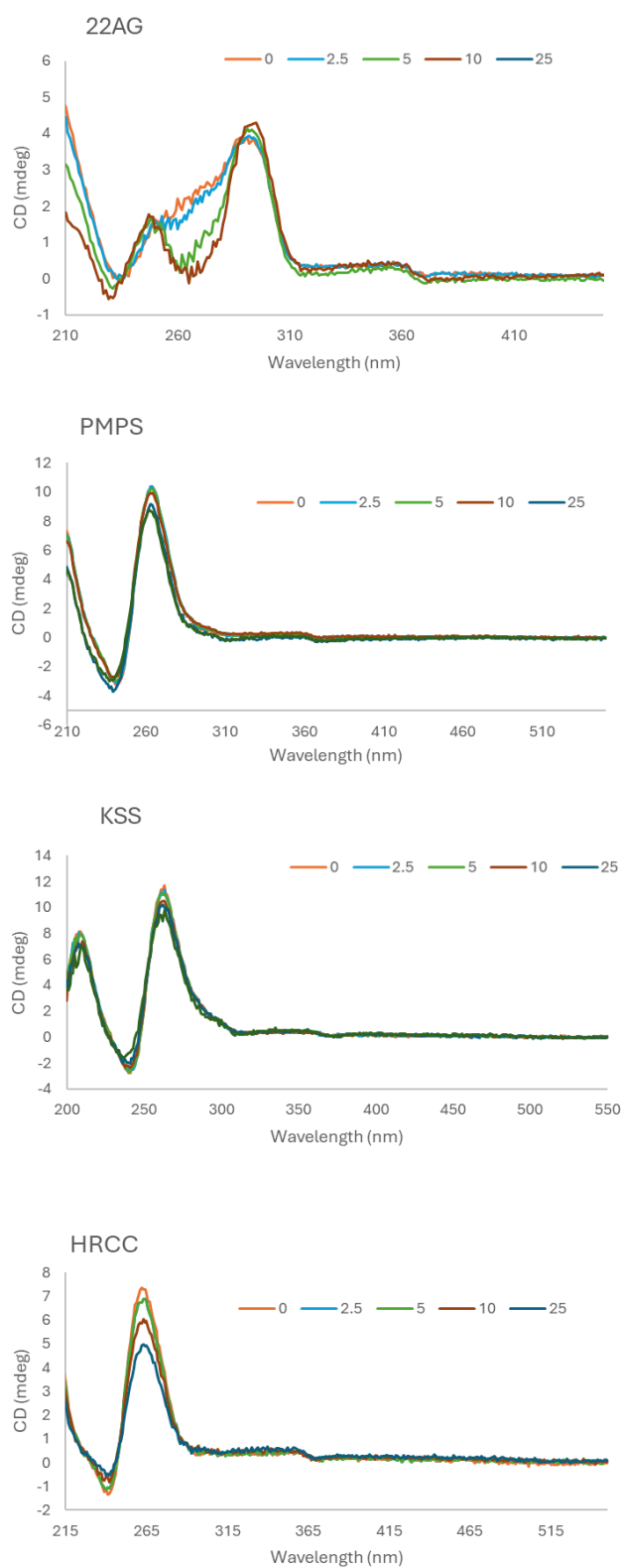


Figure 3.14. Circular dichroism response of mitoG4s (5 μM) to increasing concentrations of Ru-TAP-PDC3 (0-25 μM).

To probe if Ru-TAP-PDC3 may have a photodestructive impact on mitoG4 structures, irradiation studies in solution were performed. The samples were irradiated over the course of 60 mins at 470 nm to a total light dose of 15 J/cm² (Fig. 3.15). This light dose was selected to mirror cellular studies where prolonged irradiation can be detrimental to cell health. Though previously discussed fluorescence studies indicate PET does indeed occur, irradiation studies indicate a negligible change to G4 structure. As mentioned, [Ru(tap)₂(nⁿ)] complexes reportedly form covalent photoadducts with guanine, however it should be noted that these solution-based studies are often exposed to significant light doses that are not sufficiently defined or cannot be replicated in live cells.^{50,51} To this end, solution based photoirradiation experiments were not explored further.

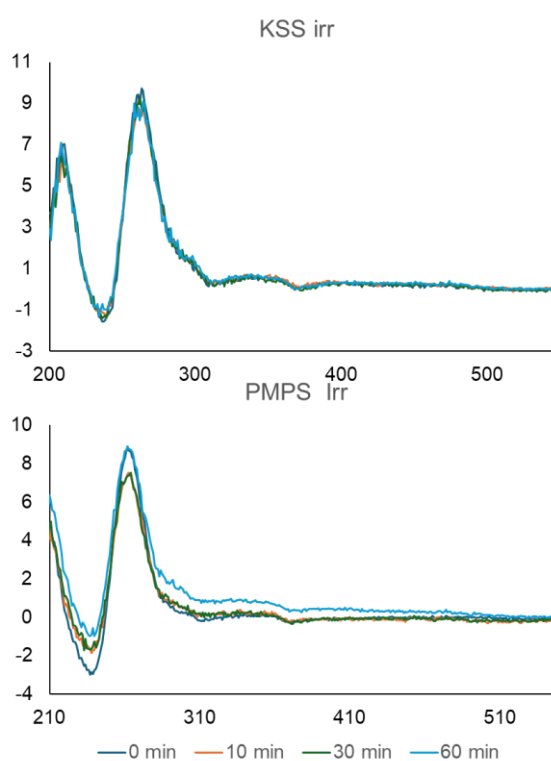


Figure 3.15 Photofluorescence studies of Ru-TAP-PDC3 when bound to KSS and PMPS. Excitation was provided by a 470 nm LED array. For a total light dose of 15 J/cm² after 60 min.

3.4 Cellular Uptake, and Localisation

The complex was examined in HeLa and HEK-293 cells using confocal fluorescence microscopy. HeLa cells were selected for this study due to their mtDNA rich mitochondria.⁵² To investigate cellular uptake and localisation, HeLa cells were incubated with Ru-TAP-PDC3 at varying concentrations between 30 μ M to 200 μ M for 24 h and 50 μ M was selected as the optimum concentration for imaging. Thereafter, confocal imaging was employed to assess cellular uptake of 50 μ M Ru-TAP-PDC3 over time (3-24 h), and a 24 h incubation found to be optimum i.e., this concentration provided sufficient emission intensity and low toxicity under imaging conditions. Uptake was tested in HEK-293 cells at 50 μ M (24 h).

To evaluate the localisation of Ru-TAP-PDC3 in HeLa and HEK-293 cells, co-localisation studies were conducted using well-established commercial probes. The complex was found to localise mainly in the mitochondria of HeLa cells after a 24 h incubation at 50 μ M, however following 48 h incubation, the accumulation of the complex in the mitochondria was reduced significantly with a decrease in Pearson's coefficient value with BioTracker 488 mitochondrial dye from 0.60 ± 0.11 at 24 h incubation to 0.33 ± 0.04 at 48 h. This may be due to the impact of the complex on mitochondrial membrane integrity after prolonged uptake. Conversely, the complex was found to suffer endosomal entrapment in HEK-293 non-cancer cells, localising mainly in the late endosomes, with a Pearson's coefficient of 0.49 ± 0.14 , after 24 h. Partial localisation of the complex was also confirmed in lysosomes (LysoTracker Green) with a Pearson's value of 0.39 ± 0.01 . Interestingly, the localisation of Ru-TAP-PDC3 in the mitochondria of HEK-293 cells at 24 h incubation was found to be similar to that of HeLa cells at 48 h incubation, with a Pearson's coefficient value of 0.30 ± 0.04 with the commercial mitochondrial dye, BioTracker 488. In the latter case this is likely attributed to late arrival to the mitochondria from the endosome.

As Ru-TAP-PDC3 emission is observed in cells, we employed the anti-G4 antibody BG4 to investigate mitoG4 binding. BG4 is capable of labelling G4s throughout the cell including nuclear, cytoplasmic RNA and recently has been shown to label mitoG4s.⁵³ Co-staining with BG4 and Ru-TAP-PDC3 can be seen in Fig. 3.16 (B), with a Pearson's coefficient of 0.63 ± 0.07 indicating the complex is binding to G4s. While colocalization is evident from this Pearson's value, it should be noted that there are areas of the cells where BG4 and Ru-TAP-PDC3 foci do not overlap. This could be a result of BG4 binding to RNA G4s and the partial lysosomal localisation of Ru-TAP-PDC3.

3.4.1 STED Imaging

The suitability of Ru(II) complex luminophores to super-resolution and in particular STED imaging has been reported by our group.⁵⁴ In STED microscopy super-resolution is achieved by selectively depleting fluorophores surrounding the focal region using stimulated emission by a laser source coincident with the red tail of the emission.⁵⁵

We compared the STED and confocal images of Ru-TAP-PDC3 in HeLa cells incubated with 50 μM of the complex for 24 h. Live HeLa cells were imaged using both confocal and STED microscopy. A 660 nm depletion laser was selected for STED imaging, which aligns with the ³MLCT emission of Ru-TAP-PDC3 at ca. 630 nm. A comparison of confocal, gated STED and deconvolved STED images of a selected region of interest (ROI) of a Ru-TAP-PDC3 treated HeLa cell showed a clear improvement in resolution of the foci (Fig. 3.16 (C)). The improved resolution was confirmed using plot profiles, where the peaks of the foci are better resolved.

Although Ru-TAP-PDC3 foci measure ($\sim 0.6 \mu\text{m}$) and therefore do not surpass the diffraction limit of confocal microscopy ($\sim 0.25 \mu\text{m}$) the finer detail captured using STED microscopy suggests the complex is a good candidate for high-resolution imaging.⁵⁶

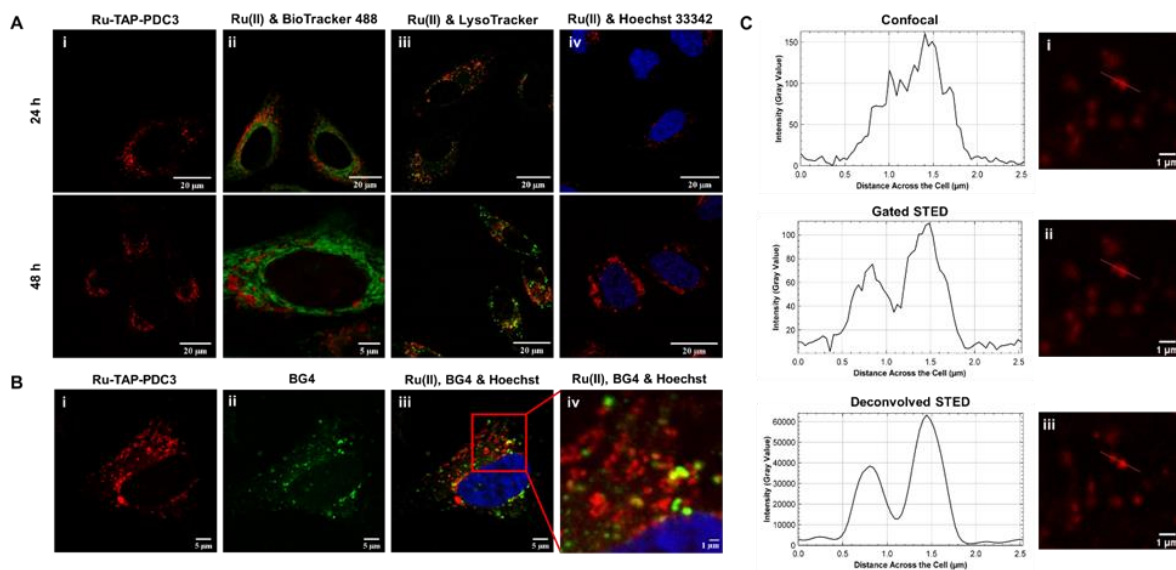


Figure 3.16 Confocal imaging of (A)(i) Ru-TAP-PDC3 (50 μ M) after 24 or 48 h incubation and overlaid with (ii) BioTracker 488, (iii) LysoTracker green and (iv) Hoechst 33342 and (B) shows fixed HeLa cells with (i) Ru-TAP-PDC3 (50 μ M, 24 h), (ii) BG4 and (iii & iv) the overlay with Hoechst 33342. Scale bars read 5 μ m (i, ii & iii) and 1 μ m (iv). (C) Depicts (i) confocal, (ii) gated STED and (iii) deconvolved STED images of Ru-TAP-PDC3 (50 μ M, 24 h) in a live HeLa cell with corresponding profile plots (generated using ImageJ).

3.4.2 Phototoxicity of Ru-TAP-PDC3 Depends on Cell Line and Localisation

The cytotoxicity of Ru-TAP-PDC3 was studied in HeLa and HEK-293 cells confirmed that Ru-TAP-PDC3 has negligible toxicity with an IC_{50} of >200 μ M in both cell lines. Contrastingly, Ru-TAP-PDC3 was found to be highly phototoxic in the test cell lines and interestingly, this effect is time dependent at a low irradiation dose of 5 J/cm². As the exact dark IC_{50} value could not be determined, the PI values of the complex are likely to be significantly higher than reported here in Table 3.3. Maximum phototoxicity was observed in HeLa cells following 24h incubation with Ru-TAP-PDC3 where a PI of >89 at was measured under weak, 5 J/cm² irradiation and >161 under 15 J/cm² irradiance power. By

comparison after 48h incubation with the complex the PI values decreased to >69 (24 h) and >170 (48 h) at 15 J/cm². Varying incubation time had minor impact on the IC₅₀ values. Given the mitochondrial localisation diminishes between 24 h and 48 h, the data suggest that mitochondrial localisation is not essential for phototoxic effect.

Overall PI values are impressive, compared to PSs reported under comparable conditions including clinically approved PSs.^{57,58} For example, Delaey *et al.* have reported a PI of >10 for Photofrin in HeLa cells at a total irradiation dose of 5 J/cm², under very similar conditions to the lower irradiation studies in normoxia reported here.⁵⁹ It is important to note that although we tried to match the irradiance power to this study, direct comparison of PI values across studies is challenging due to experimental variability for example irradiation dose and light source being used.^{12,59}

Table 3.3 Cytotoxicity and phototoxicity results of Ru-TAP-PDC3 in 2D Hela cells under normoxic and hypoxic conditions and 3D spheroids

Cell Model	Incubation	^a Light IC ₅₀ (μM)	^b Light IC ₅₀ (μM)	Dark IC ₅₀ (μM)	^a PI	^b PI
2D hypoxic	24 h	>100	16 ± 1.17	>200		>13
2D normoxic	24 h	2.24 ± 0.04	1.24 ± 0.04		>89	>161
	48 h	2.93 ± 0.01	1.17 ± 0.04		>69	>170
3D	24 h	23.07 ± 3.31		36.61 ± 0.13	1.6	

[a] Phototoxicity studies at 5 ± 0.54 J/cm² and [b] 15 ± 1.6 J/cm², where the phototoxic index (PI) is the ratio of dark IC₅₀ to light IC₅₀

3.4.3 Ru-TAP-PDC3 as an Effective Photosensitiser in a Hypoxic Environment

Since Ruthenium complexes bearing TAP ligands are potentially capable of an oxygen independent phototoxicity, we wanted to examine the impact of a hypoxic environment on the phototoxic capacity of Ru-TAP-PDC3 in HeLa cells. Cobalt chloride (CoCl_2), a popular chemical hypoxia model for *in vitro* studies, was selected for phototoxicity studies as with this method, the stabilisation of hypoxia inducible factor-1 alpha subunit (HIF-1 α) is maintained for some hours after treatment, allowing a greater window for experiments when compared to low-oxygen induced hypoxia.⁶⁰ The chemical induction of hypoxia with CoCl_2 was confirmed in our test cells using an enzyme linked immunosorbent assay to detect HIF-1 α in cell lysates.⁶¹

Under the low irradiation conditions of 5 J/cm², a hypoxic IC₅₀ of >100 μM was determined, indicating oxygen plays a significant role in phototoxicity observed under lower intensity light. This can be attributed to the mechanisms underlying ¹O₂ generation and photoinduced electron transfer (PET). A single molecule of Ru-TAP-PDC3 can produce multiple molecules of ¹O₂ through a turnover reaction, allowing for potent phototoxic effects. In contrast, the recovery of the Ru(II) centre, crucial for PET, may be slow or irreversible. Moreover, PET requires direct interaction of Ru(II) with guanine. This results in a lower effective concentration and consequently a higher light intensity requirement. As ¹O₂ scavenger gel electrophoresis studies indicated that Ru-TAP-PDC3 should display phototoxicity under hypoxic conditions (Appendix B), additional studies were completed at a higher irradiation dose of 15 J/cm² and the cells again treated with up to a maximum concentration of 100 μM . Ru-TAP-PDC3 notably displayed strong phototoxicity towards HeLa cells with a PI of >13 (light IC₅₀, 16 $\pm$ 1.17 μM) under these conditions. 15 J/cm² irradiation intensity remains a modest light dosage and well within

intensities reported for PDT studies. The data indicates that under hypoxic conditions there may be a light intensity threshold to observe oxygen independent PET and that ROS generation contributes to toxicity in the presence of oxygen.

3.4.4 Ru-TAP-PDC3 Treatment Impacts Mitochondrial Membrane Potential of HeLa Cells

The impact of photolysis on the mitochondrial membrane potential ($\Delta\Psi_M$) was investigated, using the commercial dye JC-1. The fluorescence intensities of JC-1 (475 nm excitation) were recorded at 530 nm (green) and 590 nm (red), and the ratio of red/green intensity calculated for each test condition.⁶² The ratios were represented as a percentage relative to untreated control cells (Fig. 3.17 (E)) where a decrease in the red/green ratio indicates mitochondrial depolarisation.^{63–65}

By determining the $\Delta\Psi_M$ of the HeLa cells immediately after irradiation, we were able to capture the direct response of the cells to Ru-TAP-PDC3 when activated by 470 nm light and its impact on mitochondrial function. There is a significant decrease in $\Delta\Psi_M$ of HeLa cells treated with Ru-TAP-PDC3 (3 μ M for 24 h) immediately after photoirradiation, with a decrease of 51% following a total irradiation dose of 5 J/cm², 74% at 15 J/cm² under normoxic conditions and 69% at 15 J/cm² under hypoxic conditions. The data clearly indicate photoactivation of Ru-TAP-PDC3 causes significant mitochondrial depolarisation. Control studies with cells treated with Ru-TAP-PDC3 that were maintained in the dark confirmed there was minimal disruption to the mitochondrial membrane potential without the irradiation step. A positive control completed with mitochondrial membrane disruptor, CCCP (carbonyl cyanide m-chlorophenyl hydrazone; 20 μ M, 2 h) resulted in a 37% decrease in $\Delta\Psi_M$ relative to untreated control cells incubated in the dark.

To further investigate the potential mtDNA damage caused when Ru-TAP-PDC3 is activated by light, PicoGreen (Invitrogen) was selected as a commercial mtDNA stain.

PicoGreen stains both nuclear dsDNA and mtDNA (punctate cytoplasmic staining) in live cells and co-localisation of PicoGreen was previously confirmed with commercial mitochondrial targeting dyes.⁶⁶ Fluorescent signal in the cytoplasm arising from PicoGreen emission is directly correlated to the quantity mtDNA nucleoids within a cell, diminishment of this signal can be indicative of mtDNA depletion. HeLa cells treated with Ru-TAP-PDC3 for 24h (50 μ M) were counterstained with 2 μ L/mL PicoGreen. As shown in Fig. 3.17 (A-C), after 5 minutes irradiation of the Ru(II) complex (405 nm), a distinct reduction of PicoGreen emission in the cytoplasmic region is observed. This marks a reduction in the number of mitochondrial nucleoids, indicating mtDNA damage and depletion is occurring. Importantly, this result supports localisation in of Ru-Tap-PDC3 to the mitochondria and that direct binding of mtDNA to the Ru(II) complex occurs. Control studies confirmed the results are not caused by photobleaching or photoreactions caused by PicoGreen, and again confirms that a combination of the ruthenium complex and light exposure leads to mtDNA damage.

3.4.5 Irradiation of Ru-TAP-PDC3 Treated HeLa Cells Induces Apoptosis

To assess the mechanism of cell death when Ru-TAP-PDC3 is activated by light, the FAM FLICA poly-caspase assay kit (ImmunoChemistry Technologies) was used. The FLICA assay labels active caspase enzymes that are present during apoptosis to establish if cell death is by apoptosis. The results from the FLICA polycaspase activity assay (Figure 3.17 (D)) indicate that apoptotic pathways in HeLa cells are being triggered when Ru-TAP-PDC3 is activated with 470 nm light. Activation of caspases is a central event in apoptosis thus, the upregulation of active caspase enzymes is an indicator that cells are undergoing apoptosis. A positive control was prepared by treating the cells with 1 μ M staurosporine for

3 h and the percentage of apoptotic cells was determined relative to this control. At 3 μ M Ru-TAP-PDC3, similar levels of apoptosis can be observed for normoxic cells irradiated at a dose of 5 J/cm² and hypoxic cells irradiated at a 15 J/cm². Hypoxia is often associated with pro-survival responses in cells because of the stabilisation and activation of HIF-1 α . The pro-survival effect may explain why, although the immediate response to irradiation (% apoptotic cells) is similar in both normoxic (5 J/cm²) and hypoxic (15 J/cm²) environments, the hypoxic light IC₅₀ is significantly higher than the light IC₅₀ under normoxic conditions. The higher irradiation dose (15 J/cm²) resulted in roughly 30% more apoptotic cells (normoxic) in comparison to the positive control at both 1 and 3 μ M of Ru-TAP-PDC3. Control cells treated with the Ru-TAP-PDC3 and kept in the dark during the irradiation step remained healthy with $\leq$ 10% apoptotic cells. Apoptosis can occur through two pathways: the intrinsic (mitochondrial) pathway, and the extrinsic (death receptor) pathway.^{67,68} The intrinsic pathway of apoptosis is initiated following DNA damage and results in a decrease in $\Delta\Psi_M$, thus apoptotic cells exhibiting reduced $\Delta\Psi_M$ are thought to become apoptotic through the intrinsic pathway.^{31,69} Combining the results of the studies with the JC-1 assay kit, that indicate that the $\Delta\Psi_M$ of HeLa cells is reduced by Ru-TAP-PDC3 when activated by light and apoptosis confirmed by the FAM-FLICA assay, we can infer that apoptosis is occurring through the intrinsic pathway under normoxic and (chemically induced) hypoxic conditions.

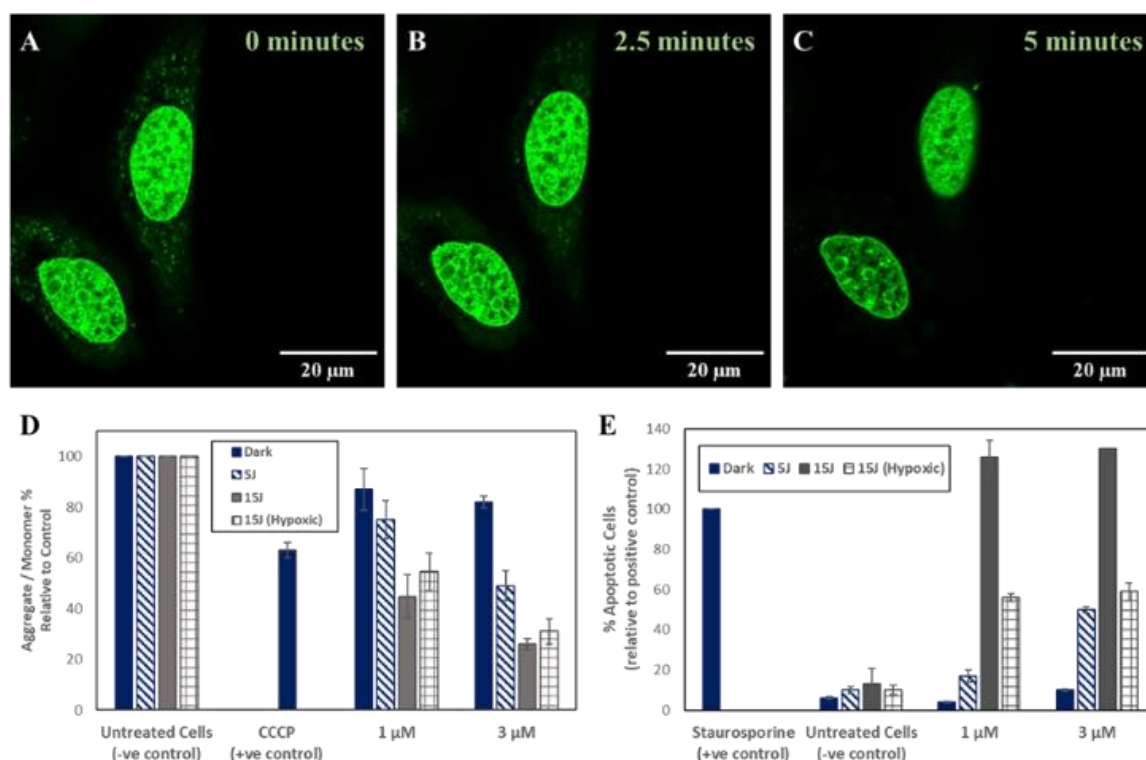


Figure 3.17 PicoGreen in HeLa cells pre-treated with Ru-TAP-PDC3 (50 μ M, 24 h) **(A)** prior to irradiation at 405 nm and after **(B)** 2.5 minutes or **(C)** 5 minutes of continuous irradiation. PicoGreen was excited at 495 nm with emission collected between 520-530 nm. **(D)** FAM-FLICA polycaspase assay and **(E)** JC-1 mitochondrial membrane potential assay results and controls in HeLa cells (24 h incubation). Results are calculated as a percentage of controls; FLICA assay results are % apoptotic cells relative to a positive control (1 μ M Staurosporine, 3 h) and JC-1 results are JC-1 monomer / aggregate % relative to untreated cells. 5J or 15J in figure legend indicates a total irradiation dose of 5 J/cm² (0.5 h) or 15 J/cm² (1.5 h) using a 470 nm LED.

3.4.6 3D Cell Studies

As 3D cell cultures are a more representative model of the tumour microenvironment in comparison to cell monolayers, we assessed if the phototoxicity of Ru-TAP-PDC3 carries through from 2D to 3D cell models, by examining HeLa multicellular tumour spheroids (MCTS). Spheroids typically produce weaker luminescence signal intensity compared to cell monolayers due to their 3D nature and light scattering effects, therefore using a higher concentration of the complex can enhance visualisation and improve imaging quality.

Confocal fluorescence imaging at 100 μM Ru-TAP-PDC3 confirms uptake of the complex into HeLa spheroids after 24 h incubation. Despite the high cytotoxicity of the complex, the overall appearance of the MCTS were healthy under the imaging conditions (lack of DRAQ7 uptake) suggesting the cytotoxic effects occurring within the MCTS are not apparent during imaging (Fig. 3.18). For MCTS toxicity studies, the HeLa spheroids were $\geq 500 \mu\text{m}$ in size and appear to have developed a necrotic core. While conventionally, the hypoxic zones within MCTS would contribute to diminished oxygen availability and thus lower efficacy of Ru-TAP-PDC3, it appears the complex did not penetrate throughout the entire spheroid. It is important to note that the complex emission is reduced when bound to DNA so it is possible some uptake cannot be visualised using confocal microscopy. As a result, it is not possible to judge if this influences the phototoxicity of the complex. The penetration depth of the LED light throughout the MCTS could impact the light IC_{50} of the complex, causing a higher light IC_{50} value of $23 \pm 3.31 \mu\text{M}$ in HeLa MCTS at 5 J/cm^2 (470 nm). Remarkably, the dark IC_{50} value is significantly higher than in cell monolayers, giving a PI of 1.6. Control studies with untreated HeLa cells at 15 J/cm^2 ($2 \times 7.5 \text{ J/cm}^2$) indicated this irradiation dose was toxic to the spheroids, limiting phototoxicity studies to beneath this dose. The significant increase in dark IC_{50} value in spheroids compared to cell monolayers ($>200 \mu\text{M}$) could be the result of the layered structure of the MCTS, with a lower number of proliferating cells than in monolayers, meaning the cells in the MCTS may struggle to recover from Ru-TAP-PDC3 penetration in comparison to the actively proliferating cell monolayers. The difference in PI values between cell monolayer and MCTS studies highlights the importance of 3D cell studies. Notable differences in the penetration or toxicity patterns of luminescent probes have been reported previously.^{15,70} It is important to note that IC_{50} and PI values are not the only indicators of phototoxic potential reported for spheroid studies of this nature. For example, Liu *et al.* demonstrated the phototherapeutic capacity of their ruthenium drug, RuAzNM (PI = 8.3 at $2 \times 6 \text{ J/cm}^2$, 465 nm), in HepG2 liver cancer MCTS using growth curves. Further, Raza *et al.* studied

the live/dead (propidium iodide) cell response to light and changes in cell morphology and cytoskeleton filament structures in C8161 human melanoma MCTS with their ruthenium TAP complex, $[\{Ru(TAP)_2\}_2(tpphz)]^{4+}$.^{71,72} These studies show that although IC_{50} values are informative, phototherapeutic potential can be further investigated through a variety of additional studies such as measuring MCTS growth or morphological changes over time.

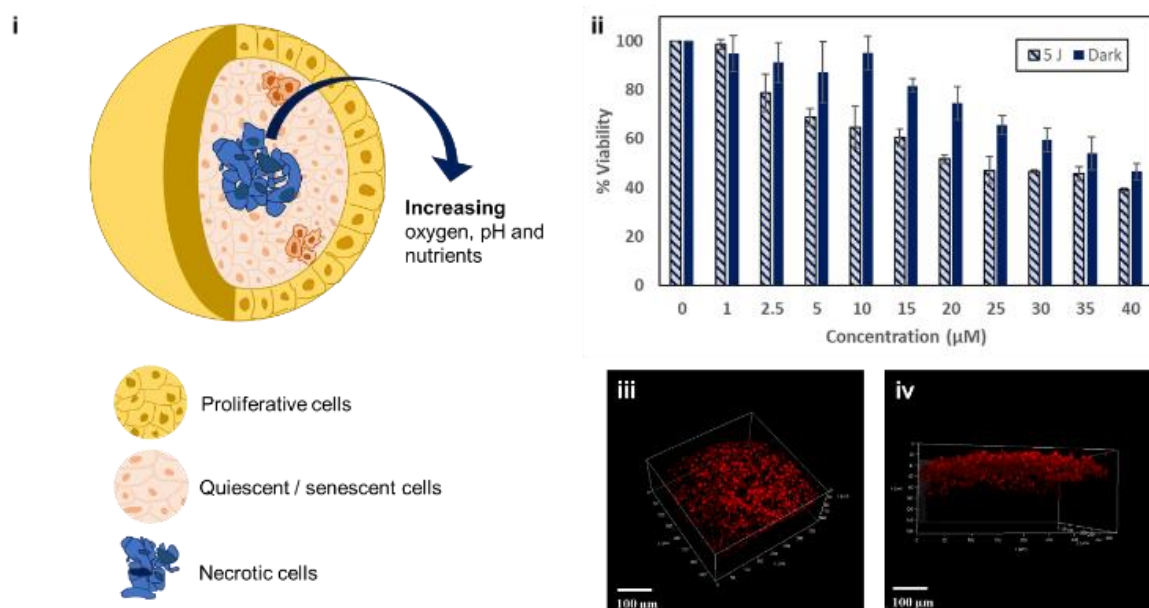


Figure 3.18 (i) Graphic of a 3D spheroid, (ii) viability of HeLa MCTS treated with varying concentrations of Ru-TAP-PDC3 for 24 h and irradiated at a total dose of 5 J/cm² compared to control MCTS kept in the dark. (iii) and (iv) show 3D reconstruction images of Ru-TAP-PDC3 in a live HeLa spheroid (100 μM , 24 h). Scale bars measure 100 μm .

3.5 Conclusion

This chapter presented a tetraazaphenanthrene bearing Ru(II) therapeutic that undergoes PET with DNA independently of O₂. The complex was found to bind strongly to DNA with a preference for mitoG4s over dsDNA. Ru-TAP-PDC3 was found to easily permeate the cell membrane and preferentially accumulate in the mitochondria of HeLa cells at 24 h incubation, with a decrease in mitochondrial localisation after a 48 h incubation. The compatibility of this Ru(II) complex with STED imaging showcases its potential as a fluorophore for high-resolution imaging. The phototherapeutic effect of this complex in HeLa cells is confirmed by a notable loss of $\Delta\Psi_M$ mtDNA depletion and ultimately apoptosis upon irradiation at 470 nm. The complex shows negligible dark toxicity with an IC₅₀ value >200 μ M in both HeLa and HEK-293 cells. Under hypoxic conditions in HeLa cells Ru-TAP-PDC3 maintained its phototherapeutic potential, although somewhat reduced. HeLa MCTS studies showed a decreased PI value of 1.6, which highlight the need for studies with both cell monolayers and MCTS to determine phototoxic potential. These results suggest that Ru-TAP-PDC3 is a great candidate for phototherapy and can be activated under both normoxic and hypoxic cellular conditions.

3.6 Materials and Methods

Reagents

Chemicals were purchased either from Sigma-Aldrich (Merck) or Fluorochem and used without further purification. Oligonucleotides were purchased from Eurofins genomics and used without further purification. Thiazole orange was purchased from VWR.

Synthetic Procedures

Phendione-DC3, 5,6-diamino-1,10-phenanthroline, tetraazaphenanthrene and $[\text{Ru}(\text{tap})_2\text{Cl}_2]$ were synthesised as previously reported.^{25,40,73}

tpphz-PDC3

Phendione-PDC3 (100 mg) and diamino phenanthroline (18 mg) were added to a RBF and dissolved in dry MeCN, a catalytic amount of glacial acetic acid was added. The solution was brought to reflux for 18hrs under argon. A beige precipitate formed and was vacuum filtrated. This product was washed with DI water and diethyl ether to yield 65 mg, 50% yield. ^1H NMR (600 MHz, DMSO- d_6): 12.18 (s, 2H), 10.32 (s, 2H), 10.27 (d, $J = 7.1$ Hz, 2H), 9.93 (d, $J = 7.5$ Hz, 2H), 9.88 (s, 2H), 9.30 (s, 2H), 8.96 (d, $J = 8.3$ Hz, 2H), 8.59 (d, $J = 8.8$ Hz, 2H), 8.55 (d, $J = 7.9$ Hz, 2H), 8.30 (t, $J = 7.98$ Hz, 2H), 8.11 (m, 4H), 4.78 (s, 6H). m/z calculated = 377.1271, found 377.1306

Ru-TAP-PDC3

$[\text{Ru}(\text{tap})_2\text{Cl}_2]$ (50 mg) and silver triflate (45 mg) were added to a RBF and refluxed in water for approx. 3 hours. The solution was cooled, and 20 ml of acetone was added. This solution was filtered through a bed of celite to separate the insoluble AgCl from the aqueated ruthenium intermediate. The filtrate was reduced to dryness under pressure and an equimolar equivalent of tpphz-PDC3 was added and dissolved in 4/1 ethylene glycol/water mixture. This solution was refluxed for 24 h before cooling to room

temperature. The solution was treated with an aqueous NH_4PF_6 solution to precipitate out the product as red solids. The solids were filtered and washed with DI water. The crude product was dissolved in acetonitrile and filtered through a bed of celite. The Acetonitrile solution was concentrated under vacuum and added to a stirring solution of sat. aqueous NH_4PF_6 to precipitate the product as a PF_6 salt before vacuum filtration and washing with DI water and diethyl ether. Purification was performed on preparative TLC C-18 silica plates with the mobile phase 80/20/1 ($\text{MeCN}/\text{H}_2\text{O}/20\%\text{KNO}_2$), yielding 60 mgs (48%). ^1H NMR (600 MHz, CD_3CN) 11.50 (s, 2H), 10.33 (d, $J = 8.0$ Hz, 2H), 10.10 (d, $J = 8.28$ Hz, 2H), 10.06 (s, 2H), 9.52 (s, 2H), 9.04 (m, 4H), 9.02 (d, $J = 3.0$, 2H), 8.69 (s, 3H), 8.43 (d, $J = 9.12$ Hz, 2H), 8.38 (d, $J = 3.0$ Hz, 2H), 8.35 (dd, 2H) 8.32 (d, $J = 2.8$ Hz, 2H), 8.26 (m, 5H), 8.03 (m, 4H), 4.70 (s, 6H). m/z calculated = 305.0690, Found 305.0712

Absorbance measurements were made using a Jasco V670 spectrophotometer, data was manipulated using Jasco Spectra Manager software and MS Excel. Luminescence studies were performed on a Varian Cary Eclipse Fluorimeter. TCSPC measurements were made using a PicoQuant FluoTime 100 Compact TCSPC system with a 450 nm laser from a PicoQuant PDL800-B Source and an external Thurlby Thander TGP110 10 MHz pulse generator. TCSPC spectra was collected up to 10000 counts, and decays were analysed and fit with PicoQuant Fluofit software. Thizole orange displacement studies were performed on Clariostar Plus plate reader. Raman spectra were obtained using a Horiba LabRam instrument using a 473 nm diode laser excitation. Raman Spectra were analysed and manipulated with NGSlabSpec5 software. Circular Dichroism measurements were made using a Chirascan series. High resolution mass spec was performed by the University of Bath mass spec facility. ^1H -NMR Spectra were recorded on a 600 MHz Bruker spectrometer and processed using the Bruker topspin software. Mass Spectrometry was performed by the University of Bath, Mass Spectrometry facility.

Stock concentrations of Ru-tap-PDC3 were kept as chloride salts at 2 mM in deionised water and kept at -20°C. Oligonucleotides were purchased from Eurofin Genomics and stored at -20°C. To prepare G4s, mitoG4s and hairpin DNA, DNA was annealed at 95°C before cooling slowly and overnight storage at 4°C. The buffer used for Absorbance, Fluorescence and CD experiments was a 10 mM potassium phosphate buffer with 100 mM KCl with pH 7.

DNA Binding Studies

Absorbance and luminescence titrations were performed in tandem using an adapted literature protocol. DNA stock concentrations were maintained at 1 mM in KPi and titrated into a 10 μ M solution of Ru-tap-pdc3 in KPi buffer. When an aliquot of DNA was titrated, the solution was mixed before measurements were recorded. Luminescence measurements were recorded using slit widths of 10 nm with an excitation at 465 nm. All measurements were performed in triplicate.

Thiazole orange displacement studies were performed using the previously reported procedure. The different DNA oligonucleotides tested were 22AG, KSS, PMPS, HRCC and ds26. Displacement curves were completed in triplicate. Thiazole Orange was excited at 490 nm and emission was recorded from 515 nm to 795 nm ensuring no contribution from Ru-TAP-PDC3. Values were recorded as the concentration required to displace 50% of bound Thiazole Orange.

Circular Dichroism

Circular dichroism measurements were recorded using a G4 solution annealed at 5 μ M in Kpi buffer. Ru-tap-pdc3 was titrated (2.5-25 μ M) for each G4 structure. CD spectra were recorded from 550 to 200 nm and are represented as the average of two accumulations. Titrations were recorded in triplicate.

Uptake in Live Cell Monolayers

HeLa and HEK-293 cells were incubated in 25cm² or 75cm² cell culture flasks (Corning) with Dulbecco's Modified Eagle Medium (DMEM) (Gibco) with L-glutamine supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin (Sigma-Aldrich). Cells were maintained at 37°C with 5% CO₂ and detached from the flask at 80-90% confluency using a 1X Trypsin-EDTA solution (Gibco). Cells were counted using the EVE automated cell counter (NanoEnTek) and seeded at 1x10⁵ or 2x10⁵ cells/mL for HeLa and HEK-293 cells respectively in an 8 chamber μ -slide (ibidi) for cell imaging. HeLa cells were left to grow and attach for 24 h at 37°C and HEK-293 cells were allowed 48 h for optimum cell attachment and growth. Cells were rinsed with 1X PBS (Gibco) and stained with Ru-TAP-PDC3 at 50 μ M for 24 h or 48 h. Cells were washed twice with PBS and covered with phenol red free DMEM medium (Gibco) with L-glutamine and 10% FBS for imaging. Live cell uptake was also studied in HeLa cells from 4 to 8 hours, i.e., no wash step was performed, and cells were imaged at 2 h intervals after treatment with 50 μ M Ru-TAP-PDC3. All imaging studies were completed using a Leica TSP DMI8 confocal microscope with 63x oil immersion lens and heated stage at 37°C, unless stated otherwise.

Localisation in Live Cell Monolayers

Co-localisation studies were performed in HeLa and HEK-293 cells with BioTracker 488 mitochondrial dye and LysoTracker Green DND. Additional co-localisation studies were completed in HEK-293 cells with Rab7a-GFP (Cell Light, late endosome stain) and Golgi-RFP (Cell Light) and in HeLa cells with Hoechst 33342.

Cells were treated with Ru-TAP-PDC3 at 50 μ M for 24 or 48 hours, washed twice with 1X PBS and imaged in phenol red free media with 10% FBS with a 63x oil immersion lens

unless stated otherwise. Ru-TAP-PDC3 was excited using a 405nm laser and emission collected between 580-750nm.

The conditions for co-localisation studies are as follows: BioTracker 488 (100 nM, 20 min) Ex 488 nm; Em 515-525 nm; LysoTracker Green DND-26 (50 nM, 1 h) Ex 504 nm, Em 510-520 nm; Rab7a-GFP (15 μ L/mL, 24 h) Ex 488 nm, Em 495-540 nm, Golgi-RFP (10 μ L/mL, 24 h) Ex 555 nm, Em 580-590 nm and Hoechst 33342 (1 μ g/mL, 10 minutes) Ex 405, Em 425-575 nm. Co-localisation analysis was performed using ImageJ and reported as the average Pearson's coefficient values with standard deviation.

STED Imaging

HeLa cells were seeded (1.5×10^5 cells / mL) in a 35 mm glass-bottom culture dish at a total volume of 1.5 mL media and grown for 24 h at 37°C and 5% CO₂. The cells were then incubated for a further 24 h with 50 μ M Ru-TAP-PDC3 in media in the absence of light. The Ru/media solution was removed, and the cells washed twice with 1X PBS. For STED imaging (and confocal fluorescence images taken for comparison) a 100x oil immersion objective was used. A 405 nm laser was used to excite Ru-TAP-PDC3 and a 660 nm depletion laser (50% laser power) was used to facilitate STED imaging, emission was collected between 570-655 nm. Images were scanned at 1024 x 1024 pixels. A line accumulation of 32 was used to eliminate as much background as possible, while maintaining cell viability / brightness for the duration of both the confocal and STED imaging. Each cell area was imaged only once, due to the phototoxicity of Ru-TAP-PDC3 under the selected imaging conditions. Images were deconvolved using Huygens Professional deconvolution software.

Effect of Ru-TAP-PDC3 on mtDNA (PicoGreen Studies)

HeLa cells were prepared for imaging as per routine confocal studies, treated with Ru-TAP-PDC3 for 24 h at 50 μ M, washed twice with 1X PBS and counterstained with PicoGreen at 2 μ L/mL for 30 minutes. The PicoGreen-media solution was removed, and the cells washed twice with 1X PBS and imaged in fresh phenol red free DMEM. Confocal images were captured after 0, 2.5 or 5 minutes of continuous irradiation using a 405 nm laser (total irradiation dose 0.5 J/cm²). The fluorescent images of PicoGreen were acquired in test cells using a white light laser at 495 nm excitation and emission collected between 520-530 nm. A control study was conducted prior to any mtDNA damage studies exciting the cells at 470 nm or 405 nm for 5 minutes to optimise the irradiation conditions for Ru-TAP-PDC3 to ensure there was negligible photobleaching of PicoGreen and excitation at 405 nm was shown to have minimal effect on PicoGreen emission.

Cytotoxicity Studies

HeLa and HEK-293 cells were seeded in clear cell culture treated 96 well plates (Nunc) at 1×10^4 and 2×10^4 cells per 100 μ L respectively and left to grow and attach for 24 h at 37°C and 5 % CO₂. For 24 h dark toxicity studies, HeLa cells were treated with 200, 175, 150, 100, 75, 50, 25 and 10 μ M Ru-TAP-PDC3 and HEK-293 cells treated with 200, 100, 50, 25 μ M. The cells were treated in triplicate and the plates incubated for 24 h in the dark at 37°C. Cell viability of all plate-based studies was assessed using the alamar blue assay. A 10% (v/v) Resazurin-media solution (alamarBlue, Invitrogen) was added to the wells and incubated at 37°C for 5 h in the absence of light. Absorbance was measured using a BMG LABTECH CLARIOstar plate reader at 570 nm and 600 nm (corrected for background subtraction). Cell viability is presented as a percentage (%) compared to untreated control wells. Plates were performed in duplicate.

Phototoxicity Studies

Phototoxicity and dark control plates were performed as described above. HeLa cells were treated with Ru-TAP-PDC3 at 5, 4.5, 4, 3.5, 3, 2.5, 2, 1.5, 1 and 0.5 μM for 24 h or 48 h to allow uptake and HEK-293 cells incubated with the same concentrations for 24 h. The wells were then emptied and filled with 100 μL phenol red free media. Cells were irradiated at a total dose of $5 \pm 0.54 \text{ J/cm}^2$ (0.5 h at $2.81 \pm 0.30 \text{ mW/cm}^2$) or $15 \pm 1.6 \text{ J/cm}^2$ (1.5 h at $2.81 \pm 0.30 \text{ mW/cm}^2$) using a 470 nm LED (TeleOpto LEDA-X LED driver and array). Control plates were incubated in the dark during the irradiation period (0.5 or 1 h). After irradiation, the cells were incubated in the dark overnight to allow for cell recovery. Irradiation conditions were tested on untreated cells to confirm cells remained viable at this dose and each phototoxicity plate included untreated test wells for cell viability comparison. Irradiation dose was determined using a power meter (Edmund Optics, Coherent LaserCheck™). Cell viability was determined using the alamar blue assay as per the cytotoxicity protocol.

Hypoxic Phototoxicity Studies

A critical limitation in application of Type II PDT reagents in solid tumours is their requirement for molecular oxygen to generate cytotoxic $^1\text{O}_2$. Hypoxia frequently characterises the tumour microenvironment, limiting the ambient oxygen concentration, and thus ROS generation where molecular oxygen in the PDT mechanism.⁷⁴ Hypoxia inducible factors (HIFs) play an integral role in regulating genes involved in activities such as angiogenesis or cell survival, with HIF-1 α being the most widely investigated of the HIFs.^{75,76} HIF-2 α and the lesser studied HIF-3 α are also activated at low oxygen concentrations.⁷⁷ Studies have shown that incubating cells with CoCl_2 under normoxic conditions can block the degradation of HIF-1 α by inhibiting prolyl hydroxylases, thus allowing HIF-1 α to accumulate, mimicking a hypoxic environment.^{78,79}

HeLa cells were seeded and treated with Ru-TAP-PDC3 as in the phototoxicity study protocol above, using a higher concentration range of Ru-TAP-PDC3 i.e. 100, 75, 50, 25, 10, 7.5, 5, 2.5, 1, 0.5 μM . Ru-TAP-PDC3 was removed from the wells after a 24 h incubation and instead of replacing with phenol red free media and irradiating immediately, a 300 μM solution of Cobalt (II) Chloride hexahydrate in cell culture media was added to the cells for 4 h to mimic hypoxia. The CoCl_2 solution was removed from the wells and quickly replaced with phenol red free media. Cells were irradiated immediately using a 470 nm LED at a total irradiation dose of $5 \pm 0.54 \text{ J/cm}^2$ or $15 \pm 1.6 \text{ J/cm}^2$ and a dark control plate performed simultaneously. A control study was performed with 300 μM CoCl_2 added to the cells for 4 or 24 h in the dark prior to any hypoxic phototoxicity studies to confirm this method was not toxic towards HeLa cells. The alamar blue assay was used to determine cell viability.

HIF-1 α Human ELISA Kit

An enzyme linked immunosorbent assay (ELISA) was completed to confirm the induction of hypoxia, based on the levels of hypoxia inducible factor 1 subunit alpha (HIF-1 α) in HeLa cells. A commercial ELISA kit (HIF-1 α human ELISA kit, Invitrogen) was used to assess HIF-1 α expression and RIPA (radio-immunoprecipitation assay) lysis buffer (ThermoFisher), was employed to obtain cell lysate samples for the assay. Cells were lysed on ice; the cells were washed with ice cold PBS, followed by the addition of lysis buffer and a cell scraper (Corning) used to ensure complete removal of attached cells. The ELISA was performed stepwise as per the manufacturer's protocol and a standard curve produced using known stocks of HIF-1 α (pg/mL) to calculate the relative amount of HIF-1 α in the cells under each condition.

The conditions tested were 100 μM or 300 μM CoCl_2 (4 h incubation), hypoxia by coverslip (2 h incubation) and untreated HeLa cells. The 300 μM CoCl_2 protocol resulted in greater

HIF-1 α expression in HeLa cells than 100 μ M CoCl₂ or a 2 h incubation using the coverslip method, where the entire cell surface is covered by a sterile glass coverslip, resulting in oxygen depletion.^{72,80}

FAM-FLICA Polycaspase Activity: Fluorescence Assay

The polycaspase assay detects multiple activated caspases. The FLICA reagent, FAM-VAD-FMK, used for this assay is comprised of a recognition peptide sequence, valine-arginine-aspartic acid (VAD), along with a fluoromethyl ketone (FMK) group, which forms a covalent bond with active caspases. This interaction results in green fluorescence from the carboxyfluorescein (FAM) component of the reagent.⁸¹

HeLa cells were seeded at 3×10^5 cells/mL in a 96 well plate (Nunc) at a total volume of 100 μ L per well and incubated overnight at 37°C with 5% CO₂. The following control populations were prepared for each cell line: negative (untreated) control and positive control cells treated with Staurosporine (1 μ M/3 h). Experimental populations were exposed to the Ru-TAP-PDC3 at 1 and 3 μ M (above and below the phototoxic IC₅₀) for 24 h and either placed in the dark or irradiated for 30 minutes using a 470 nm LED (5 ± 0.54 J/cm²). All samples were subsequently incubated with the FLICA dye for 60 min at 37°C and washed using the buffer provided as per manufacturer's protocol. Aliquots were analysed in triplicate (3×100 μ L) in a black bottomed 96-well plate using BMG Labtech ClarioSTAR plate reader set at 488 nm excitation and 520 nm emission.

JC-1 – Mitochondrial Membrane Potential: Fluorescence Assay

$\Delta\Psi_M$ maintenance is essential for ATP production, and any change to it can substantially impact mitochondrial function so it is a useful marker of metabolic damage to this

organelle. HeLa cells were seeded at 1×10^5 cells/mL in a 96 well plate (Nunc) at a total volume of 100 μ L per well and incubated overnight at 37°C with 5% CO₂. The following control populations were prepared: negative (untreated) control and positive control cells treated with carbonyl cyanide m-chlorophenyl hydrazone (CCCP, 20 μ M/2 h). Experimental populations were exposed to Ru-TAP-PDC3 at 1 and 3 μ M (above and below the normoxic light IC₅₀) for 24 h and either placed in the dark or irradiated for 30 minutes (total irradiation dose of 5 ± 0.54 J/cm²) or 1.5 hours (total irradiation dose of 15 ± 1.6 J/cm²) using a 470 nm LED. All samples were subsequently incubated with the JC-1 dye for 30 minutes at 37°C and washed using 1X PBS. Aliquots were analysed in triplicate (3×100 μ L) in a black bottomed 96-well plate using BMG Labtech ClarioSTAR plate reader set at 475 nm excitation and 530 nm & 590 nm emission. Resulting data are expressed as the percentage of the ratio of J-aggregates to J-monomers (590 nm / 530 nm) indicative of the loss of mitochondrial membrane potential relative to the ratio in untreated dark control cell populations. The term 'J-aggregates' in the JC-1 assay represents the accumulation of the JC-1 dye in mitochondria with normal $\Delta\Psi_M$. The red fluorescence intensity of the J-aggregates at 590 nm is linked to the hyperpolarisation of the mitochondrial membrane. The green fluorescence emitted by the monomeric form of the dye at 530 nm indicates metabolically stressed cells and mitochondrial depolarisation i.e., a decrease in $\Delta\Psi_M$.

Spheroid Imaging Studies

Spheroids were seeded in a spheroid perfusion μ -slide (ibidi) at 2×10^5 cells/mL and allowed to grow and compact for 72 h before being treated with 100 μ M Ru-TAP-PDC3 for 24 h. The Ru-media solution was replaced with fresh phenol red free media prior to imaging. Spheroid images were captured at different planes in the z direction from below to above the spheroid and were acquired using a 40x oil immersion lens unless stated otherwise.

Spheroid Toxicity Assays

Spheroids were seeded in a clear BIOFLOAT™ round bottom 96 well plate (Sarstedt) at 2×10^4 cells per well, centrifuged using the BMG Labtech CLARIOstar platereader for 5 minutes at 700 rpm and allowed to grow and compact for 96 hours. A 2X concentrated solution of Ru-TAP-PDC3 was added in a 1:1 ratio (TAP:media) to the spheroid wells already containing 100 μ L phenol red free media and incubated for 24 h. Prior to irradiation 50% of the media/Ru solution was removed, replaced with fresh phenol red media to dilute the Ru-TAP-PDC3 (this step was repeated as necessary, with multiple dilution steps required for higher concentrations), and brought to a total volume of 100 μ L. Phototoxicity studies in HeLa spheroids were performed as per the cell monolayer protocol with an irradiation dose of 5 ± 0.54 J/cm² using a 470 nm LED. The CellTiter-Glo 3D cell viability assay (Promega) was used to assess cell viability. The assay was performed as per the manufacturer's protocol with the CellTiter-Glo 3D reagent added to the spheroid wells in a 1:1 ratio. This is a luminescence-based assay, and results were obtained using the BMG LABTECH CLARIOstar plate reader.

3.7 References

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Chapter 4 A Guanine Quadruplex Targeted Ru(II) Peptide Conjugate

Contributions: All cellular studies in this chapter were performed by Rhianne Curley.

4.1 Abstract

This chapter explores a Ru(II) polypyridyl peptide conjugate for its potential use as a G4 targeting probe. A 22 amino acid peptide, derived from the N-terminal domain of the RHAU helicase protein was selected due to its reported selectivity for parallel G4s. Conjugation of the RHAU peptide to $[\text{Ru}(\text{bpy})_2(\text{PIC-COOH})]^{2+}$ yielded the novel probe, Ru-RHAU. A series of DNA and RNA spectroscopic binding studies were performed to examine the DNA and RNA G4 binding capacity of Ru-RHAU and establish the photophysical response of the metal complex.

The complex showed high affinity binding to DNA and RNA G4s with the highest affinity for the parallel G4 CMYC. Furthermore, the complex showed excellent stabilisation of the RNA G4, N-RAS, as demonstrated by CD melting studies. When compared to the parent complex, a known DNA intercalator, $[\text{Ru}(\text{bpy})_2(\text{PIC-COOH})]^{2+}$, Ru-RHAU had a notably different response on association with duplex DNA, indicating that conjugation may prevent the metal centre from intercalation of dsDNA. Live cell studies showed conjugation probe promoted live cellular uptake into the cytoplasm where it was found to cause stress granules formation in a time dependent and concentration dependent manner. Confirmation that the foci evident in the cytoplasm were bona fide stress granules was supported by immunofluorescence co-localisation studies with well-established SG biomarkers TIA-1 and G3BP1.

4.2 Introduction

RNA G4s are an attractive target for the development of novel G4 probes, due to their importance in transcription and post translational modification as well as viral RNA containing pG4s. As RNA is located in different organelles such as the nucleus and mitochondria in addition to the cytoplasm, G4 probes targeting these structures are not necessarily required to transverse the nuclear or mitochondrial membrane, a limiting feature for many G4 targeted ligands. RNA helicase associated with AU-rich element (RHAU) is a helicase protein responsible for unwinding and resolving nucleic acids G4s and is essential for cellular homeostasis. RNA helicases can be categorised as DEAD box or DEAH box depending on the amino acid sequence of their helicase domain. RHAU can be categorised as a DEAH box helicase and is often referred to as DHX36, since that is the gene that is responsible for encoding it.¹

The RHAU helicase is present in both the cytoplasm and nucleus, though in differing forms.^{2,3} The nuclear form contains a slightly longer helicase domain with an additional 14 amino acids making the entire protein 1008 amino acids. In addition to the central helicase domain, RHAU also contains a C-terminal domain (approx.400 AA) and N-terminal domain (approx. 200 AA).⁴

The N-terminal of RHAU has been well researched due to its role in RNA binding. Studies investigating the N-terminal domain have determined a conserved 13 AA acid sequence required for G4 binding.⁵ This RHAU specific motif (RSM) occurs between the amino acids 54-66. Interestingly, deletion of the glycine rich region of RHAU prevents localisation of the protein to SGs.⁶

Crystallisation of RHAU bound to a DNA quadruplex has provided valuable structural insight into its binding and subsequent unwinding of G4s (Fig. 4.1).⁷ Crucially, this article revealed that the RSM forms an α -helix when it binds to parallel G4s. The paper provides

further insights into the mechanical functions of the helicase, as when G4 binding occurs, rearrangements of the helicase core are induced and the DNA single strand is pulled, resolving the quadruplex.

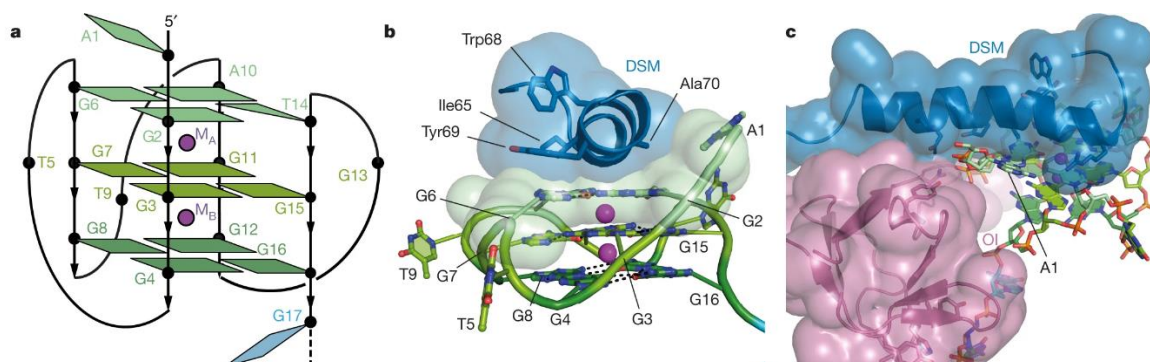


Figure 4.1, a Schematic of the DHX36-bound all-parallel G-quadruplex. b, The DSM stacks on the 5' (top) non-canonical quartet. Transparent spheres represent van der Waals radii. c, The DSM and the OI loop of the OB domain flank A1. (Adapted from Ref 7).

Initial investigations were focused on altering the length of the amino acid with peptides 13 AA to 53 AA. The 18 AA sequence, RHAU 18 (SMHPGHLKGREIGMWYAKKQ) proved to be an effective binder and stabiliser of G4s.⁸ NMR studies indicate that this peptide can bind to parallel G4s through π -stacking on the top of tetrads and through electrostatic interactions with the anionic phosphate backbone. Importantly NMR studies indicate the peptide folds into an alpha helix upon parallel G4 binding. To increase stability, a cyclic variant of RHAU 18 was synthesised called RHAU23c, where the peptide retained its alpha helix and became more resistant to peptidase degradation.⁹ Di Antonio *et al.* have since reported the 22 AA RHAU based sequence, DM039, that indicated high selectivity for the DNA quadruplex C-MYC based on anisotropy experiments (Fig. 4.2 (C)).¹⁰ The group attempted to preserve helicity through hydrocarbon stapling of the peptide, but this resulted in diminished G4 selectivity.

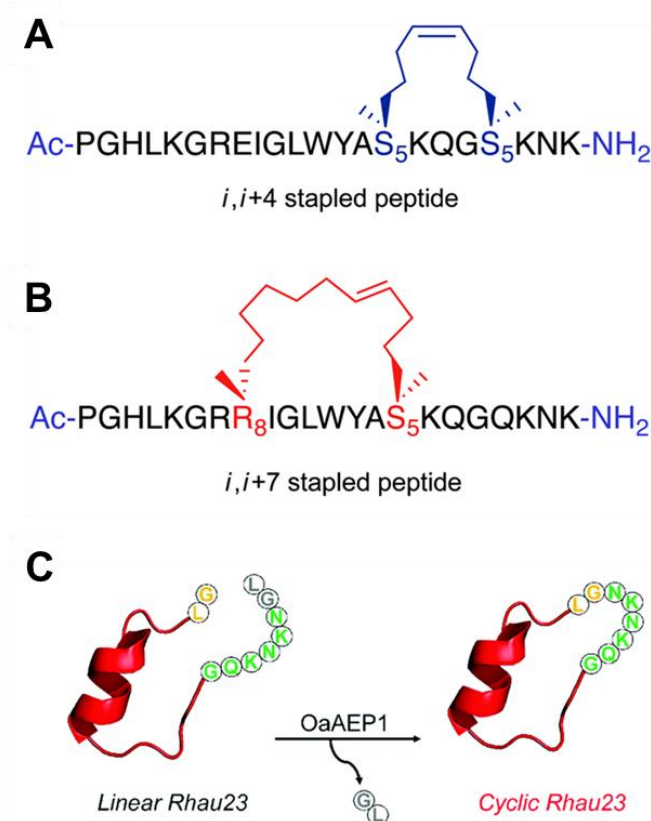


Figure 4.2 (A) *i, i + 4* hydrocarbon stapled peptide **DM083**, (B) *i, i + 7* hydrocarbon stapled peptide **DM102**. (Adapted from Ref. 10). (C) Schematic representation of the cyclization of Rhau23 by OaAEP1. (Adapted from Ref. 8 and 9).

Dang *et al.* explored RHAU conjugations to fluorescent proteins, this paper investigated a series of RHAU-Cyan Fluorescent protein (CFP) conjugations.¹¹ The series explored four conjugations of CFP to RHAU amino acids of differing lengths all of which show micromolar affinity. Conjugations to both the C-terminal and N-terminal ends of RHAU were explored with no significant differences in selectivity reported. This could be a promising imaging tool to visualise cellular G4s, but it does require genetic encoding for to be expressed in cells posing a barrier for live cell imaging. As these cellular studies have not been further investigated, it does leave open questions regarding CFP-RHAU conjugates feasibility as an analytical tool. Simpler conjugations to well established

fluorophores could be capable of similar binding affinity and live cell uptake but there is currently a lack of papers exploring this possibility.

An aspect of RHAU derived peptides that is also underexplored is their relationship with stress granules.¹² Stress granules are non-membranous biocondensates of mRNA and proteins that form through stress induced liquid-liquid phase separation. In addition to their role in cellular survival and homeostasis, their unregulated formation has strong links to neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS), frontotemporal dementia (FTD) and Alzheimer's disease, making SGs a potential target for novel therapeutics.^{13–15} They are composed primarily of 40S ribosomal subunits, poly(A)⁺ mRNA and RNA binding proteins but SGs can include additional biomolecules including protein kinases, RNA and cytoskeletal proteins. An example of SG components is illustrated in Fig. 4.3.¹⁶ G3BP1 and TIA-1 are of particular interest as the biomarkers commonly used to assess SG formation in cells using immunofluorescence techniques.¹⁶ In addition to SGs there are a number of other condensates, for example, P bodies that a storage site for untranslated mRNAs or the nucleolus that is responsible for rRNA production and ribosome assembly.¹⁷ The occurrence of these alternative cellular condensates makes the confirmation of bona fide SGs essential for their research.

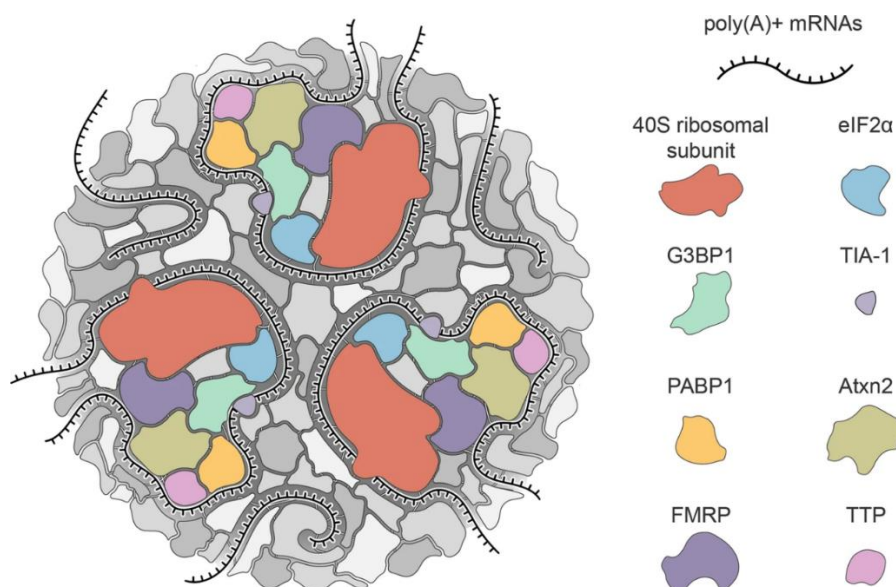


Figure 4.3 Stress granules structure and their respective components. They include eukaryotic translation initiation factor 2 subunit alpha (eIF2 α), ras GTPase-activating protein-binding protein 1 (G3BP1), T-cell intracellular antigen-1 (TIA-1), polyadenylate-binding protein 1 (PABP1), ataxin-2, fragile X mental retardation protein (FMRP) and tristetraprolin (TTP). The unspecified shapes coloured in grayscale represent the remaining proteins counted among the numerous SG protein components described so far. (Adapted from Ref. 16)

It is well established that the N-terminal domain of RHAU is not only responsible for DNA/RNA binding, but it also participates in recruitment to stress granules.⁶ Currently it is thought that RHAU associates with RNA during mRNA turnover regulation during stress. Due to this translocation towards SGs, RHAU peptides derived from the N-terminal domain could be prove to valuable tools for bioconjugates to target SGs.¹²

While it is clear that SGs are of great importance to cellular homeostasis and survival under stress conditions, there is currently a lack of tools to investigate and visualise their composition, assembly and disassembly in a live cell environment to further elucidate their function and unravel their connection to rG4s. Liu *et al.* have reported a biomimetic protocellular study that visualises the capacity for G4s to undergo liquid-liquid phase separation when interacting with proteins, providing an insight into nucleation and

assembly of SGs.¹⁸ Typically, stress granules are visualised immunostaining colocalisation with biomarkers that comprise stress granules, commonly G3BP1, TIA-1 or eIF4G. The drawback of immunofluorescence techniques to study dynamics is the requirement of cell fixing that dramatically alters the cell membrane structure and as SGs are dynamic structures, fixed cellular studies cannot provide adequate insight into their function. There has been a small number of probes that reportedly colocalise with stress granules though none are commercially available. Shao *et al.* designed and synthesised TASG, a chromophore that undergoes an increase in fluorescence when bound to RNA or G3BP1. The probe has limited fluorescence when in nonstressed cells but after cell undergo stress, the probe was found to concentrate in SGs, visualising their formation in the cytoplasm.¹⁹ TASG was also found to stain the nucleoli. Iida *et al.* synthesised a series of G4 probes that similarly stained the nucleoli of cells though with a high level of background noise.²⁰

This chapter aims to develop a novel Ru(II)-RHAU peptide conjugate (Ru-RHAU) probe to study stress granules in live cells. The DNA and RNA binding of a novel Ru-RHAU is evaluated, as well as its potential as a fluorescent probe towards studying the dynamics of stress granule assembly and disassembly.

4.3 Results

4.3.1 Synthesis and Photophysical Characterisation

The 23 AA sequence, DM039, derived from the RHAU helicase was selected to bioconjugate with a $[\text{Ru}(\text{bpy})_2(\text{Pic-COOH})]^{2+}$ (Ru-PIC) (Fig. 4.4). The peptide was selected due to its high selectivity for parallel G4s and its 5+ cationic charge, which was deemed important to promote cellular uptake. To ensure a successful conjugation, a hexyl amino linker was included in the synthesis of the peptide as a conjugation site. The peptide was synthesised by Celtek peptides and provided at a purity of greater than 95%. The parent complex was selected to exploit its long-lived excited state and well established emission properties in both aqueous and organic solvents, to facilitate tracking in a cellular environment. Bipyridine was selected as an auxiliary ligand due to its reduced DNA binding capacity in comparison to alternatives such as phenanthroline.²¹ This decision was aimed to promote DNA binding through the peptide as opposed to non-selective binding that may occur from the metal complex. It is also noteworthy that our group has reported successful cell uptake of Ru(II)-peptide conjugates that were similarly derived with $[\text{Ru}(\text{bpy})_2(\text{Pic-COOH})]^{2+}$ as the parent complex.^{22,23}

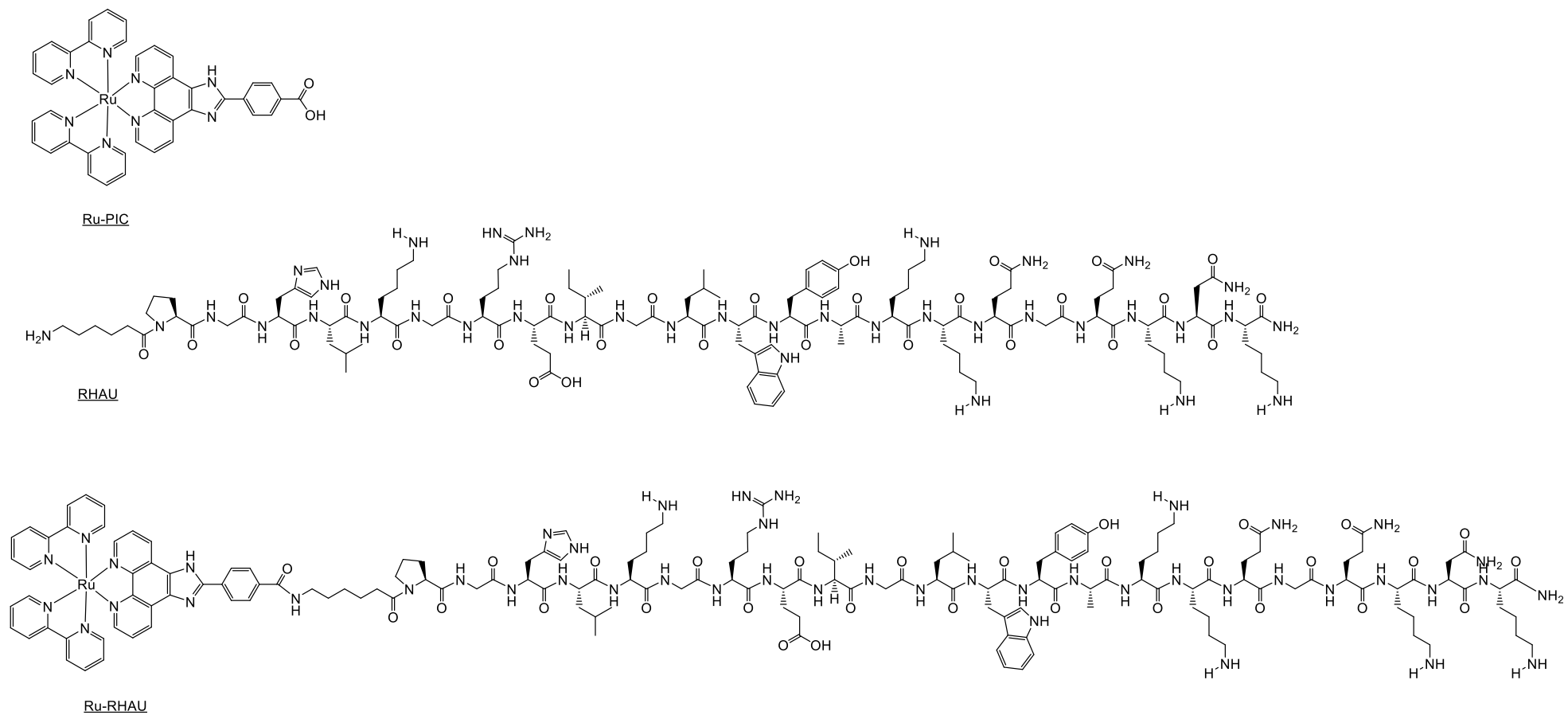


Figure 4.4 Structure of the parent complex (Ru-PIC), the peptide (RHAU) and the conjugate (Ru-RHAU)

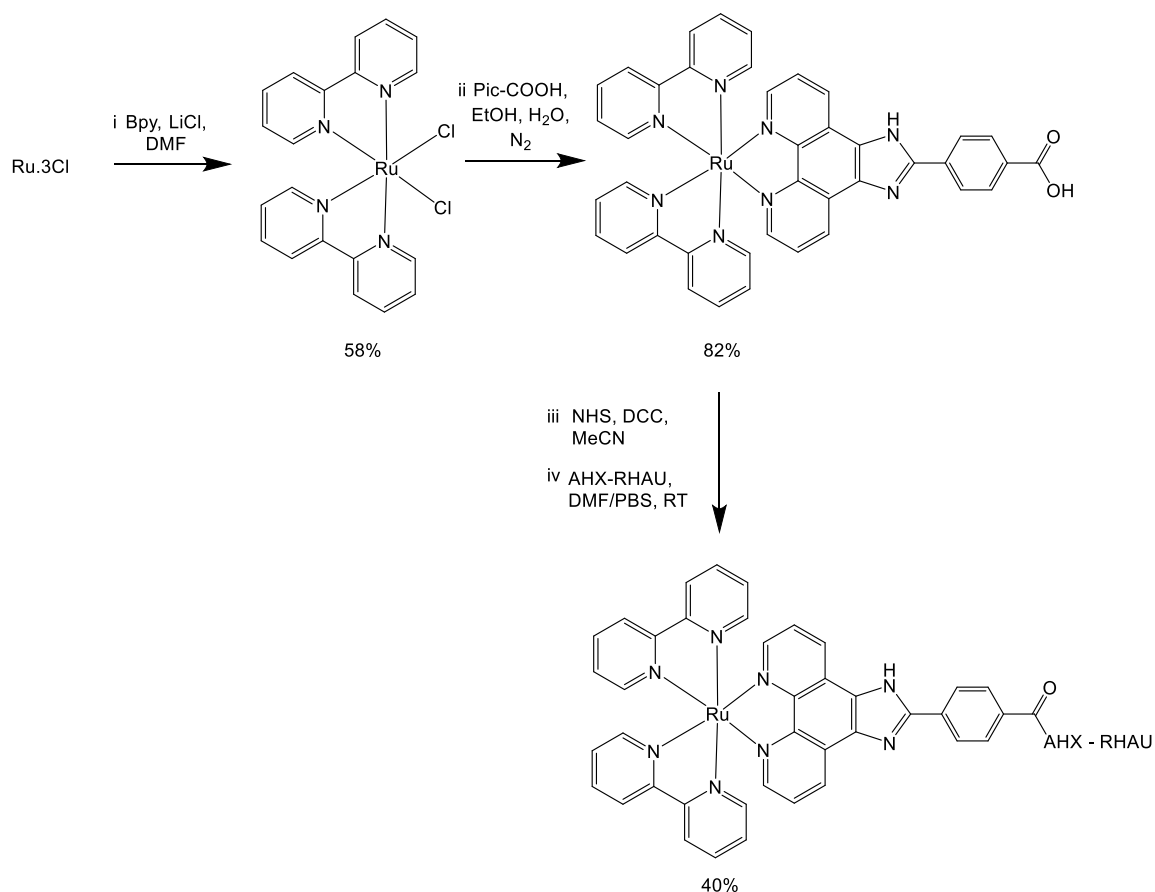
[Ru(bpy)₂Cl₂] was synthesised as previously reported by Sullivan *et al.* where RuCl₃ was dissolved in 10 ml of anhydrous DMF with 2,2-bipyridine and lithium chloride.²⁴ The solution was refluxed for 8 h under N₂, followed by precipitation in acetone, being filtered and washed. Purification by silica chromatography (MeOH/DCM) yielded fractions that were evaporated under pressure to yield pure [Ru(bpy)₂Cl₂] as a dark purple solid.

To synthesise Pic-COOH the method developed previously by the Keyes group was used.²² Phendione, carboxybenzaldehyde and ammonium acetate were added to a RBF in acetic acid and refluxed for 6 h. The solution was cooled, and water was added to precipitate the product before filtering and washing with water, methanol and diethyl ether. The ligand was used without further purification.

Pic-COOH was easily complexed to [Ru(bpy)₂Cl₂] by refluxing in MeOH before purification on silica column with an 80/20/1 (MeCN/ H₂O/ 20% w/v KNO₃) mobile phase. Fractions of the purified product were collected and combined before evaporation under vacuum. [Ru(bpy)₂(Pic-COOH)]²⁺ was dissolved in minimal acetone before precipitating the complex as a PF₆ salt by stirring in an aqueous ammonium hexafluorophosphate solution. A portion of [Ru(bpy)₂(Pic-COOH)]²⁻ was converted to a chloride salt for use as a water soluble parent complex in photophysical and biophysical studies. Conversion to a chloride salt was achieved by dropwise addition of the complex dissolved in a minimal quantity of acetone to a stirring solution of TBAC in acetone. The chloride precipitate was filtered and washed with copious quantities of acetone.

Ru-RHAU was synthesised using conjugation methods previously reported in our group.²³ [Ru(bpy)₂(Pic-COOH)]²⁺, N-hydroxysuccinimide (NHS) and dicyclohexyl carbodiimide (DCC) were added to a round bottom flask with 10 ml of anhydrous MeCN and stirred. Formation of the NHS intermediate was monitored by TLC, when complete the solution was evaporated and redissolved in 1 ml DMF. The RHAU peptide sequence was dissolved in 2 ml of PBS and added to the RBF. The solution was stirred overnight. After

18 h the solution was purified using a C18 silica prep plate. The band identified as the conjugated product, Ru-RHAU, was isolated. Ru-RHAU dissolved by MeCN and filtered through a bed of celite before evaporation under pressure until approx. 1 ml of solvent remained. Ru-RHAU was then yielded as PF_6 salt by slow addition to stirring water saturated with ammonium hexafluorophosphate. The dark red ppt. was filtered and washed with water followed by diethyl ether. Conversion to a chloride salt was completed by dissolving the complex in minimal MeCN before stirring overnight on amberlite chloride exchange resin. The complex was evaporated to dryness, dissolved in MeOH and filtered in order to remove resin. After filtration, the Ru-RHAU solution was evaporated to dryness yielding the final product.



Scheme 4.1 Synthetic scheme underwent to obtain Ru-RHAU.

The NMR was obtained in by dissolving the complex in CD₃OD and adding D₂O to promote hydrogen-deuterium exchange in order to simplify the spectra (Fig. 4.5).

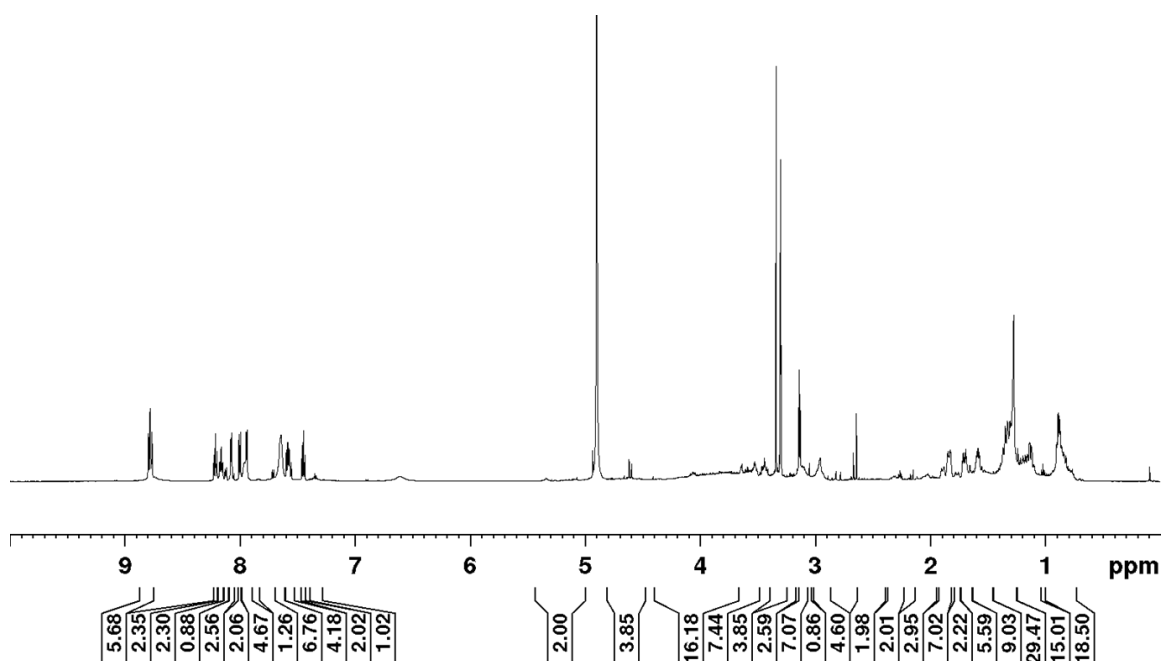


Figure 4.5 ¹H NMR of Ru-RHAU (CD₃OD, D₂O).

To further confirm successful conjugation, the RHAU peptide, Ru-PIC parent complex and Ru-RHAU conjugate were analysed by HPLC. Chromatograms of the three compounds were obtained using the same method by using a gradient system H₂O/MeCN with 0.1% TFA. The chromatograms are illustrated in Fig. 4.6. Peak detection was recorded at 280 nm and 450 nm using a photodiode array detector. Fig. 4.6 shows the peaks obtained and their respective retention times at 280 nm. Uv-Vis spectra of each peak was also recorded. The peptide RHAU had a retention time of 7.8 minutes. This chromatogram also contained a broad feature at the beginning of the run at 2.2 min attributed to the mobile phase. By comparison, Ru-PIC had a retention time of 14.3 mins. The conjugate

displayed a clearly resolved peak at 10.5 min, importantly the solution purity was determined to >95% based on peak area when measured at 280 nm.

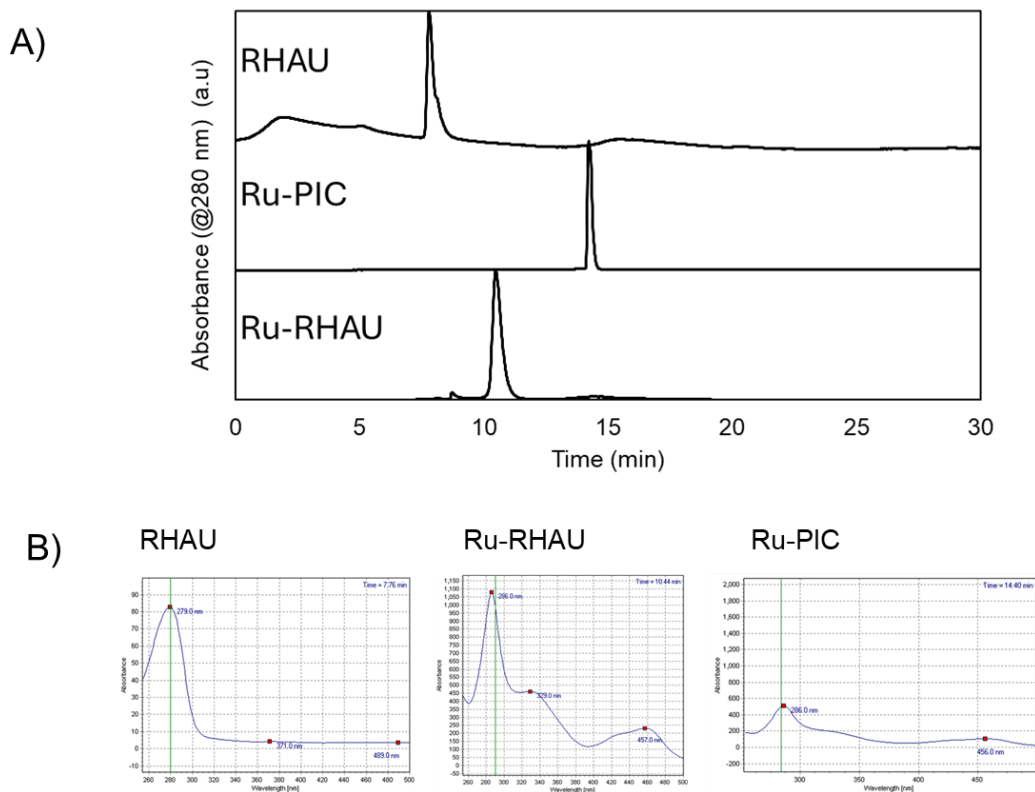


Figure 4.6 (A) HPLC chromatograms of RHAU, Ru-PIC and Ru-RHAU. (B) absorbance spectra gathered from the PDAD from 280 to 500 nm.

As shown in Fig. 4.7, Ru-RHAU has absorbance features at 285 nm, 340 nm and 465 nm, in all solutions. The complex has an emission maxima at 610 nm for D.I, KPi, and PBS buffer and an emission maxima of 621 nm in MeCN. Ru-RHAU has a mono exponential decay in D.I water of 0.4872 μ s.

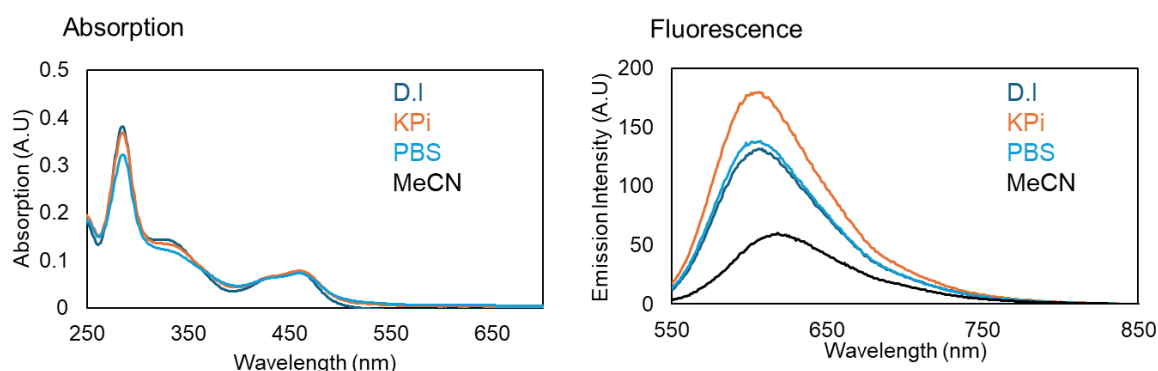


Figure 4.7: Absorption and emission spectra of Ru-RHAU in distilled water, PBS and KPi (10 mM potassium phosphate, 100 mM KCl) at 5 μ M.

4.3.2 DNA and RNA Guanine Quadruplex Binding of Ru-RHAU

To investigate the DNA and RNA binding capabilities of Ru-RHAU, a series of absorbance and luminescence titrations were performed by the addition of DNA or RNA oligonucleotides to a solution of Ru-RHAU in buffer. The oligonucleotides investigated are the guanine quadruplex forming sequences KRAS, CMYC, 22AG and NRAS (parallel, parallel, mixed-hybrid and parallel RNA) and double stranded duplex DNA (ctDNA and ds26) (Table 4.1). The DNA G4 oligonucleotides were annealed in a Kpi buffer (potassium phosphate 10 mM with additional potassium chloride 100 mM) at a DNA strand concentration of 1 mM. Whereas RNase free buffer was used to prepare stocks of the RNA oligonucleotides with a strand concentration of 0.1 mM, similarly with the addition of 10 mM of potassium phosphate and 100 mM potassium chloride. Confirmation of G4 formation was completed by analysis using circular dichroism.

Table 4.1 Panel of oligonucleotides selected for testing.

Name	Secondary structure	Sequence 5'→3'	Ref.
22AG	Mixed hybrid	AGGGTTAGGGTTAGGGTTAGGG	25
KRAS	Parallel	AGGGCGGTGTGGGATAGGGAA	26
CMYC	Parallel	TGGGGAGGGTGGGGAGGGTGGGGAAGG	27
Pu24T	Parallel	TGAGGGTGGTGAGGGTGGGGAAGG	25
NRAS	Parallel	UGUGGGAGGGGCGGGUCUGGGUGC	28
ds26	Double Stranded	CAATCGGATCGAATTTCGATCCGATTG	29
ctDNA	Double stranded		

Representative absorption titration graphs of Ru-RHAU binding to the series of DNA is illustrated in Fig. 4.8. All DNA titrations showed a decrease in absorption of the MLCT associated region of Ru-RHAU. The Ru-RHAU MLCT absorption peak at 465 nm underwent the greatest hypochromic shift. To aid visualisation of changes in the absorption spectra, inset graphs in Fig. 4.8 show the expanded MLCT region from 400 nm to 500 nm. Here these graphs reflect that Ru-RHAU is binding at different rates to the series of oligonucleotides. Importantly, the response of Ru-RHAU binding with DNA was a hypochromic shift for all DNA sequences tested and the response was saturable in all cases. This allows for the application of the McGhee Von-Hipple equation to extract binding affinities.¹⁰ This binding model has been reported previously for Ru(II) polypyridyl complexes and applied to both changes in emission and absorbance.^{31,32} It should also be considered that the affinities are extracted from the MLCT region, meaning they reflect the impact of the environmental change induced by binding to DNA on the metal complex and are a less direct measurement of peptide binding. However, it is noteworthy that the additional electrostatic interaction provided by the RHAU peptide conjugation are expected to aid in stronger binding for Ru-RHAU, in comparison to the parent complex. For experiments with RNA, it is assumed that the oligonucleotides are completely folded into G4s and no degradation of RNA has occurred.

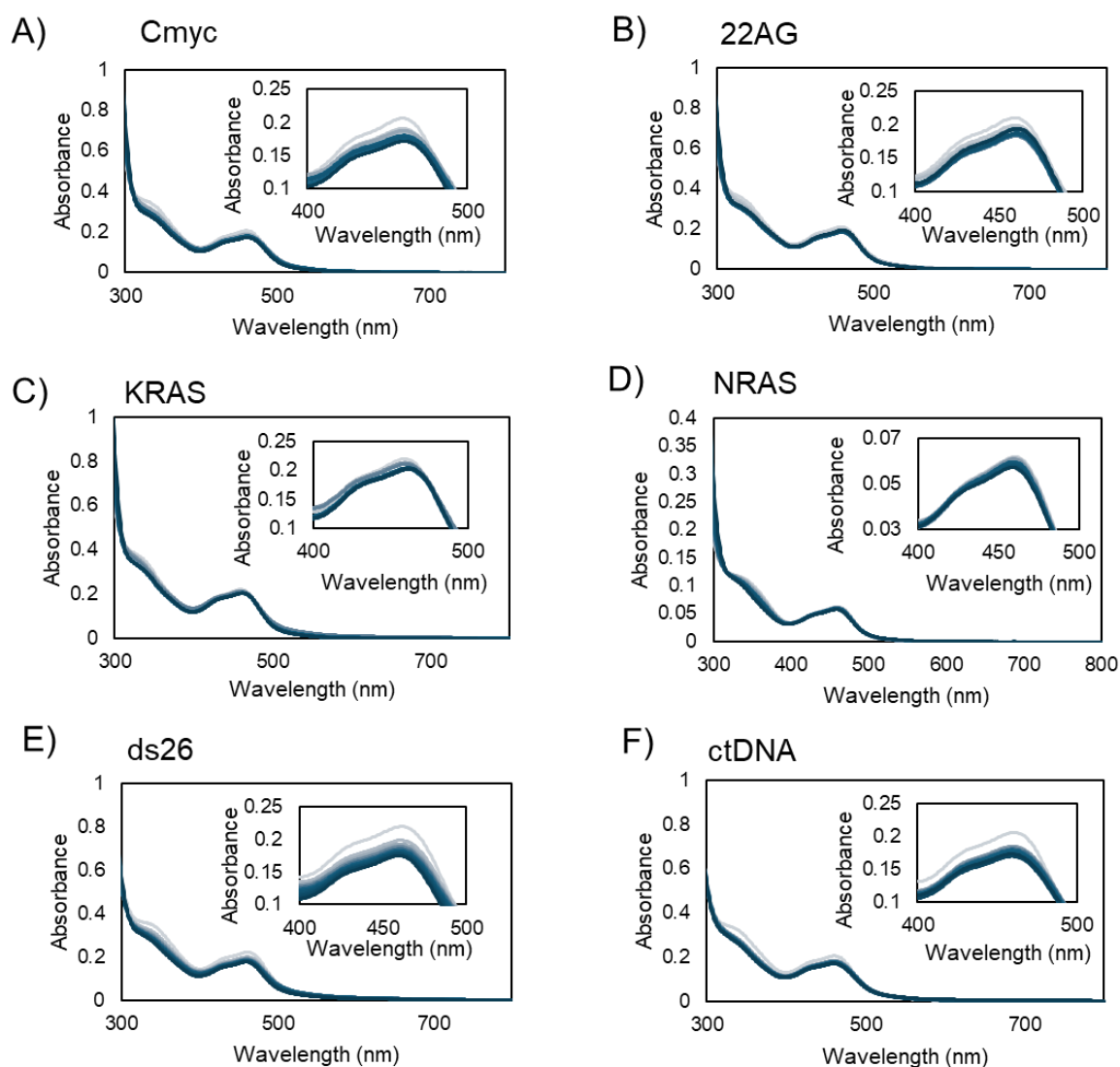


Figure 4.8 Representative absorption spectra obtained from a series of DNA titrations. Ru-RHAU (10 μ M) was titrated with the DNA G4s CMYC, KRAS, 22AG (0- 30 μ M) and the duplex DNA ds26 and ctDNA (0-50 μ M bp). Ru-RHAU (2 μ M) titration with the rG4 NRAS (0-6 μ M) in RNase free buffer. Insets of all graphs show the expanded MLCT region from 400 to 500 nm.

Fig. 4.9 illustrates the binding curves (blue data points) and their respective binding models (Orange lines) used to obtain binding constants tabulated in Table 4.2. Ru-RHAU had the greatest binding affinity for the parallel quadruplex CMYC with a K_b of 1.5×10^7 . As the unmodified RHAU peptide has reportedly preferential binding to CMYC, this likely indicates that the complex is binding through the peptide via the same binding mode thus the affinity is similarly the greatest for parallel structures compared to the other DNA topographies tested. KRAS and 22AG had binding affinities of 1.0×10^6 and 5.2×10^6 respectively, this binding affinity is similar to literature reports of Ru-Pip complexes. As the RHAU peptide preferentially binds to parallel G4s, it is unsurprising that the complex maintained a higher binding for the parallel G4s in comparison to the mixed hybrid G4, 22AG. Binding to ctDNA and ds26 showed affinities of 6.2×10^5 and 2.5×10^5 respectively. These values are lower in comparison than DNA G4s which are approximately a factor of 10 higher. Attempts to apply the McGhee-Von Hippel to the absorption titrations for NRAS were unsuccessful, in this case, binding models were applied to fluorescent response of the complex producing a K_b similar to DNA G4s of 1.9×10^6 .

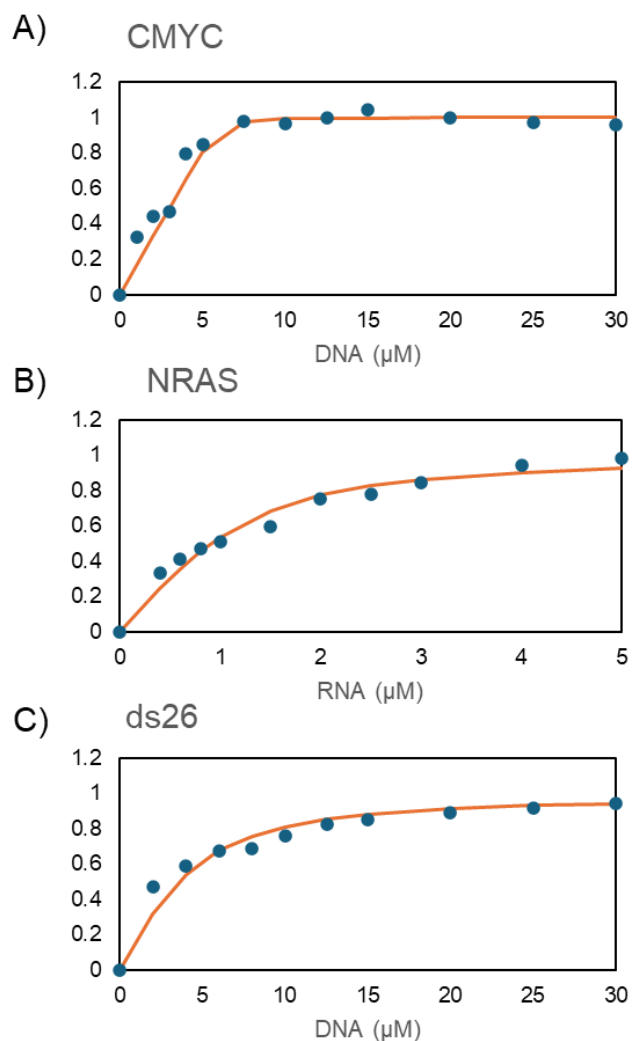


Figure 4.9: Representative absorption titrations of Ru-RHAU with CMYC, NRAS and ds26 and their respective binding curves.

In order to examine the impact of DNA binding on the luminescence of Ru-RHAU, emission measurements were conducted in tandem with the absorption titrations. 490 nm was selected for excitation to minimize any contribution to absorbance cross section changes to the emission intensity changes measured. Interestingly, different emission responses were observed between duplex and G4 binding. When binding to G4s, the luminescence of Ru-RHAU elicits a hyperchromic effect whereas when bound to ctDNA and dsDNA a hypochromic effect is evident. The hyperchromic responses when Ru-RHAU

was bound to the parallel G4s KRAS, Pu24T, and CMYC were approximately 62%, 43% and 34% respectively and 25% for the rG4 NRAS. Whereas a hypochromic effect was observed at 20% and 32% when binding at saturation to ctDNA and ds26 respectively (Fig. 4.11). This phenomenon has been noted in previous reports investigating the interaction between Ru(II) complexes containing pip-like ligands and their binding to G4s.³³ The enhanced luminescent intensity has been attributed to increased π delocalisation due to π - π stacking between the aromatic surfaces of the Pip ligand and DNA when binding to G4s in comparison to duplex DNA.^{34,35} The decrease in emission intensity when Ru-RHAU is associated with dsDNA may also be the result of protection from H₂O in combination with the small decrease in absorbance cross section observed in the absorption spectra at 490 nm.

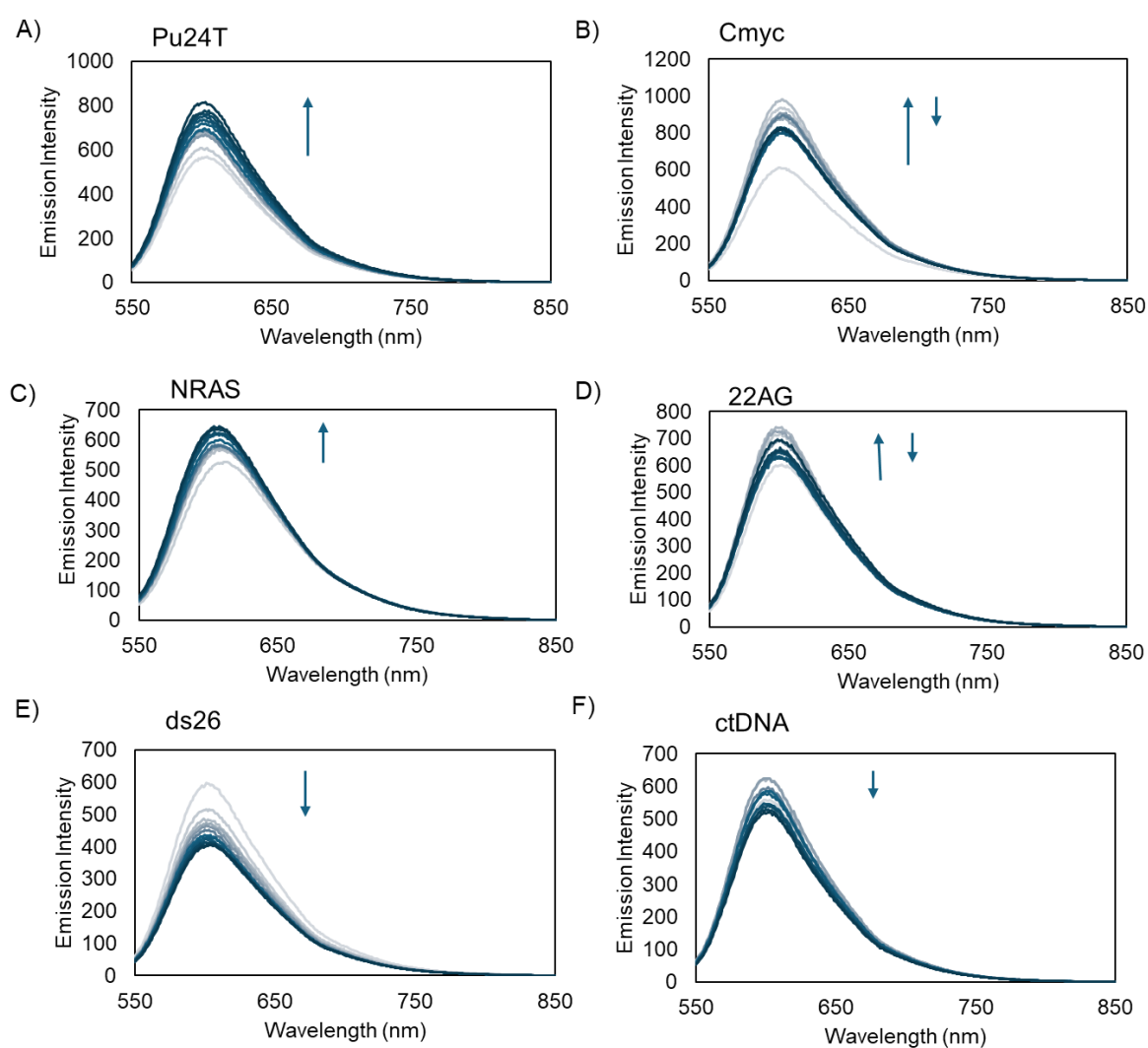


Figure 4.10 Representative emission spectra obtained from a series of DNA titrations. Ru-RHAU (10 μ M) was titrated with the DNA G4s CMYC, KRAS, 22AG (0- 30 μ M) and the duplex DNA ds26 and ctDNA (0-50 μ M bp). Ru-RHAU (2 μ M) titration with the rG4 NRAS (0-6 μ M) in RNase free buffer. Ru-RHAU was excited at 490 nm

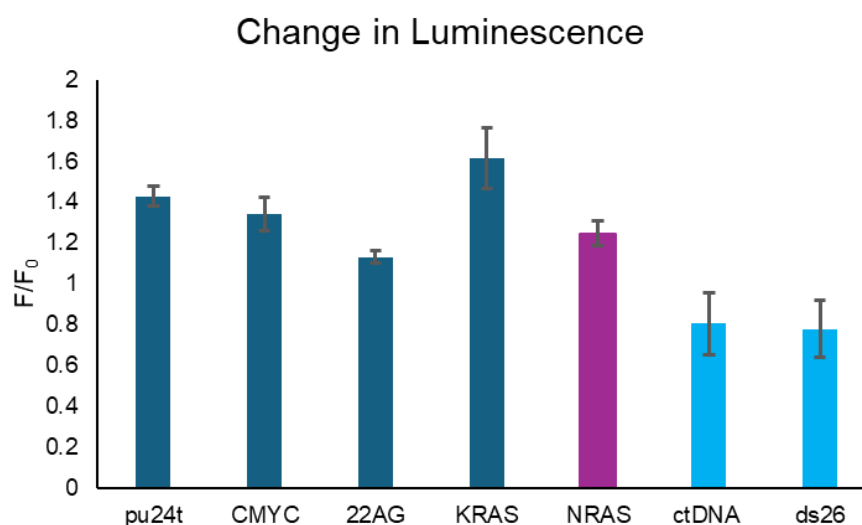


Figure 4.11 Changes in emission intensity in response to binding with DNA and RNA oligonucleotides at saturation. (n=2)

4.3.3 DNA and RNA Guanine Quadruplex Binding of Ru-PIC

In order to gain a clearer understanding of the binding modes and mechanisms undergone of G4 and duplex DNA binding to Ru-RHAU, studies were performed with the parent complex, Ru-PIC (Fig. 4.12). Titrations were performed in an analogous manner CMYC, ctDNA and NRAS. Absorption titrations against CMYC showed a decrease in the extinction of the MLCT particularly at 465 nm. Notably, titrations for Ru-PIC required higher concentrations of C-MYC to saturate in comparison to Ru-RHAU, indicating a reduced binding affinity. Interestingly titrations with ctDNA did not elicit a strong response in the MLCT absorption. Interestingly negligible changes in the absorption spectra were observed when NRAS.

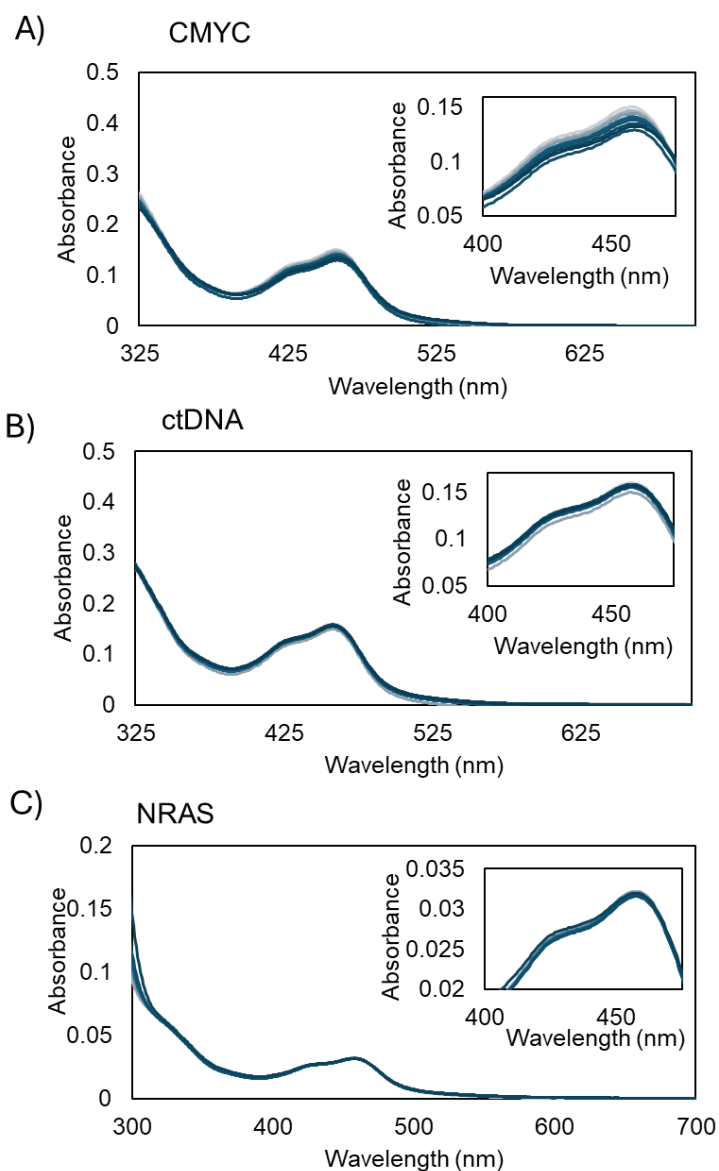


Figure 4.12 representative absorption spectra of Ru-PIC (10 μM) when titrated with CMYC (0-30 μM) and ctDNA (0-50 μM). The inset figures show expanded MLCT regions from 400 to 500 nm.

Fluorescence titrations were carried out in parallel with the absorbance titrations to examine the impact of DNA binding on the luminescence intensity of the complex (Fig. 4.13). The complex was excited at 490 nm as there was minimal change in absorption features at this wavelength. Interestingly, there is a remarkably different luminescent response of the parent complex compared to the conjugate when bound to ctDNA. ctDNA

elicits a considerable increase in the parent Ru-PIC luminescence on association unlike the conjugate that elicited a decrease in intensity. This is indicative of different binding modes between the two complexes. It should be considered that Ru(II) complexes containing Pic-like ligands and their derivatives have been reported to intercalate between base pairs, the aromatic stacking involved and induced rigidity in this binding mode could be responsible for the difference in intensity. Whereas the Pic ligand of Ru-RHAU is conjugated to a large peptide that could be posing a steric block towards intercalation. An alternative binding mode could be groove binding promoted through electrostatic interactions between the cationic metal complex, cationic peptide that carries a +5 charge and the anionic DNA backbone. When Ru-Pic was titrated with CMYC, a similar increase in luminescence intensity was observed.

Remarkably, upon titration with NRAS, negligible changes in fluorescence were observed. This likely indicates that no significant binding to NRAS is occurring. This could be the result of poor binding as a result of the differences in DNA G4s and RNA G4s. RNA G4s are known to be significantly more stable, it is possible that the increased stability prevented any perturbing of the G4 to facilitate Ru-PIC binding.³⁶

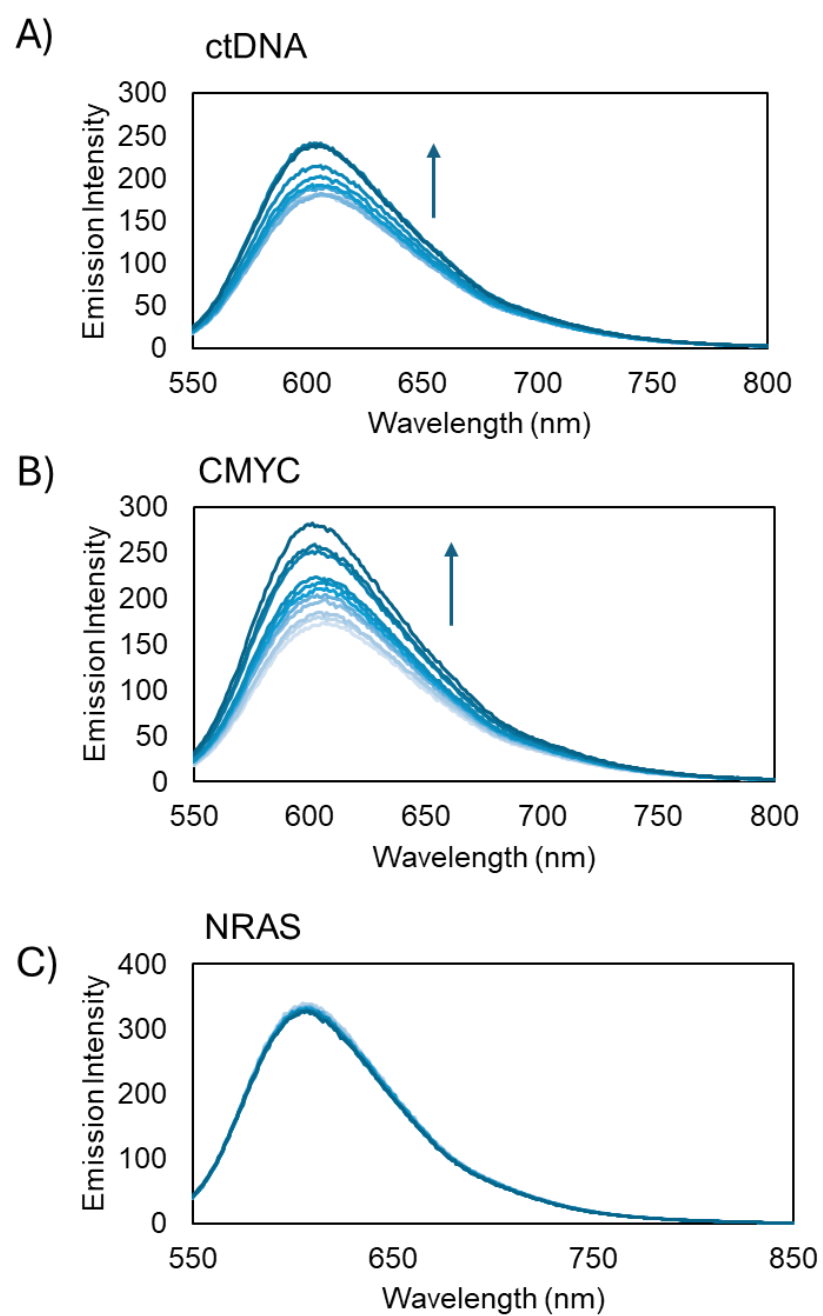


Figure 4.13 Representative luminescence spectra of Ru-PIC ($10\ \mu\text{M}$) when titrated with CMYC ($0\text{--}30\ \mu\text{M}$) and ctDNA ($0\text{--}50\ \mu\text{M}$). Excitation at $490\ \text{nm}$.

4.3.4 Influence of Ru-RHAU on the Thermal Stability of G4s

In order to determine the impact of Ru-RHAU towards on thermal stability of DNA CD, melting studies were carried out using circular dichroism to monitor changes to the topography of the DNA. For melting studies with the G4s KRAS, Pu24T and 22AG, a buffer solution of 10 mM potassium phosphate and 100 mM KCl was used. Due to the intrinsically high stability of CMYC and N-RAS, a weaker stabilising buffer was required, comprised of 10 mM lithium cacodylate and 90 mM of lithium chloride. The melting temperature (T_m) of the quadruplexes were first determined in buffer by measuring their CD signal from 225 nm to 290 nm with increasing temperatures from 20 °C to 95 °C at a rate of 1 °C a minute. A CD spectrum was measured for every increase in degree Celsius. CD spectra were analysed using the global 3 chircan software to obtain melting temperatures for the conditions outlined above. To investigate the impact of Ru-RHAU on stabilisation, the complex was incubated with DNA at a molar equivalency of [2]:[1], [Ru-RHAU]:[G4]. An incubation time of 10 mins was allowed to ensure sufficient binding had occurred, based on previous spectroscopic data.

In the case of the parallel DNA G4s CMYC, Pu24T and KRAS, notable stabilisation was evident (Fig. 4.14 and 4.15). Binding to Pu24T caused a substantial increase in T_m of 17.6 (± 0.6) °C. KRAS resulted in an increase of 10.9 (± 1.0) °C. Similarly, CMYC also had an T_m of 9.0 (± 0.5) °C. Although strong binding affinity of Ru-RHAU to 22AG was calculated based on changes in absorption titrations discussed in previous sections, binding to Ru-RHAU did not result in significant stabilisation where only an increase of 2.9 (± 0.6) °C was evident. Interestingly a change in CD signal was evident for 22AG after Ru-RHAU binding over the course of the melting study. A new peak emerged at 265 nm and a decrease of the characteristic 290 nm mixed hybrid peak was evident. As mentioned in previous chapters, changes at these wavelengths for 22AG have been attributed to conformational changes from mixed hybrid to parallel topology, although it does appear that there is likely

a mix of the two conformations given ellipticity at 290 nm is still evident over the course of the measurement. The change in topology is likely to also cause changes in the thermal stability of the quadruplex which could explain why no significant changes in T_m were observed. The most significant increase in melting temperature was evident for the rG4 NRAS where an increase in melting temperature of $26.4 (\pm 0.3) ^\circ\text{C}$. As RHAU derived peptides are typically specific to parallel G4s, it is unsurprising that Ru-RHAU stabilises the parallel G4s tested but does not appear to increase the thermal stability of 22AG. This could be further indication that the Ru-RHAU is indeed binding to G4s through the peptide. Schneekloth *et al.* have reported a small molecule microarray to target the NRAS rG4. The best hit defined by SAR studies was capable of inhibiting translation and increasing the thermal stability of NRAS by $6.2 ^\circ\text{C}$ at a binding ratio of 2:1, ligand to G4, under similar conditions Ru-RHAU is significantly more thermally stabilising in comparison.³⁷ The thermal stabilising impact of the myb peptide discussed in the introduction of this chapter was also investigated, where it was found to increase the T_m of CMYC by $5 ^\circ\text{C}$. Ru-RHAU in comparison was had a greater thermal stabilising impact by increasing the T_m by $9.0 (\pm 0.5) ^\circ\text{C}$

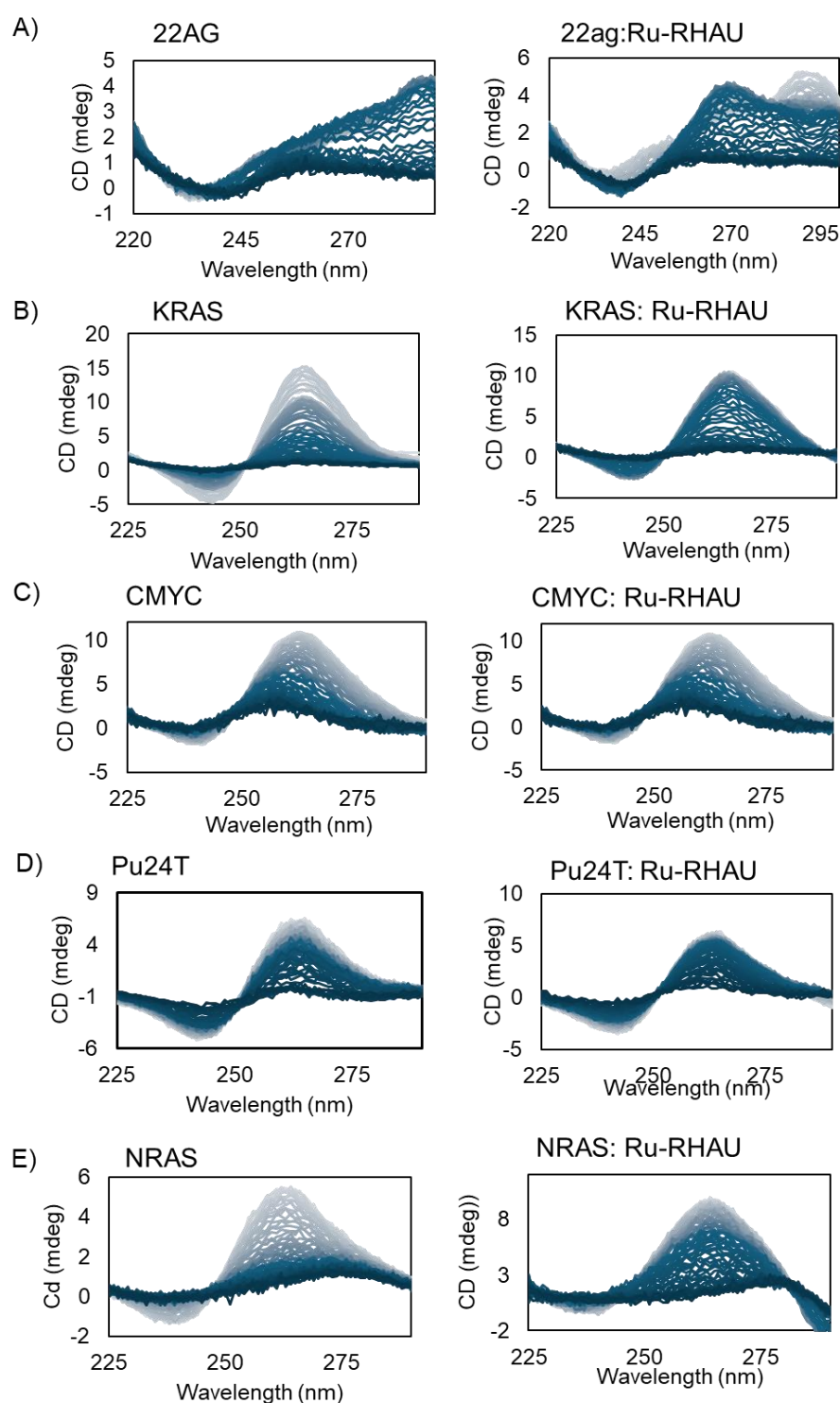


Figure 4.14: Melting curves of G4s at (5 μ M) and when bound to Ru-RHAU (10 μ M), (A) 22AG, (B) KRAS, (C) CMYC, (D) PU24T and (E) NRAS. Spectra are measured for every increase in 1 $^{\circ}$ C from 20 $^{\circ}$ C to 94 $^{\circ}$ C across a window of 225 nm to 290 nm.

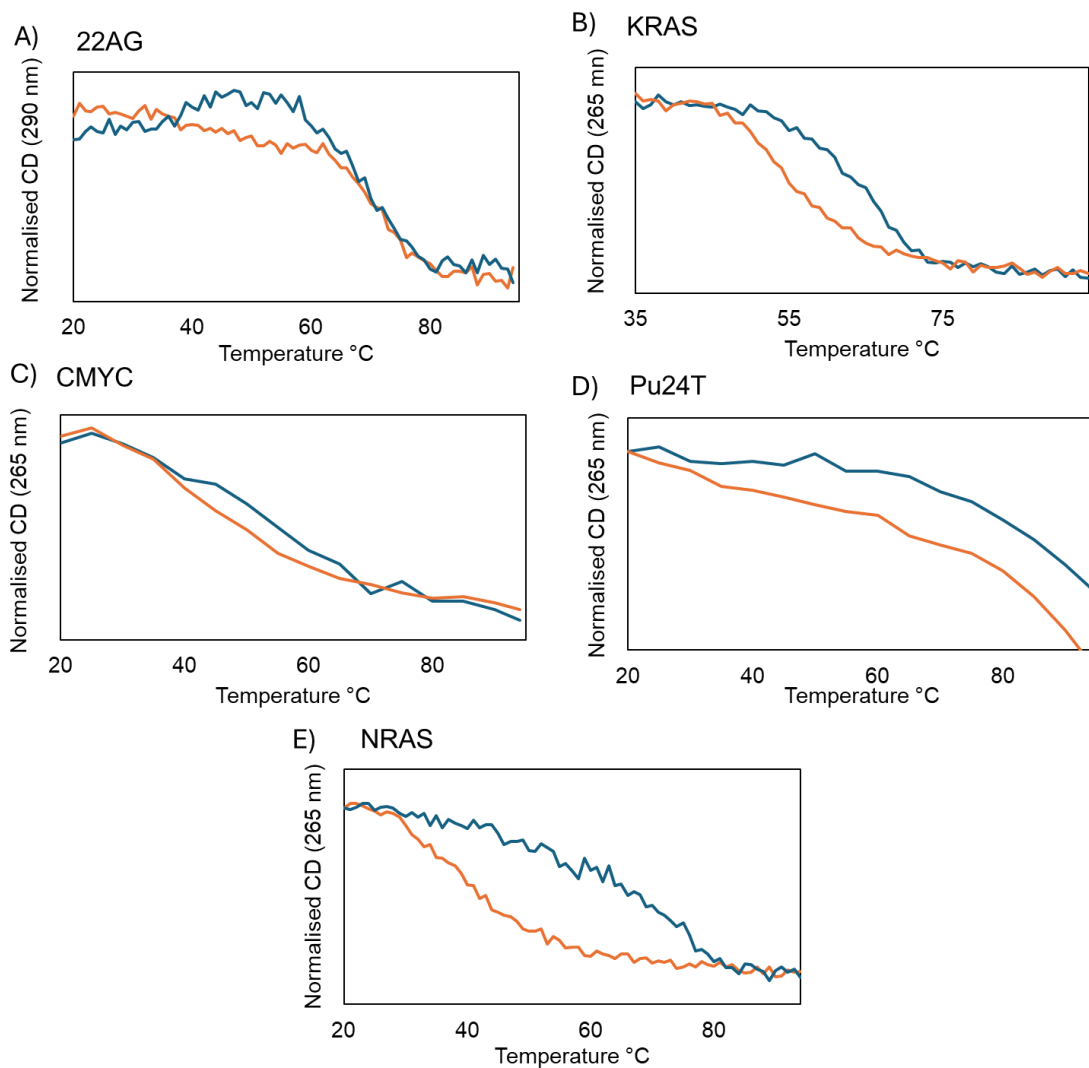


Figure 4.15 Comparison of thermal stability of G4s (5 μ M) when unbound (orange) and bound (blue) to Ru-RHAU (10 μ M).

Table 4.2 Binding affinities of Ru-RHAU to a series of oligonucleotides and their respective error as sum of squared residuals (SSR), changes in fluorescence with saturated binding (F/F_0) and increases in thermal stability ΔT_m ($^{\circ}\text{C}$).

Sequence	Binding affinities (M^{-1})	SSR (%)	F/F_0	ΔT_m ($^{\circ}\text{C}$)
Pu24T	3.2×10^5	4.0	1.43 (± 0.5)	17.6 (± 0.6)
CMYC	1.5×10^7	6.5	1.34 (± 0.08)	9.0 (± 0.5)
KRAS	1.0×10^6	5.3	1.62 (± 0.15)	10.9 (± 1.0)
22AG	5.2×10^6	3.7	1.13 (± 0.03)	2.9 (± 0.6)
NRAS	1.9×10^6	5.2	1.25 (± 0.06)	26.4 (± 0.3)
ctDNA	6.2×10^5	4.3	0.80 (± 0.15)	N.D.
ds26	2.5×10^5	3.9	0.68 (± 0.14)	N.D.

4.3.5 Influence of Ru-PIC on The Thermal Stability of CMYC and NRAS

To further probe the interaction of Ru-RHAU with NRAS and CMYC, melting studies were conducted with the parent Ru-PIC complex and the unconjugated RHAU peptide. These additional melting curves are presented in Fig. 4.16. Titrations were conducted using the conditions previously stated, with changes in ellipticity observed at 265 nm and normalised. When NRAS was incubated with 2 molar equivalents of Ru-PIC, interestingly the complex did not have any impact on the thermal stability. In contrast, when 2 molar equivalents of RHAU were incubated a significant increase in melting temperature of 27.5 (± 0.7) $^{\circ}\text{C}$ was observed. When considering the increased thermal stability from Ru-RHAU binding in comparison to the parent Ru-PIC and the lone peptide, it is clear that Ru-RHAU in a similar manner to the lone peptide. This is indicative of Ru-RHAU binding to and stabilising NRAS through the peptide as opposed to the metal centre. It may also help explain the reduced binding affinity of Ru-RHAU to NRAS in comparison to CMYC and KRAS. As the complex binds through the peptide and not the metal centre, the

corresponding changes to the MLCT may not be adequately reflected in binding affinity. Melting studies with CMYC were performed, both Ru-PIC and RHAU increased the melting temperature by 9.1 (± 0.3) °C and 14.2 (± 0.8) °C respectively. In contrast to NRAS, all ligands tested on CMYC produced a similar increase thermal stability, though the unconjugated RHAU peptide did elicit the strongest response by a small margin.

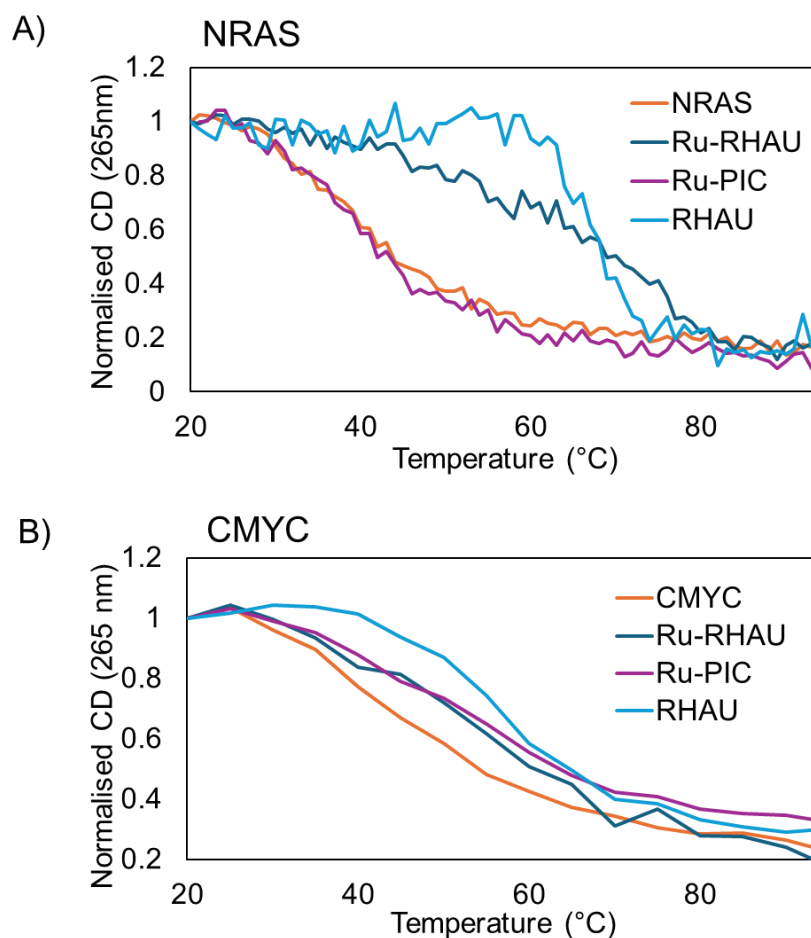


Figure 4.16 Thermal stability of NRAS and CMYC when unbound (orange) and bound to Ru-PIC (purple), RHAU (cyan), Ru-PIC (navy).

4.3.6 Impact of Nucleic Acid Binding on the Lifetime Decay of Ru-RHAU

To investigate if binding had an impact on the lifetime of Ru-RHAU, a series of TCSPC experiments were performed. Lifetimes were recorded in triplicate, except in the case of rG4s where they were recorded in duplicate. Measurements were made after giving sufficient time for binding of Ru-RHAU to DNA by monitoring changes in absorbance with respect to the MLCT region after addition of DNA or RNA. Further, measurements were completed at multiple binding ratios of Ru-RHAU with DNA (Table 4.3).

When measured in aqueous solutions, the complex exhibited monoexponential decay, with the lifetimes 487 ns, 580 ns and 584 ns in distilled water, KPi and PBS respectively. When bound to DNA G4s a notable increase in lifetime is evident, in addition to the emergence of a second decay occurs. Lifetimes were measured with Ru-RHAU (5 μ M) in the presence of DNA G4s at 5 μ M (1 eq) and 10 μ M (2 eq).

Generally, when bound to DNA G4s, a long lifetime component of the decay of approx. 1 microsecond emerges in addition to a shorter lifetime of approx. 500 ns, which is close to the lifetime of the free complex in solution. More specifically when Ru-RHAU was bound to 1 mol. eq. of 22AG, a τ_1 of 1.005 μ s and a τ_2 of 0.5276 μ s with an intensity weighted amplitude of 76% and 24% respectively. Upon the addition of a further mol. eq. of DNA a τ_1 of 1.0364 μ s and a τ_2 of 0.4856 μ s with an intensity weighted amplitude of 82% and 18% respectively was observed. Interestingly the second lifetime is notably similar to the free conjugate in solution and is likely the result of a portion of the solution containing unbound Ru-RHAU. This is supported by the decrease in amplitude of τ_2 when an additional molar equivalent of DNA G4 is added. A similar behaviour was evident for CMYC, where a τ_1 of 1.0728 μ s and a τ_2 of 0.5182 μ s with an intensity weighted amplitude of 49% and 51% respectively for 1 mol. eq. of DNA was observed. When bound to 2 mol. eq. the amplitude of τ_1 increased to 75% and τ_2 decreased to 25%. These changes in amplitude are reflected in the increasing average lifetime upon the addition of DNA from 0.7891 μ s to 0.8574 μ s.

The lifetime of the conjugate was also studied with dsDNA, where the lifetime also displayed a biexponential decay. When bound to an equimolar equivalent of ctDNA a τ_1 of 0.7683 μs and a τ_2 of 0.3879 μs was observed. When bound to 5 mol. eq. of ctDNA lifetimes observed increased, with a τ_1 of 0.9516 μs and a τ_2 of 0.4791 μs and an amplitude of 52% and 48% respectively and an average lifetime of 0.7248 μs .

Binding to the NRAS G4 also resulted in a biexponential decay of 0.5865 μs and 0.3539 μs when bound to 1 mol. eq. with an average lifetime of 0.4903 μs . Interestingly this lifetime is the shortest recorded at this mol. eq. when compared to the other G4s and the duplex DNA. This shorter lifetime could indicate a difference in binding mode that leaves the complex to exposed, perhaps the metal centre is more exposed. Considering the differences between rG4s and DNA G4s, it is thought that the additional hydroxyl group present in the ribose of rG4 grooves can retain more water molecules and results in increased stabilisation.³⁸ It should be considered that this difference in G4 environment could contribute to the decay of Ru-RHAU. Alternatively, the increase in stability may prevent Ru-RHAU from perturbing G4 grooves in a necessary manner for the metal centre to bind sufficiently. Another difference to consider is the inclusion of uracil instead of thymine in rG4s, the impact of which is underexplored in the literature.³⁹

Table 4.3 TCSPC results of Ru-RHAU when bound to DNA (5 μ M) and RNA (2 μ M) oligonucleotides.

DNA	μ M	Average lifetime (μ s)	$\tau_1(\mu$ s)	$\tau_2(\mu$ s)
22AG	5	0.8894	1.0051 (76%)	0.5276 (24%)
	10	0.9373	1.0364 (82%)	0.4856 (18%)
Pu24T	5	0.9491	1.0218 (81%)	0.4339 (19%)
	10	0.9897	1.0998 (81%)	0.5128 (19%)
KRAS	5	0.9692	1.0291 (91%)	0.3982 (9%)
	10	0.9870	1.0934 (82%)	0.5049 (18%)
Cmyc	5	0.7891	1.0748 (49%)	0.5182 (51%)
	10	0.8574	0.9969 (75%)	0.4334 (25%)
NRAS	2	0.4903	0.5856 (60%)	0.3539 (40%)
	4	0.5324	0.9067 (23%)	0.4221 (78%)
ds26	5	0.6408	0.7236 (74%)	0.4026 (26%)
	15	0.6602	0.7308 (80%)	0.3766 (20%)
	25	0.6934	0.7967 (73%)	0.4170 (27%)
ctDNA	5	0.6932	0.7682 (80%)	0.3879 (20%)
	15	0.7284	0.8741 (64%)	0.4633 (36%)
	25	0.7248	0.9516 (52%)	0.4791 (48%)
ss17	5	0.5946		
	15	0.6170		
Distilled		0.4872		
Kpi (rnase free)		0.5332		
KPi		0.5808		
PBS		0.5844		

4.4 Cellular studies of Ru-RHAU

4.4.1 Cellular uptake of Ru-RHAU and Assembly of Stress Induced Foci

Cellular uptake was assessed by incubating HeLa cells with Ru-RHAU over varying concentrations and incubation times. Fig. 4.17 shows the uptake of Ru-RHAU with increasing concentrations from 25 μM to 40 μM after a 6 h incubation. After 6 h, the complex is successfully taken up and appears to localise within the cytoplasm across this concentration range and cells appear healthy. Notably, large foci (approx. 2 μm) become apparent at 40 μM with a 6 h incubation. Additionally, the intensity of these foci is also stronger with increasing concentration of Ru-RHAU; suggesting an increase in Ru-RHAU concentration is influencing the formation of these foci. It is apparent that the formation of these foci is dependent on concentration.

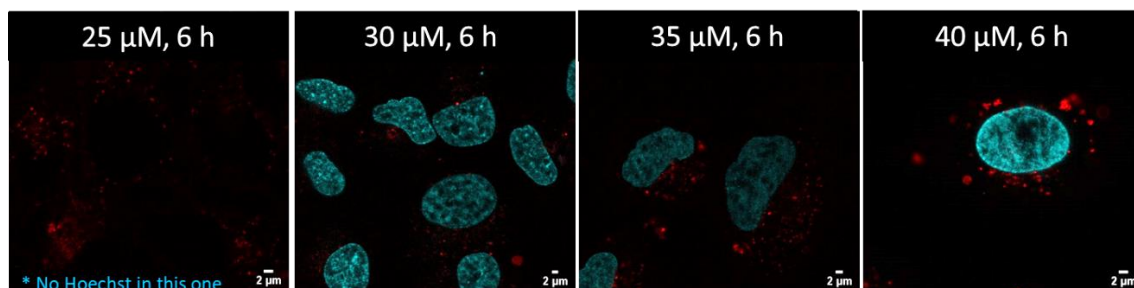


Figure 4.17: Uptake of Ru-RHAU at increasing concentrations after an incubation time of 6 h.

To further investigate the formation of these foci, additional imaging studies were performed at the same Ru-RHAU concentrations with an 8 h incubation period (Fig. 4.18). Large foci appear at 25 and 30 μM with an 8 h incubation. However, at 35 and 40 μM , cells begin to appear unhealthy based on confocal imaging. Further, the emergence of significantly larger foci becomes evident (approx. 10 μm) at these imaging conditions.

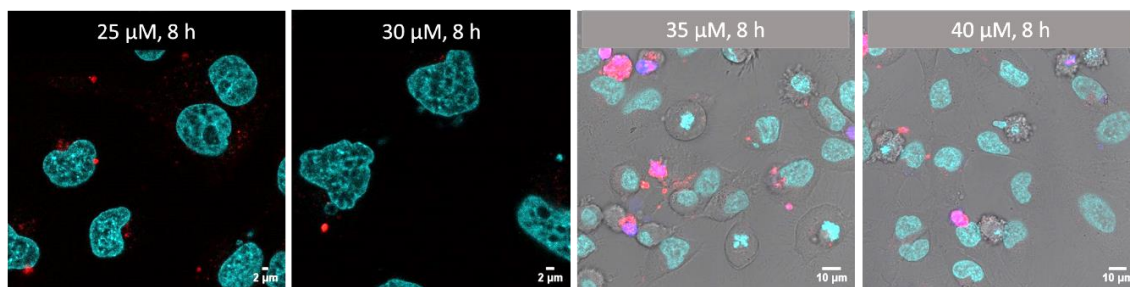


Figure 4.18: Uptake of Ru-RHAU at increasing concentrations after an incubation time of 8 h.

An Incubation time of 24 h was also investigated at 25 μM, 30 μM and 50 μM where 2 μm foci were apparent at 25 μM. To understand the impact of incubation time and concentration on cell health, cells were tested with DRAQ 7 to determine if cell death or damage could be confirmed at 50 μM (24 h). This is apparent in Fig. 4.19, where DRAQ 7 can be seen to have entered the nuclei of a number of cells. It is also evident that after a 24 h incubation, the number of larger foci 10 μm in size is more frequent. For further imaging studies, the conditions chosen were 40 μM (6 h) and 25 μM (24 h), due to the formation of notable cytoplasmic foci and to preserve good cellular health.

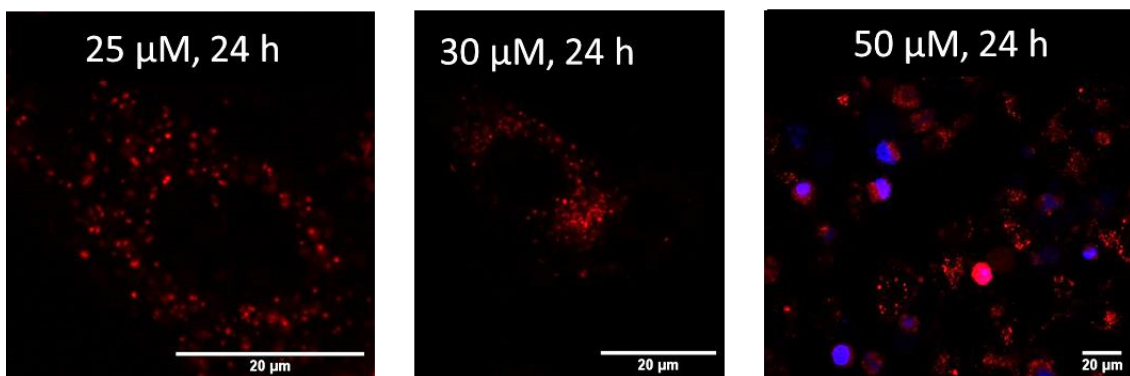


Figure 4.19: Uptake of Ru-RHAU at increasing concentrations after an incubation time of 24 h.

4.4.2 Immunofluorescence Staining of Stress Induced Foci

Considering the link between the sequestering of RHAU to SGs and the size of the foci, immunostaining was employed in order to determine if the foci were indeed *bona fide* SGs.⁴⁰ To accomplish this, anti-bodies tagged with AlexaFluor-647 were used to stain the SG localising proteins, G3BP1 and TIA-1.^{41,42} Strong colocalisation of the larger foci of Ru-RHAU with G3BP1 and TIA-1 was observed (Fig. 4.20) supporting the idea that Ru-RHAU is associated with and capable of initiating SG assembly, interacting with the most widely studied SG markers in the process. Strong overlap was observed under both selected imaging conditions, with significant amounts of G3BP1 and TIA-1 in the foci forming after a 24 h incubation of the complex in HeLa cells. Control studies were completed with the SG markers in HeLa cells under normal conditions, and heat shock (42 °C) treated. Heat shock at 42 °C induces stress in cells, providing a positive control to validate immunofluorescence studies with the chosen SG markers. The ability of Ru-RHAU to induce and image SGs in a live cell environment could allow for enhanced understanding of the role SGs play in cellular stress adaptation and disease pathogenesis.

Interestingly, the significantly large foci (10 µm) evident after 24 h, also have strong colocalisation. These could be late-stage stress granules or “pathological stress granules” that are incapable of disassembly a stressor is removed from the cell environment.

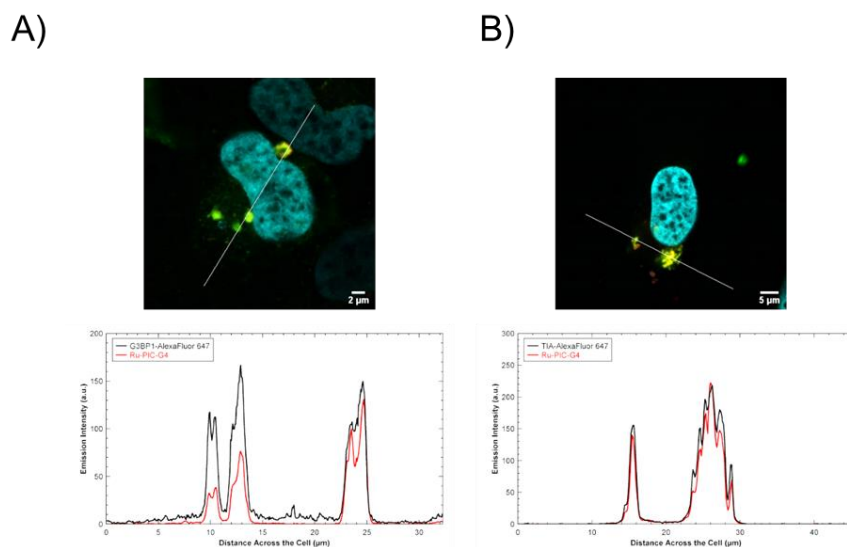


Figure 4.20 Immunofluorescent co-staining with G3BP1 Alexafluor 647 (A) and TIA-1 alexafluor647 (B) with Ru-RHAU (25 μ M) after 24 h.

To assess if Ru-RHAU is binding to rG4s in live HeLa cells and validates connection to SGs, HeLa cells treated with Ru-RHAU (40 μ M, 6 h or 25 μ M, 24 h), fixed and stained with the well-established anti-G4 antibody, BG4 (Merck).⁴³ BG4 is a single-chain variable fragment (scFv) antibody, which don't typically fold effectively in live mammalian cells and is impermeable, thus can only be used for fixed cell studies.⁴⁴ Immunofluorescence staining of BG4, can bind both DNA and RNA G4s, as a result foci is often seen in both the nucleus and cytoplasm of cells. In the BG4 channel, staining can be seen throughout the entire cell with notable foci (approx. 2 μ M) in the cytoplasm of cells when stained after an incubation period with Ru-RHAU. The increased intensity, in comparison to the control cells, is particularly of note within SGs. Similar localisation is evident in the cytoplasm for Ru-RHAU, indicating rG4 binding. Colocalisation is further visualised in Fig. 4.21, examining the emission intensity from both dyes, strong colocalisation is clear. From these results rG4 binding can be identified, which could play a role in Ru-RHAU's ability to induce SGs, supporting the link between rG4s and SGs. This link between the rG4 binding and stress granule formation may provide cellular evidence for the role of G4s in SG seeding and nucleation.

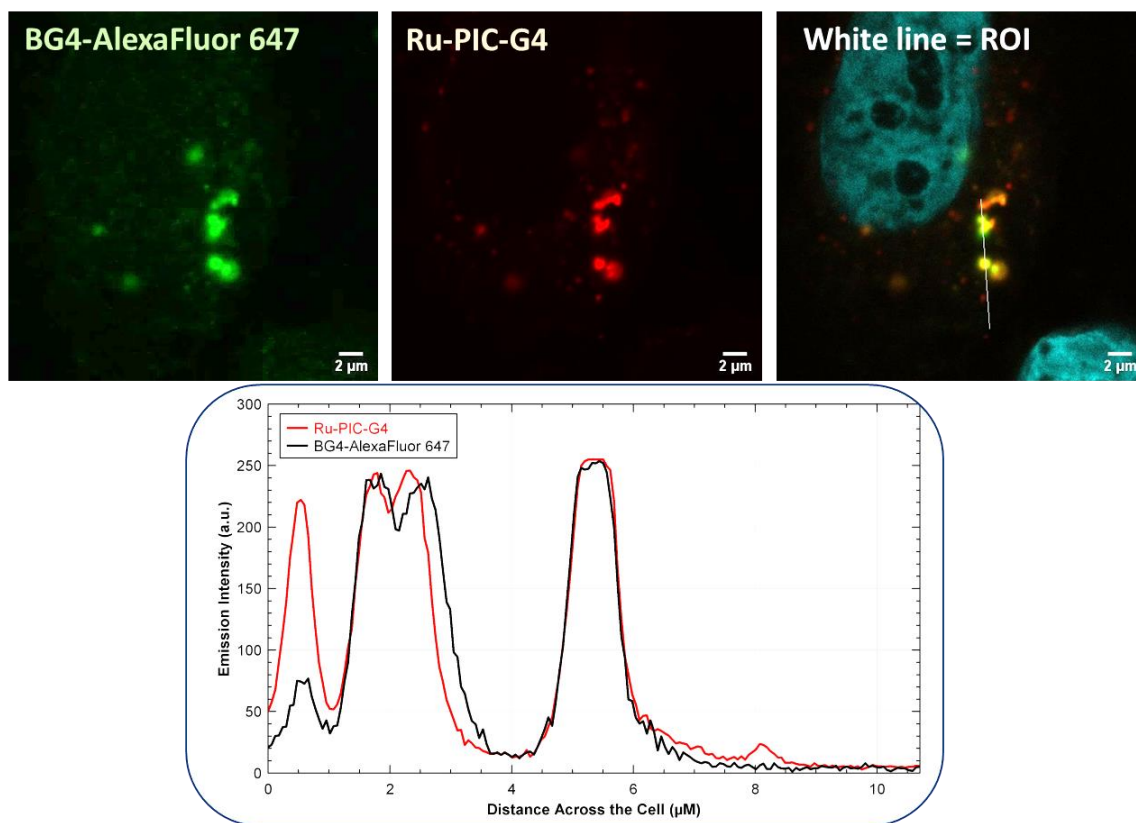


Figure 4.21: (A) Immunofluorescent co-staining of BG4 in fixed HeLa cells. (B) Staining of fixed HeLa cells with Ru-RHAU (25 μM) after 24 h incubation.

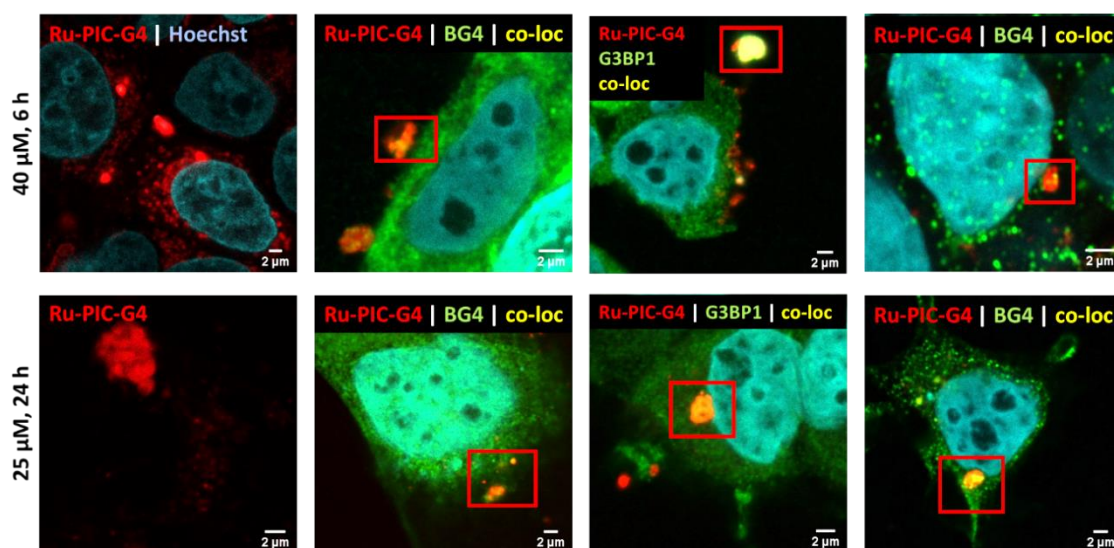


Figure 4.22 Uptake and formation of SGs in MCF7 cells after incubation with Ru-RHAU. Co-localisation studies in live cells.

It is clear from the previous cellular studies that not all of Ru-RHAU is localised to stress granules, owing to the fact that smaller foci are seen in the cytoplasm that are less than 2 μm and do not colocalise with the SG biomarkers, G3BP1 and TIA-1.

To investigate if these are localising in membrane containing organelles, additional colocalisation studies were performed with Biotracker (mitochondrial localising dye) and LysoTracker Deep Red (lysosome localising dye) (Fig. 4.23). Confocal imaging studies with commercial lysosome stain LysoTracker Deep Red confirmed that when Ru-PIC-G4 is not sequestered within large foci, it localises in the lysosomes of HeLa cells at incubation times of 6 h (Pearson's coefficient $r = 0.47 \pm 0.06$) or 24 h (Pearson's coefficient $r = 0.52 \pm 0.08$) (Table 4.4). This is not surprising as lysosomes are closely linked to SGs, with lysosomal damage reportedly causing cell stress and SG assembly.⁴⁵ Moreover, stress granule marker G3BP1 has been shown to co-localise with the LAMP-1 lysosome protein, further complicating the picture.^{46,47}

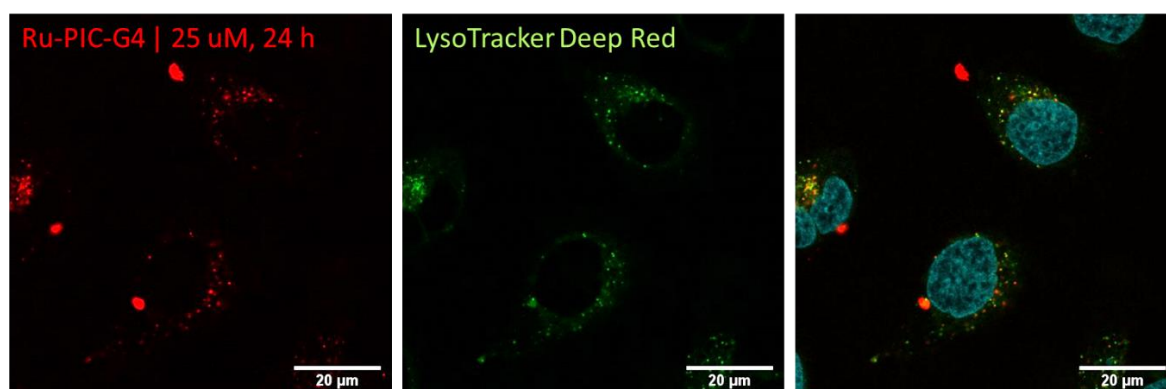


Figure 4.23 Co-staining lysotracker deep red and Ru-RHAU (25 μM) after 24 h. (Pearsons's coefficient 0.53).

4.4.3 Stress Granule Disassembly:

Recovery of SGs induced from a cell stress response is an important biological feature of SGs and should occur between 60 and 120 min. To test the recovery of live HeLa cells post Ru-RHAU treatment, cells were treated with Ru-RHAU for 6 h (40 μM) or 24 h (25

μM) and imaged every 15 minutes for 150 minutes (Appendix C). Imaging over time suggests the shorter incubation of 6 h enables the formation of early stage SGs, the majority of which disassemble after the removal of Ru-RHAU, over the course of 2 hours. After 24 h the formation of some large pathological persistent SGs was evident. These pathological SGs that do not disassemble within 150 min after the removal of the Ru(II) complex. The phase contrast images indicate the changes in cell morphology over time and indicate that as the SGs disassemble, cell damage occurs and ultimately cell death (Appendix C). Cell death as a response to SG disassembly is thought to occur from the release of caspase proteins within the granules and initiation of apoptosis.

4.4.4 Toxicity

For use as a cellular probe, it is important that Ru-RHAU has limited dark toxicity. This is required so offsite toxicity does not complicate the conclusions drawn from cellular studies, even more so when examining biological phenomena that innately put cells under stress. To this end, the toxicity of Ru-RHAU was determined. In HeLa cells, it was determined that Ru-RHAU has an IC_{50} of 59 ± 0.98 μM after a 24 h incubation and an $IC_{50} > 100$ μM after 6 h. The negligible toxicity at 6 h further confirms that Ru-RHAU is an excellent candidate as an imaging probe. It was also important to establish the impact of stress granule disassembly towards toxicity as it has been reported to induce apoptosis through the release of caspases. This was achieved by removing Ru-RHAU after an incubation of 24 h and waiting for 2 h before determining cell viability. This resulted in an IC_{50} of 28 μM. This is unsurprising considering the length time the cells were under stress and could be indicative that the stress granules provided a form of protection while incubated with Ru-RHAU.

4.5 Conclusions

In this Chapter, a rG4 targeted Ru(II) probe, Ru-RHAU, is developed. Ru-RHAU was shown to bind with high affinity to DNA and RNA oligonucleotides with the strongest affinity calculated for CMYC ($1.5 \times 10^7 \text{ M}^{-1}$). The complex also showed moderate affinity to the dsDNA oligonucleotides ctDNA and ds26 with binding affinities of $6.2 \times 10^5 \text{ M}^{-1}$ and $2.5 \times 10^5 \text{ M}^{-1}$ respectively. Importantly, melting studies with Ru-RHAU highlighted the complexes ability to strongly stabilise the rG4 NRAS by $26.4 (\pm 0.3) ^\circ\text{C}$ and DNA G4 Pu24t by $17.6 (\pm 0.6)$. Melting studies of Ru-RHAU, Ru-PIC, and RHAU with the NRAS indicated similar stabilising effects of Ru-RHAU and RHAU that could suggest similar binding modes.

Incorporation of the RHAU peptide promotes cellular uptake of the complex where localisation is evident within the cytoplasm. The results from this chapter show that Ru-RHAU can be used to induce and visualise SGs in live cells, in a concentration and time dependent manner. SG assembly was confirmed through fixed cell imaging of Ru-RHAU treated cells with SG markers G3BP1 and TIA-1, and G4 binding established by imaging with BG4. Ru-RHAU displayed moderate cytotoxicity after a 24 h incubation period (IC_{50} $59 \mu\text{M}$).

Materials and Methods

Reagents

Chemicals were purchased either from Sigma-Aldrich (Merck) or Fluorochem and used without further purification. Oligonucleotides were purchased from Eurofins genomics and used without further purification. Peptides were purchased from Celtek peptides, and provided at >95% purity as determined by HPLC.

[Ru(bpy)₂Cl₂]

[Ru(bpy)₂Cl₂] was synthesised as previously reported by Sullivan *et al.* where RuCl₃ was dissolved in 10 ml of anhydrous DMF with 2,2-bipyridine and lithium chloride.²⁴ The solution was refluxed for 8 h under N₂, followed by precipitation in acetone, being filtered and washed. Purification by silica chromatography (MeOH/DCM) yielded fractions that were evaporated under pressure to yield pure [Ru(bpy)₂Cl₂] as a dark purple solid. ¹H NMR (600 MHz, DMSO-d₆): 9.20 (d, 2 H), 8.64 (d, 2 H), 8.48 (d, 2 H), 8.11 (t, 2 H), 7.46 (2H) 7.35 (d, 2 H), 7.15 (d, 2 H), 7.05 (2 H)

Pic-COOH

To synthesise Pic-COOH the method developed previously by the Keyes group was used.²² Phendione, carboxybenzaldehyde and ammonium acetate were added to a RBF in acetic acid and refluxed for 6 h. The solution was cooled, and water was added to precipitate the product before filtering and washing with water, methanol and diethyl ether. The ligand was used without further purification. ¹H NMR (600 MHz, DMSO- d₆): 13.91 (s, 1 H), 12.70 (s, 1 H), 9.03 (dd, 2 H), 8.90 (dd, 2 H), 8.38 (d, 2 H), 8.17 (d, 2 H), 7.82 (q, 2 H).



Pic-COOH was easily complexed to $[\text{Ru}(\text{bpy})_2\text{Cl}_2]$ by refluxing in MeOH before purification on silica column with an 80/20/1 (MeCN/ H_2O / 20% w/v KNO_2) mobile phase. Fractions of the purified product were collected and combined before evaporation under vacuum. $[\text{Ru}(\text{bpy})_2(\text{Pic-COOH})]^{2+}$ was dissolved in minimal acetone before precipitating the complex as a PF_6 salt by stirring in an aqueous ammonium hexafluorophosphate solution. A portion of $[\text{Ru}(\text{bpy})_2(\text{Pic-COOH})]^{2+}$ was converted to a chloride salt for use as a parent complex in photophysical and biophysical studies. Conversion to a chloride salt was achieved by dropwise addition of the complex dissolved in a minimal quantity of acetone to a stirring solution of TBAC in acetone. The chloride precipitate was filtered and washed with copious quantities of acetone. ^1H NMR (600 MHz, $\text{DMSO}-d_6$) 9.05 (s, 2 H), 8.54 (d 2 H) 8.50 (d, 2 H), 8.38 (d, 2 H), 8.21 (d, 2 H) 8.10 (t, 2 H), 8.00 (m, 4 H), 7.85 (d, 2 H), 7.74 (q, 4 H), 7.61 (d, 2 H), 7.45 (t, 2 H), 7.22 (t, 2 H).

Ru-RHAU

Ru-RHAU was synthesised using conjugation methods previously reported in our group.²³ $[\text{Ru}(\text{bpy})_2(\text{Pic-COOH})]^{2+}$, NHS and DCC were added to a RBF with 10 ml of anhydrous MeCN and stirred. Formation of the NHS intermediate was monitored by TLC, when complete the solution was evaporated and redissolved in 1 ml DMF. The RHAU peptide sequence was dissolved in 2 ml of PBS and added to the RBF. The solution was stirred overnight. After 18 h the solution was purified using a C18 silica prep plate. The band identified as the conjugated product, Ru-RHAU, was isolated. Ru-RHAU dissolved by MeCN and filtered through a bed of celite before evaporation under pressure until approx. 1 ml of solvent remained. Ru-RHAU was then yielded as PF_6 salt by slow addition to stirring water saturated with ammonium hexafluorophosphate. The dark red ppt. was filtered and washed with water followed by diethyl ether. Conversion to a chloride salt was completed by dissolving the complex in minimal MeCN before stirring overnight on

amberlite chloride exchange resin. The complex was evaporated to dryness, dissolved in MeOH and filtered in order to remove resin. After filtration, the Ru-RHAU solution was evaporated to dryness yielding the final product. ^1H NMR (600 MHz, MeOD/D₂O) 8.78 (6H), 8.22 (t, 2H), 8.16 (t, 2H), 8.11 (d, 1H), 8.08 (3H), 8.01 (dd, 2H), 7.96 (s, 2H), 7.94 (d, 2H), 7.7 (d, 1H), 7.64 (m, 7H), 7.44 (2H) 7.35 (1H), 6.60 (3H), 4.62 (4H), 3.68-4.30 (16H), 3.58 (7H), 3.44 (4H), 3.11 (7H), 3.05 (1H), 2.95 (5H), 2.51 (2H), 2.3 (2H), 2.0 (3H), 1.86 (7H), 1.77 (2H), 1.7 (6H), 1.55 (9H), 1.35 (29 H), 1.14 (15H), 0.88 (18H).

Absorbance measurements were made using a Jasco V670 spectrophotometer, data was manipulated using Jasco Spectra Manager software and MS Excel. Luminescence studies were performed on a Varian Cary Eclipse Fluorimeter. TCSPC measurements were made using a PicoQuant FluoTime 100 Compact TCSPC system with a 450 nm laser from a PicoQuant PDL800-B Source and an external Thurlby Thander TGP110 10 MHz pulse generator. TCSPC spectra was collected up to 10000 counts, and decays were analysed and fit with PicoQuant Fluofit software

Stock concentrations of Ru-RHAU and Ru-PIC were stored at -20 °C as Cl⁻ salts in deionized water at a concentration of 1 mM and 4 mM respectively.

Oligonucleotides were purchased from Eurofins Genomics and stored at -20°C. Preparation of G4s were performed similarly to previous reports. A KPi buffer (10 mM potassium phosphate, 100 mM KCl) was used to anneal the quadruplex for all absorption, fluorescence and TCSPC measurements. Quadruplexes were annealed at 95 °C for 5 mins before cooling slowly to room temperature. DNA fluorescent and absorbance titration experiments were performed in tandem using a stock concentration of 1mM quadruplex strand concentration. RNA fluorescent and absorbance titration experiments were performed in tandem using a stock concentration of 100 μM quadruplex strand concentration.

CD melting studies were performed at an oligonucleotide concentration of 5 μ M and measured over a window of 220 – 290 nm from 20 to 94 °C. The oligos KRAS, 22AG and Pu24T were annealed in KPi buffer. Due to the increased stability of CMYC, a lithium cacodylate buffer was used (10 mM) with additional LiCl (90 mM). Circular dichroism measurements were performed on a Chirascan Series and data manipulation was performed using the Global 3 Analysis Software and MS Excel

Cellular Uptake

HeLa cells were seeded at 1×10^5 cells/mL in 8 chamber slides (Ibidi, Germany) and allowed to grow for 24 h at 37 °C with 5% CO₂. Cells were treated with Ru-PIC-G4, by diluting a 1 mM stock solution of the complex in cell culture media. After the desired incubation time e.g. 24 h, the Ru-media solution was removed, and the cells washed twice with 1X PBS (Gibco). The cells were imaged in phenol red free DMEM (Gibco) supplemented with 10% FBS. Cells were imaged using a Leica TCS DMI8 confocal microscope (63 $\times$ or 100 $\times$ oil immersion objective lens) with a heated stage at 37 °C. Ru-PIC-G4 was excited at 488 nm and the emission range set to between 550 and 800 nm. Uptake was assessed after treating the cells with Ru-PIC-G4 at 25, 30 and 50 μ M for 24 hours. DRAQ7 (3 μ M) was added each well prior to imaging to determine if the cells were damaged under the imaging conditions being tested.

To determine the mechanism of cellular uptake, the cells were pretreated with metabolic inhibitors oligomycin (Abcam) and 2-deoxy-d-glucose (Acros Organics). Cells were incubated with 5 μ M of oligomycin and 50 mM of 2-deoxy-d-glucose in cell culture media for 40 minutes in the dark at 37 °C with 5% CO₂. After the 40-minute incubation period, the metabolic inhibitors were removed, and the cells washed once with 1X PBS. A 200 μ L solution of Ru-PIC-G4 in fresh media at the desired concentration was then used to treat the cells for 24 h prior to imaging. A z-stack was performed to confirm uptake of the complex throughout the cells.

Localisation in Live HeLa Cells

Co-localisation studies were performed in HeLa cells with BioTracker 488 mitochondrial dye (100 nM, 30 min) Ex 488 nm; Em 515-525 nm and LysoTracker Deep Red lysosomal dye (100 nM, 1 h) Ex 647 nm, Em 665-675 nm.

HeLa cells were seeded in 8 chamber slides (ibidi) at 1×10^5 cells/mL and allowed to grow and attach overnight before being treated with Ru(bpy)₂-PIC-G4 at 5, 10, 15 or 25 μ M for 24 hours. After the 24 h incubation the cells were washed twice with 1X PBS and imaged in phenol red free media using a 100x oil immersion lens unless stated otherwise. Ru-PIC-G4 was excited at 488 nm and emission collected between 580-645 nm. Co-localisation analysis was performed using ImageJ software.

Table 4.4 Pearson's coefficients calculated based on colocalisation studies of Ru-RHAU.

Concentration of Ru-PIC-G4 (μ M)	Incubation time (h)	Commercial Dye	Organelle	Pearson's Coefficient $r =$
25	24	BioTracker 488	Mitochondria	0.18 ± 0.05
		LysoTracker Deep Red	Lysosomes	0.52 ± 0.08
40	6	LysoTracker Deep Red	Lysosomes	0.47 ± 0.06

Immunostaining

Coverslips were coated using poly-d-lysine for 30 min and allowed to dry completely. HeLa cells were seeded at 1×10^5 cells/mL on sterile, coated coverslips (round, $\varnothing$: 18 mm) in a 12-well tissue culture plate (Corning) and incubated for 24 h at 37 °C. Controls were fixed immediately (untreated controls) or incubated at 42 °C for 1 h to induce SGs prior to fixation and test cells were treated with Ru-PIC-G4 at 40 μ M for 6 h or 25 μ M for 24 h before fixation.

Cells were fixed with 4% paraformaldehyde (BioLegend) for 15 minutes at room temperature (RT), and subsequently permeabilized with 0.01% Triton X-100 in PBS (Invitrogen) for 5 min at RT. Cells were washed with PBS and blocked with 5% BSA in PBS (5% goat serum in PBS for BG4 studies) for 1 h at RT in the dark. The cells were incubated with Alexa-Fluor 647 conjugated G3BP1 antibody, Alexa-Fluor 647 conjugated TIA-1 antibody (Santa Cruz Biotechnology, 1:50) or anti-G4 antibody BG4 (Merck, 1:50) overnight at 4 °C. Cells stained with TIA-1 & G3BP1 were washed twice with PBS and Hoechst 33342 was added for 7 minutes at RT to stain the nucleus. BG4 stained cells were incubated with Flag-Tag (Cell Signaling Technology, 1:500, anti-rabbit mAb) for 2 hours at RT, followed by a 3 h incubation with goat anti-rabbit AlexaFluor 647 (Invitrogen, 1:500) at RT, and Hoechst 33342 (2 µg/mL) for 7 minutes at RT. Cells were washed twice with PBS between each incubation. Coverslips were mounted on glass slides using Prolong Gold Antifade (Invitrogen), incubated for 24 h at room temperature in the dark and sealed prior to confocal imaging.

Cytotoxicity Studies

HeLa cells were seeded in clear cell culture treated 96 well plates (Nunc) at 1×10^5 cells/mL and left to grow and attach for 24 h at 37 °C and 5 % CO₂. The cells were then treated with 100, 75, 60, 50, 25, 15, 10, 7.5, 5, 2.5 and 1 µM Ru(bpy)₂-PIC-G4. Six replicate wells were treated for each concentration, and the plates incubated for 24 h in the dark at 37°C. Cell viability of all plate-based studies was assessed using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay. The wells were emptied on the day of the viability assay (i.e. after 24 h Ru incubation) and replaced with 100 µL phenol red free media. 25 µL of a 2.5 mg/mL MTT solution (Invitrogen) was added per 100 µL of media and incubated at 37 °C for 3 h in the absence of light. During the 3 h incubation actively respiring cells convert the yellow MTT into formazon, a water-insoluble purple crystal. The MTT/media solution was carefully removed from the wells and

replaced with 100 μ L DMSO to solubilise the formazon. Absorbance was measured using a BMG LABTECH CLARIOstar plate reader at 550 nm and 620 nm (corrected for background subtraction), with a 5-minute shaking step in the plate reader prior to any measurements. Cell viability is presented as a percentage (%) compared to untreated control cells. Plates were performed in duplicate.

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Chapter 5 Conclusions

5.1 Conclusions

The aim of this thesis was develop novel G4 binding probes and therapeutics for applications in live cell environments. In Chapter 2, the novel G4 binding probe RuPDC3 was synthesised, designed to incorporate a ruthenium complex capable of “light switch” photophysical properties with a G4 targeting ligand. It was demonstrated that the complex could distinguish dsDNA from G4s in solution studies using both luminescence lifetime and resonance Raman spectroscopy. It was also demonstrated that RuPDC3 could disrupt G4 tertiary structure at high RuPDC3 concentrations. These in solution provided the basis for cellular studies where the aim of developing a G4 probe was achieved. In cells, it was found that RuPDC3 successfully localised in the nucleus of HeLa cells where it was used as a multimodal probe with PLIM and resonance Raman mapping.

Chapter three sets out to develop a G4 binding type-III photosensitiser capable of directly photodamaging G4s. This was achieved by implementing the tpphz-PDC3 ligand designed in chapter 2 with a Ru(II)-tap complex. The novel theranostic Ru-TAP-PDC3 was synthesised and successfully demonstrated capability to undergo PET with GMP. Additional quenching studies confirmed that the probe could undergo PET with mitoG4s. The complex showed strong binding to mitoG4s with using the established fluorescent intercalator displacement assay. Importantly, in cell studies, the probe proved to be an effective therapeutic capable of depleting mtDNA from photoirradiation in HeLa cells, inducing apoptosis in both hypoxic and normoxic conditions. In comparison to other Ru(II)-tap complexes, Ru-TAP-PDC3 was significantly more phototoxic and remained negligibly dark toxic.

In chapter 4, a Ru(II) polypyridyl probe is once again explored for use as a cellular probe, however its biological application greatly differs. The Ru(II)-peptide complex, Ru-RHAU

was similarly designed as a G4 binder where it was demonstrated to significantly stabilise the RNA G4, NRAS, whereas in comparison the parent complex had negligible impact on the thermal stability of NRAS. Further Ru-RHAU indicating a higher binding affinity towards parallel G4s over dsDNA, in some cases by a factor approximately 50. The complex localised in the cytoplasm where it was found to induce and image stress granule formation in a concentration and time dependent manner. This is an exciting prospect and could provide the basis to investigate outstanding fundamental questions regarding the formation and function of stress granules.

Chapter 6 Future Work

While this thesis was largely successful in completing the aims defined at the beginning of the thesis, there is of course a plethora of possible experiments to expand and improve our understanding of the complexes presented in thesis. Additional future work regarding RuPDC3 includes further biological experimentation to gain a greater understanding of DNA binding in a cellular context. Some experiments will include pretreatment of cells with G4 stabilisers Phen-DC3 and PDS to investigate the resonance of RuPDC3, particularly in PLIM and Raman experiments. This could be an important control to add further confirmation of G4 binding and confirm if the techniques implemented have successfully visualised G4 binding. Additional photophysical studies are currently underway using ultrafast spectroscopy and transient absorption to gain a greater understanding of what photophysical process occur in protic solutions, aprotic solutions and when bound to G4s. Similarly for Ru-TAP -PDC3, additional photophysical experiments could provide insights in the PET process that the complex undergoes. Additionally, chapter 2 uses spheroids as a model for tumours, however some deficiencies of this model have been noted, implementation of alternative models such as organoids could be more representative of cancer tumours.

Chapter 4 highlights how RHAU peptide conjugation can be utilised as a vector to transport cargo into cytoplasmic stress granules. This prospect is unexplored in literature but could provide the basis for therapeutics capable of overcoming cancer therapy resistance induced by stress granule formation. It is important to note that SGs have a diminished oxygen concentration possibly presenting a barrier for typical type II photosensitisers, however it could be opportune to target SGs with type III PSs directly undergoing PET causing photodamage to RNA and proteins. This could provide an avenue to overcome cancer resistance.

Chapter 1 discussed a number of enantiopure Ru(II) complexes that have been studied for their difference in DNA binding and biological activity. Unsurprisingly, it has been observed that different enantiomers can have considerable differences in binding affinity across different DNA secondary structures. This is evident for multiple classes of Ru(II) complexes including bimetallic, pip-bearing and dppz-bearing complexes. These factors highlight the importance of investigating enantiopure complexes in combination to typical racemic mixtures. Preliminary work was carried out to try separate the ruthenium(II) enantiomers of RuPDC3. Chiral separations were performed using a Chiralpak AD Coated column packed with an amylose tris(3,5 dimethylphenyl carbamate) stationary phase (Fig. 6.1). This sugar based stationary phase is similar to the dextrin and amylose stationary phases that have previously been reported to yield successful enantiomer separation.

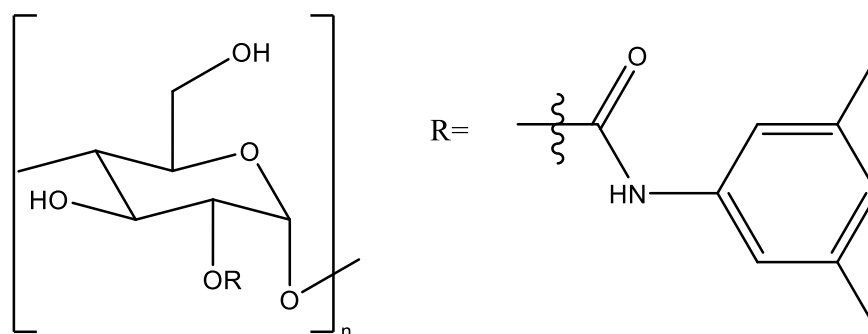


Figure 6.1 Chemical structure of the Charalpak AD stationary phase used in this chapter. The use of chiral columns is accompanied by somewhat strict operating conditions, required to preserve the stationary phase. For ChiralPak AD, it is stated to use 0-15% v/v max MeCN in MeOH. It is possible to use 100 % MeOH or 100% MeCN but transitional mobile phases are required. Another limiting factor to consider is the inclusion of additives in the mobile phase. Acid or base may be required to achieve chiral separation; however, it is recommended when using ChiralPak AD to use a V/V of less than 0.5% base or acid. Using a mobile phase of MeOH/ MeCN/ Acetic Acid/ TEA (85/15/0.4/1.2), two peaks were evident in the HPLC trace, with the first peak eluting at 9 min and the second peak eluting

at 11 min (Fig. 6.2). However, the chromatogram showed poor peak resolution and significant peak tailing, inhibiting the collection of enantiomerically pure complexes. Nonetheless, fractions were collected at the leading edge and tailing edge of the two peaks respectively to reduce cross contamination of enantiomers. The fractions collected were examined by circular dichroism and absorbance spectroscopy to establish if the two peaks were indeed the result of chiral separations.

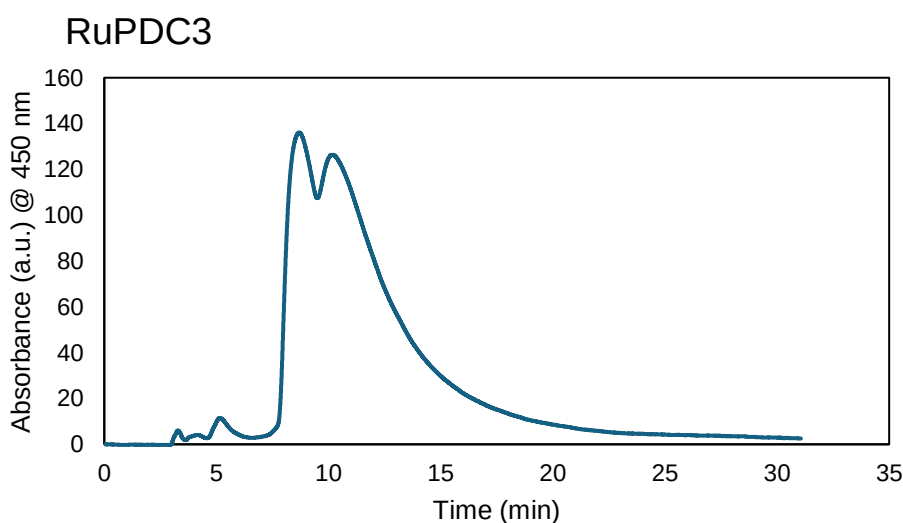


Figure 6.2 HPLC trace of RuPDC3 with a chiralpak AD column, measuring with a photodiode array @450 nm. Mobile phase: MeOH/ MeCN/ Acetic Acid/ TEA (85/ 15 /0.4 / 1.2 v/v). Flowrate = 1 ml/min

Fig. 6.3 (A) shows the CD spectrum collected from the first eluted peak. It is evident that this fraction contains an enantiomeric excess of RuPDC3 considering the significant features present in the CD spectrum, notably the peaks at 255 nm, 270 m and 300 nm, and 460 nm corresponding to LC, LC, MC, and MLCT respectively. The corresponding absorption spectra measured simultaneously is illustrated in Fig. 6.3 (B) confirming appearance of chiral features is indeed a result of an enantiomeric excess.

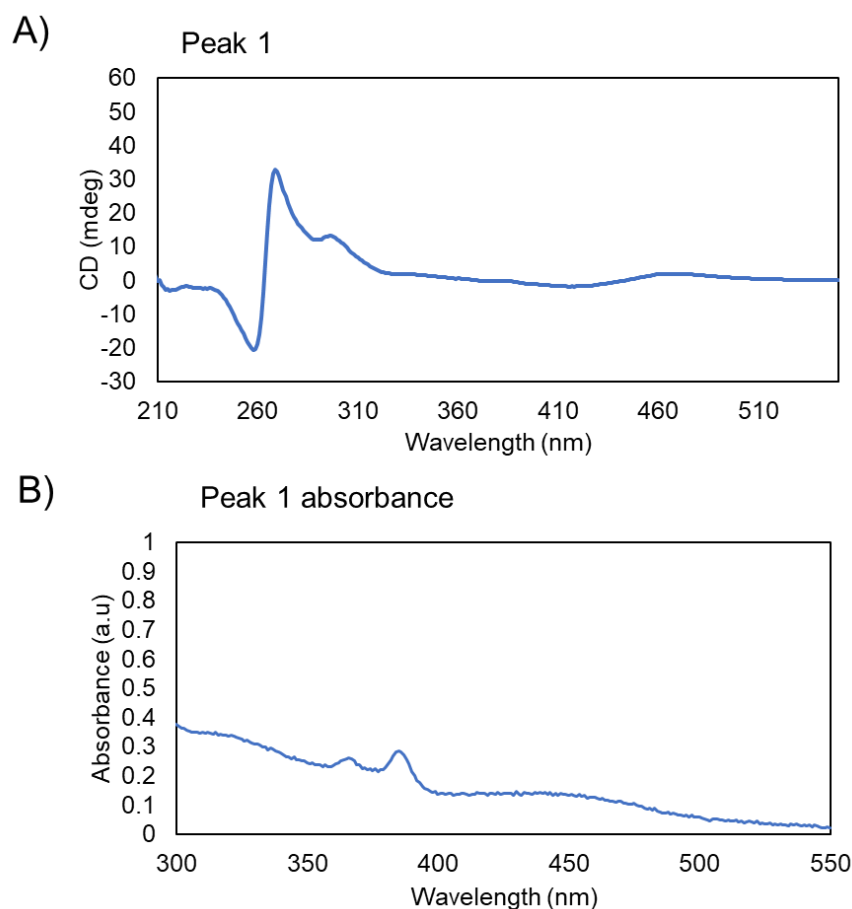


Figure 6.3 (A) CD spectra of fraction 1 of RuPDC3 in MeCN. (B) Absorption spectra of fraction 1 in in MeCN

A second fraction was collected from the tail of second HPLC peak and the respective CD spectrum is shown in Fig. 6.4 (A). Similarly, this fraction contains an enantiomeric excess of RuPDC3. Further, it is clear that this fraction contains a second enantiomer when analysing the ellipticity of the features in the CD spectra. This spectrum contains the same features at 255 nm, 270 m and 300 nm, and 460 nm as before, however the ellipticity of these features are opposite, further indicating enantiomer separation.

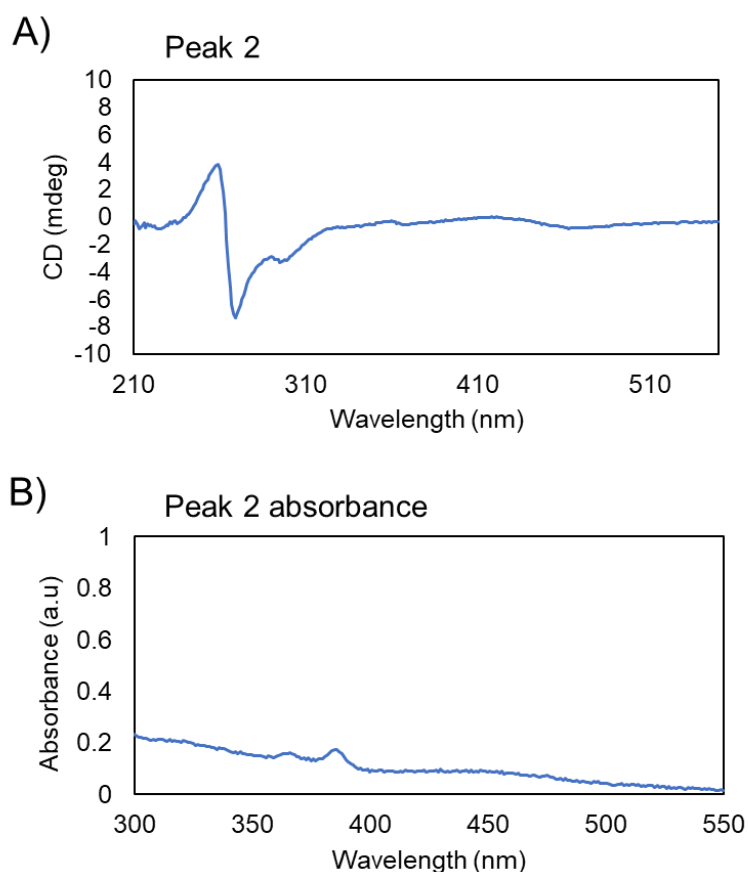


Figure 6.4 (A) CD spectra of fraction 1 of RuPDC3 in MeCN. (B) Absorption spectra of fraction 1 in in MeCN

From CD analysis it is apparent the enantiomeric purity was not sufficient to explore their photophysical properties in solution or their biological efficacy through cell studies. Future work will focus on achieving greater resolution. It is possible that chiral columns capable of enduring higher concentration of additives may facilitate better separation, though first flow rate and mobile phase will be modified to explore if sufficient resolution is possible. The use of chiral columns with alternative stationary phases could also provide the basis for improved resolution. Once pure enantiomers can be obtained, future work will include their biological profiling including cellular uptake and toxicity studies.

Appendix A

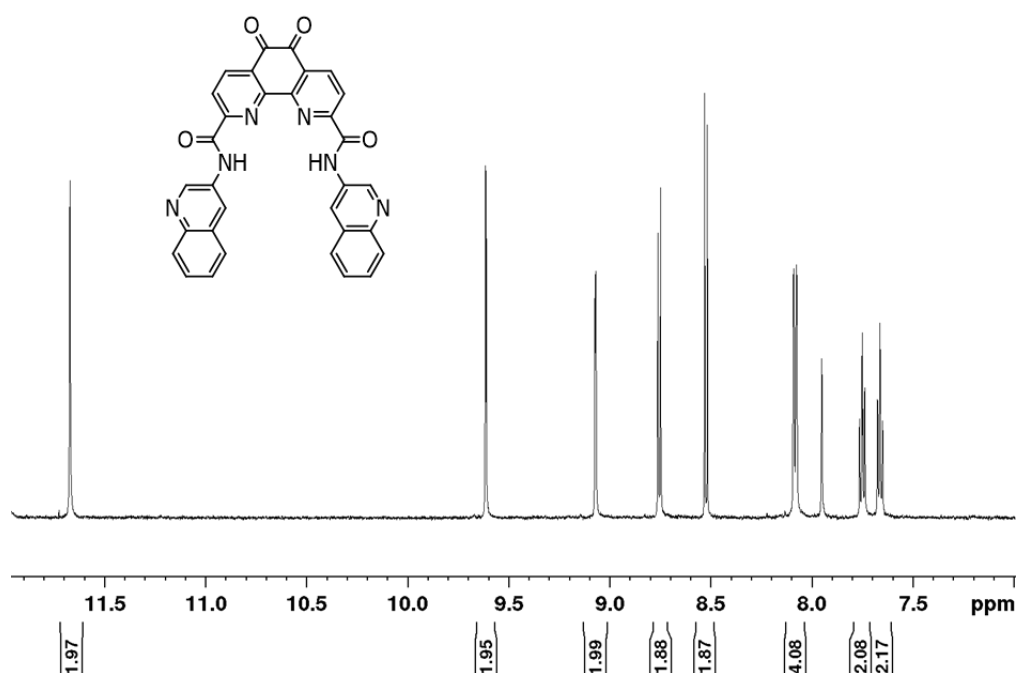


Figure A1: ¹H NMR of 2 in DMSO-d₆.

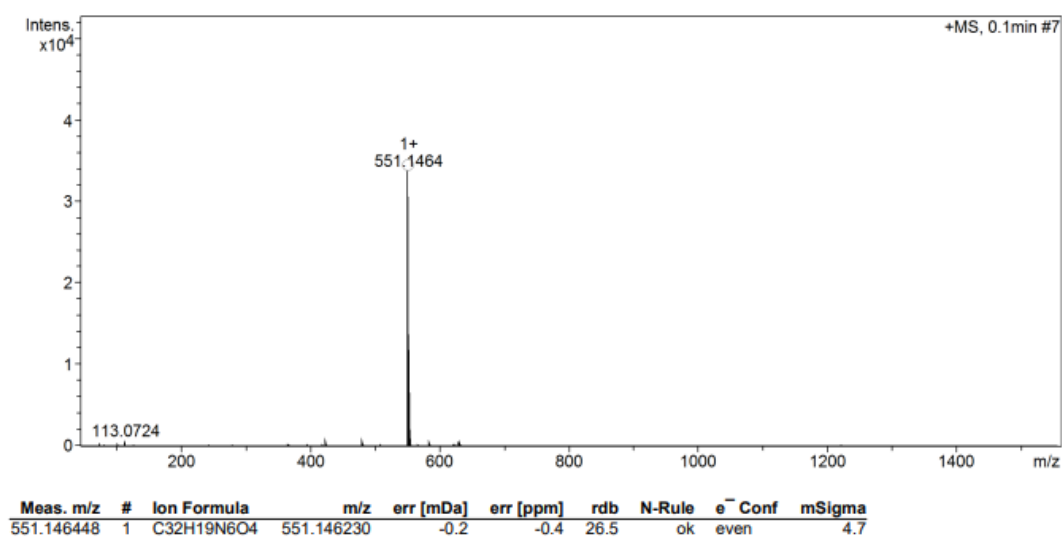


Figure A2: Mass spec of (2)

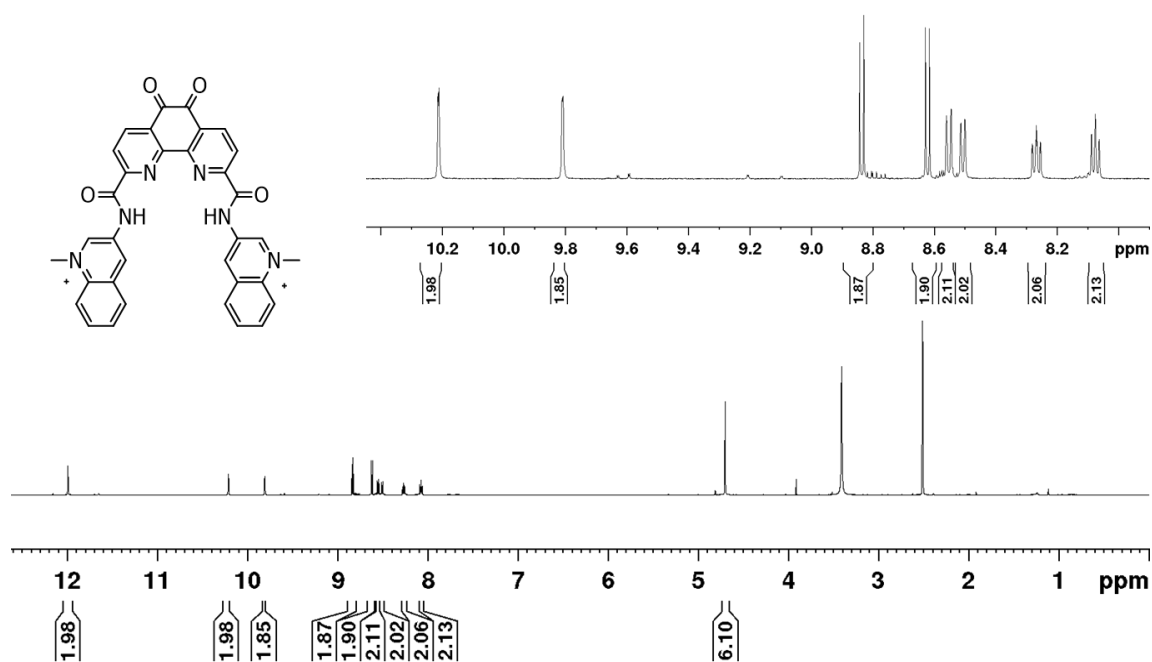


Figure A3: ^1H NMR (600 MHz) of in $\text{DMSO}-d_6$. Inset shows the aromatic region.

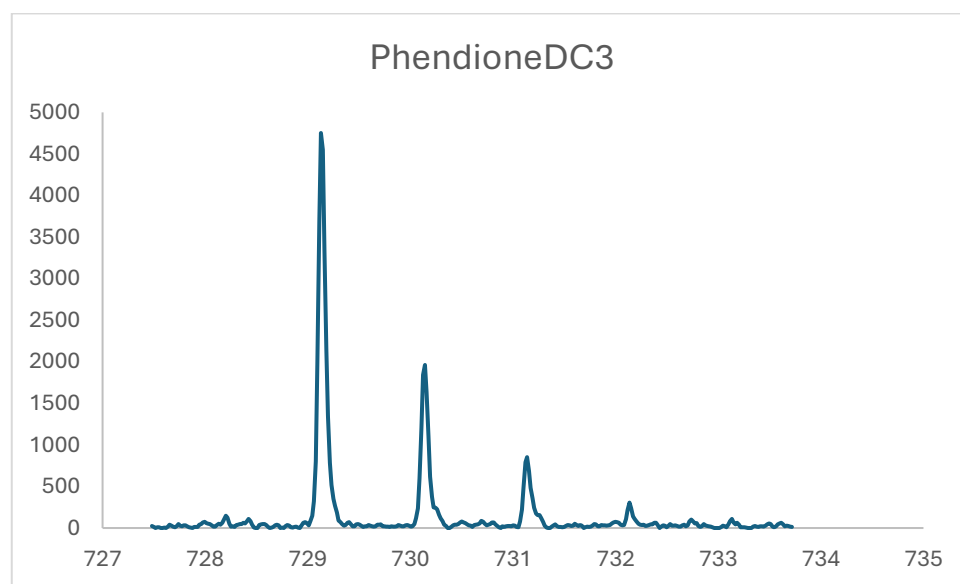


Figure A4. Mass spec of phendioneDC3.

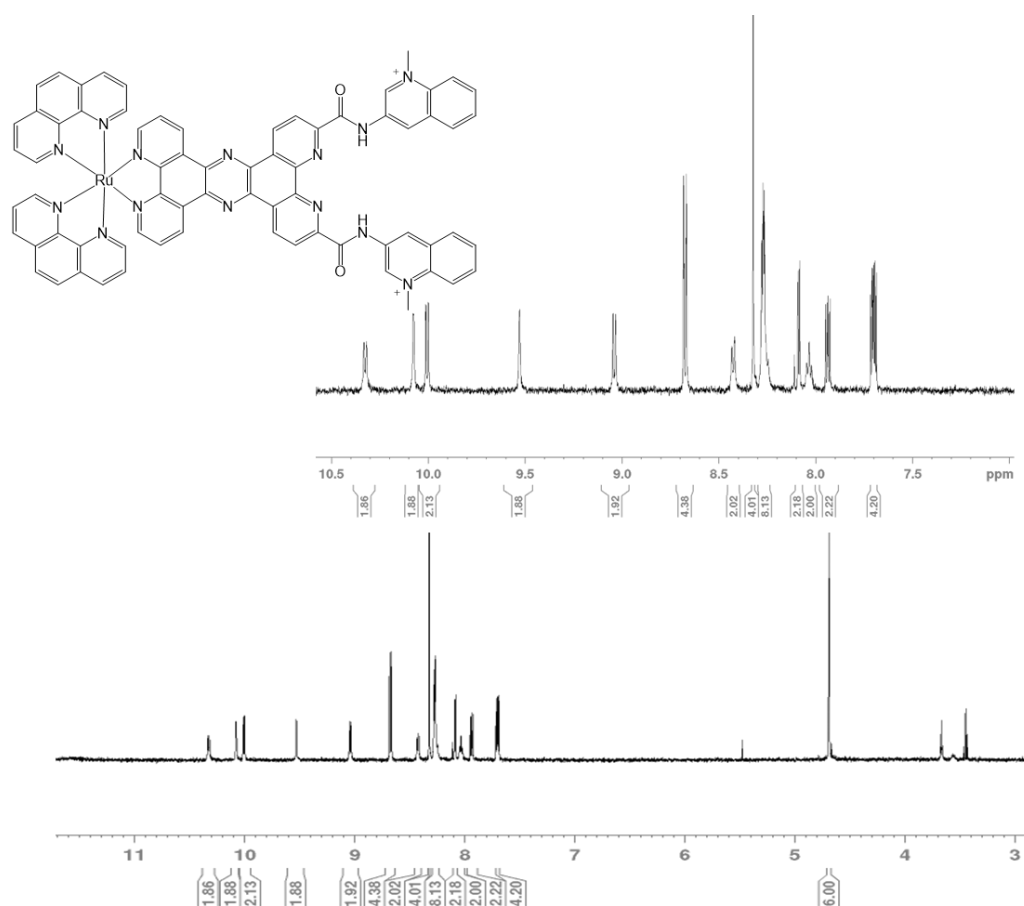


Figure A5: ^1H NMR of RuPDC3 in CD_3CN

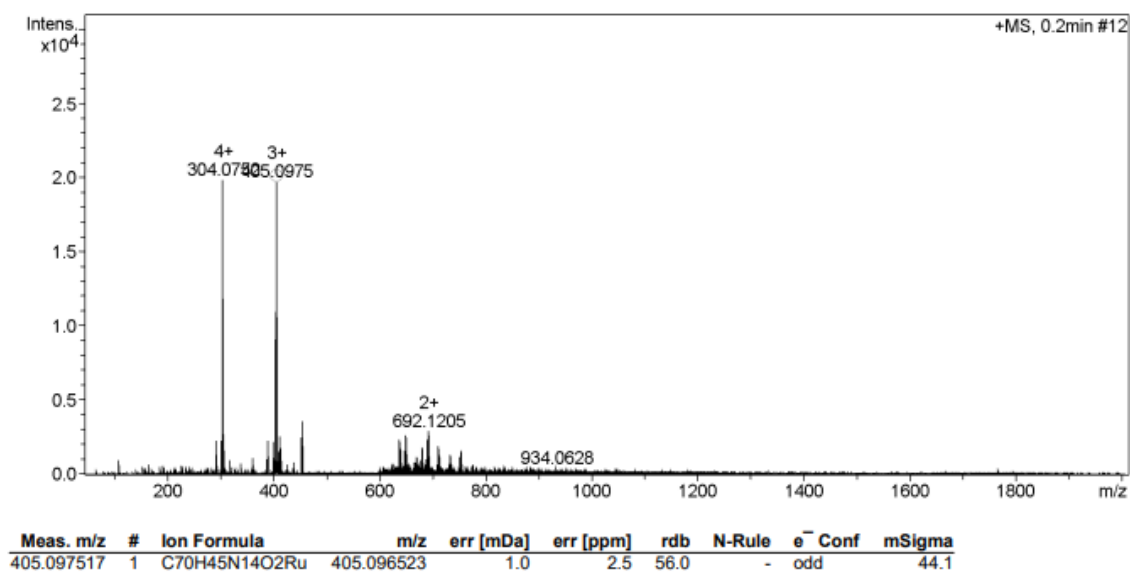


Figure A6: Mass spec of Ru-PDC3

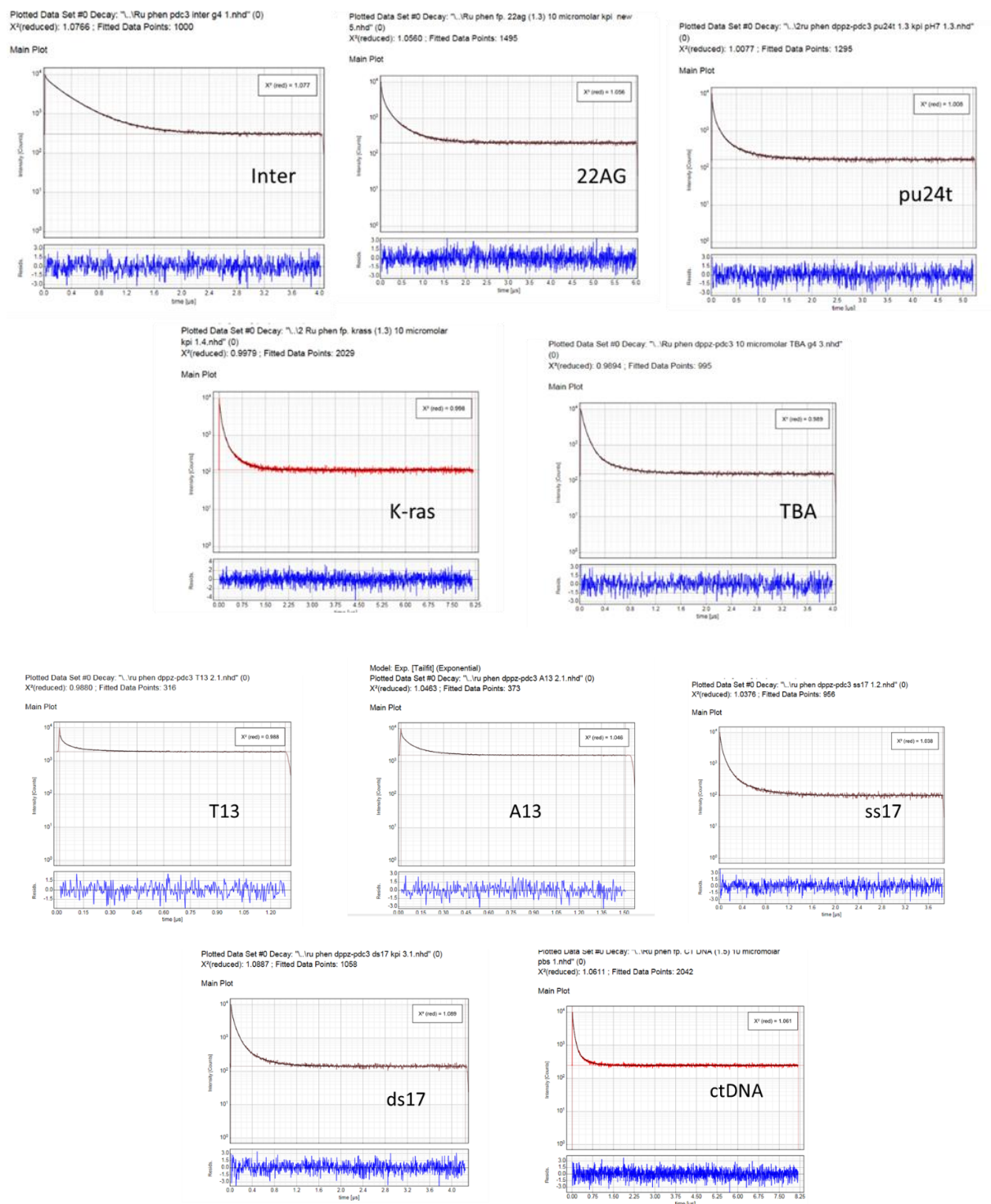


Figure A7: Lifetime decays of Ru-PDC (10 μ M) with single stranded and double stranded DNA (50 μ M)

Circular Dichroism

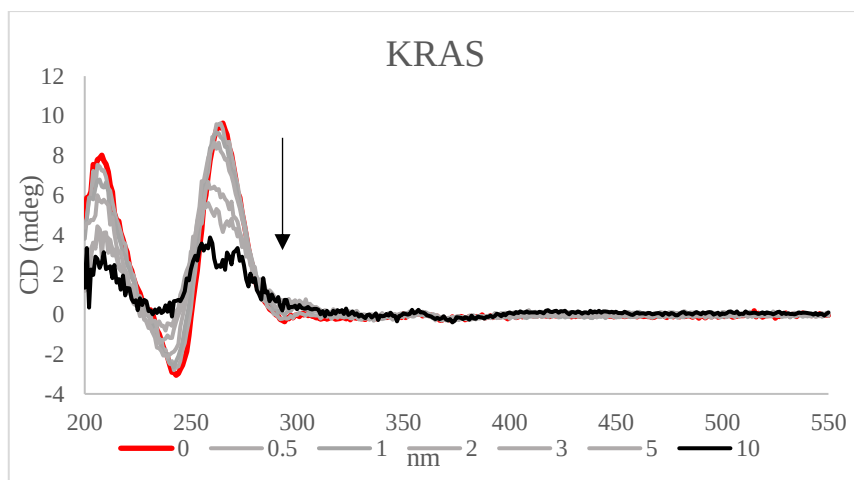


Figure A8: Structural changes to a circular dichroism spectra of KRAS (5 μM) upon addition of Ru-PDC3 (0-50 μM) in Kpi buffer. The arrow indicates the decrease in ellipticity upon titration with Ru-PDC3.

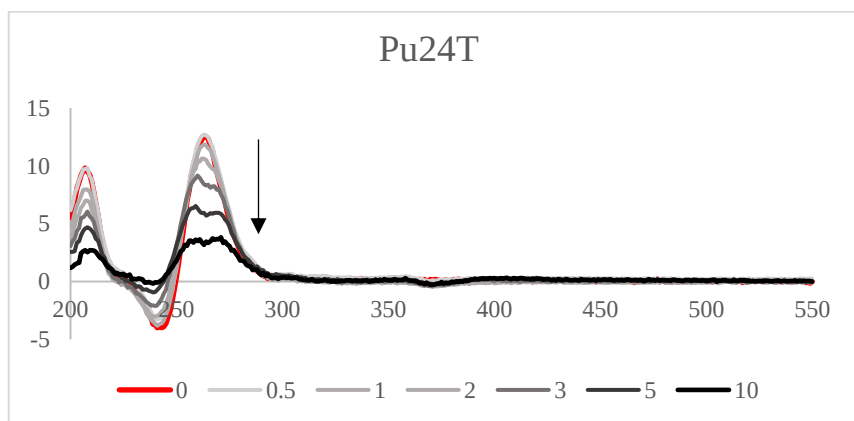


Figure A9: Structural changes to circular dichroism spectra of Pu24T (5 μM) upon addition of Ru-PDC3 (0-50 μM) in Kpi buffer. The arrow indicates the decrease in ellipticity upon titration with Ru-PDC3.

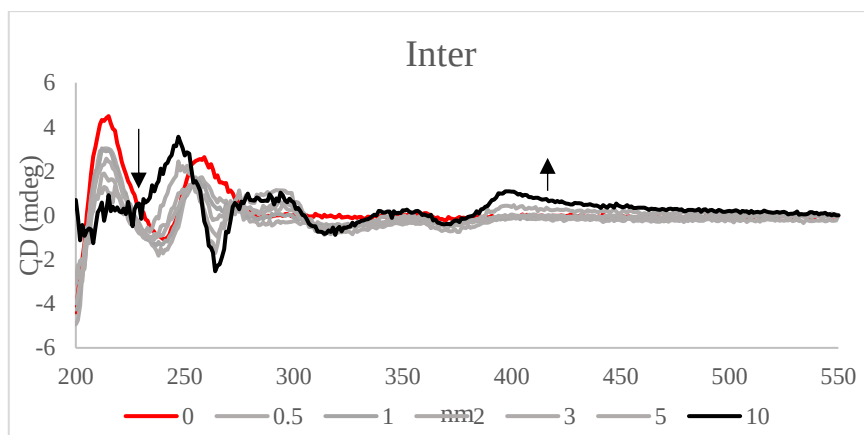


Figure A10: Structural changes to circular dichroism spectra of inter (5 μM) upon addition of Ru-PDC3 (0-50 μM) in Kpi buffer. The arrows indicate the decrease in ellipticity upon titration with Ru-PDC3 and the emergence of a ICD.

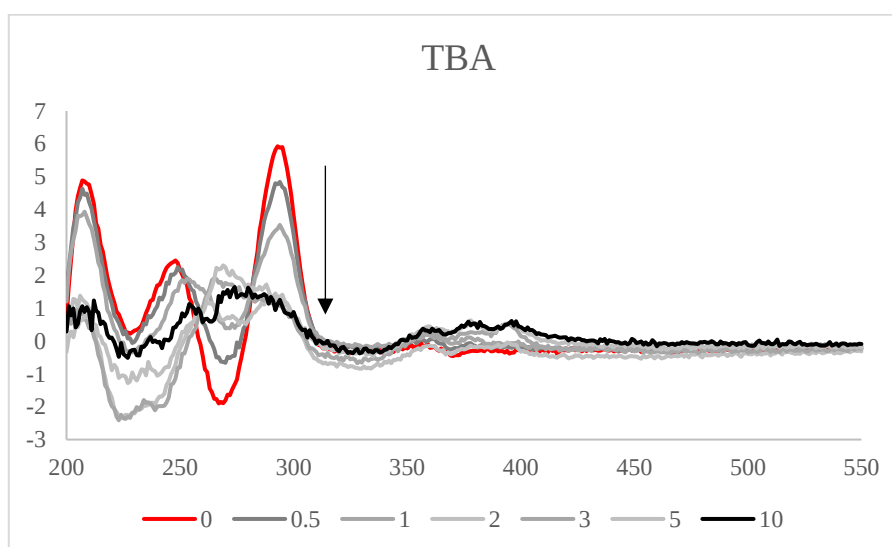


Figure A11: Structural changes to circular dichroism spectra of TBA (5 μM) upon addition of Ru-PDC3 (0-50 μM) in Kpi buffer. The arrow indicates the decrease in ellipticity upon titration with Ru-PDC3.

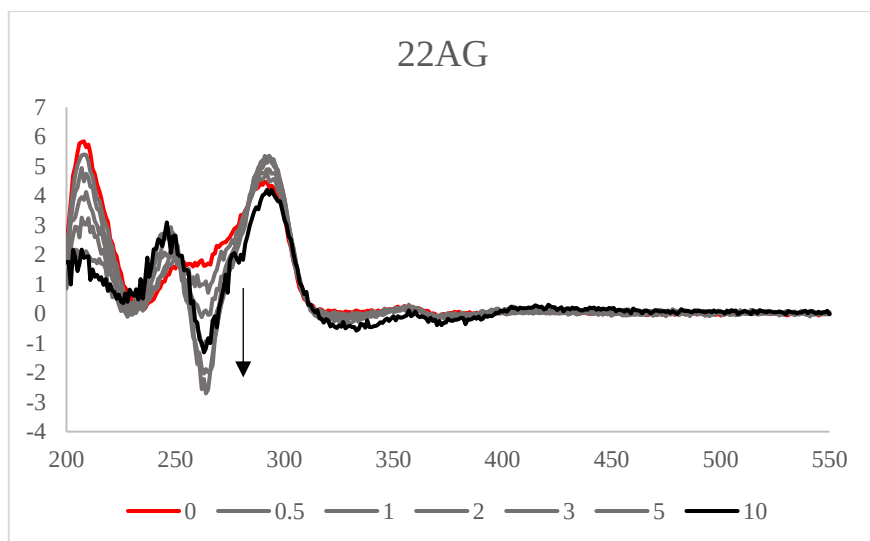


Figure A12: Structural changes to circular dichroism spectra of 22AG (5 μM) upon addition of Ru-PDC3 (0-50 μM) in kpi buffer. The arrow indicates the decrease in ellipticity upon titration with Ru-PDC3.

Table A1: Variation CD signal for each quadruplex and the wavelength at which observations are made.

	structure	(nm)	0.5	1	2	3	5	10
INTER	Intermolecular	210	-31	-34	-42	-61	-72	-95
Pu24T	Parallel	265	1	-7	-18	-32	-53	-73
KRAS	Parallel	265	-3	-8	-16	-37	-57	-71
TBA	Anti-parallel	295	-21	-43	-76	-82	-79	-82
22AG	Mixed hybrid	295	7	12	17	23	19	-5

Raman Studies

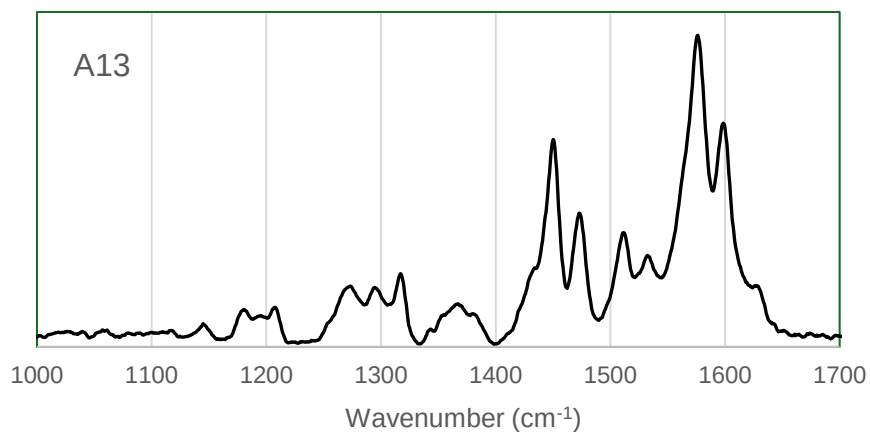


Figure A13: Ru-PDC3 (100 μ M) bound to A13 (500 μ M) in KPi buffer pH 7 (λ =473 nm)

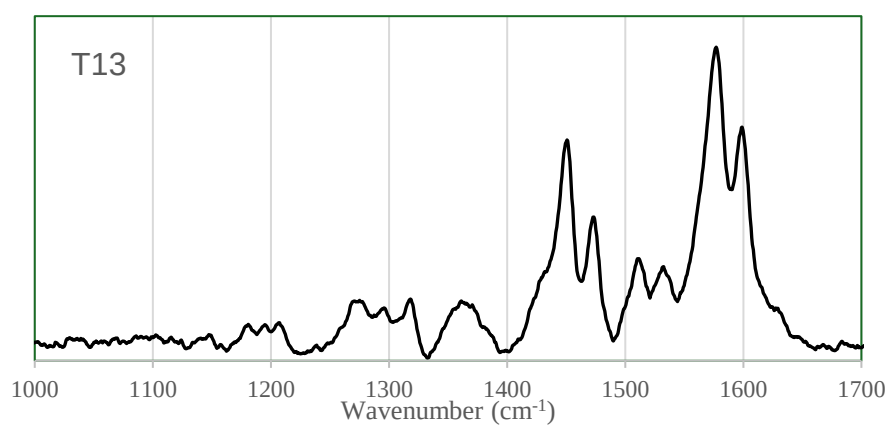


Figure A14: Ru-PDC3 (100 μM) bound to T13 (500 μM) in KPi buffer pH 7 ($\lambda=473$ nm)

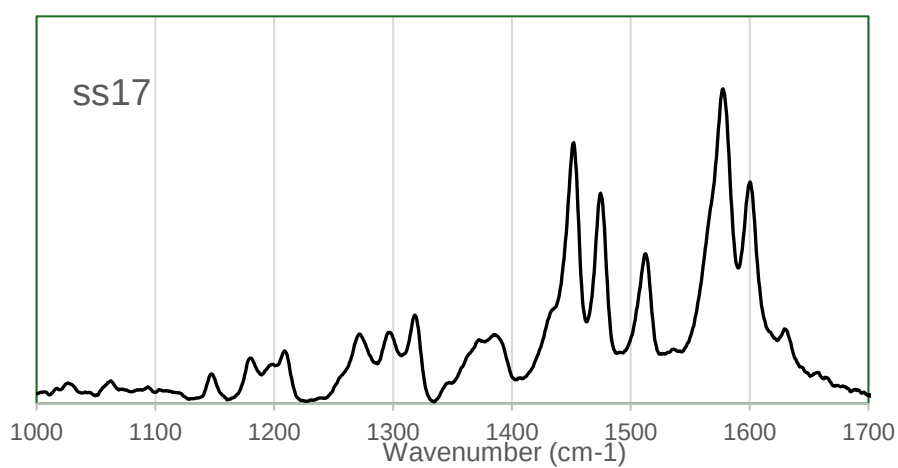


Figure A15: Ru-PDC3 (100 μM) bound to ss17 (500 μM) in KPi buffer pH 7 ($\lambda=473$ nm)

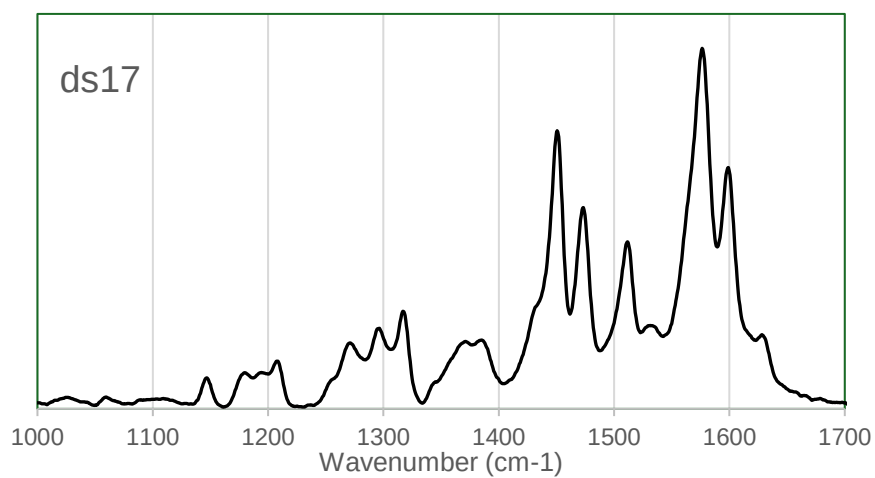


Figure A16: Ru-PDC3 (100 μM) bound to ds17 (500 μM) in KPi buffer pH 7 ($\lambda=473$ nm)

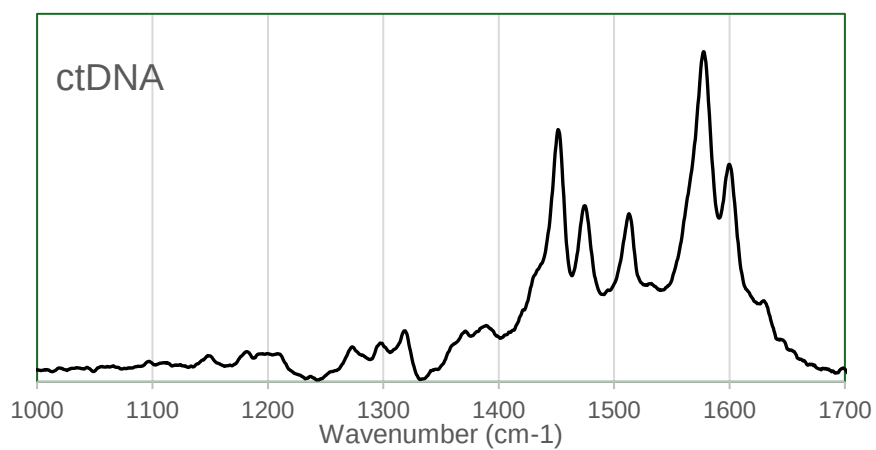


Figure A17: Ru-PDC3 (100 μM) bound to ctDNA (500 μM) in KPi buffer pH 7 ($\lambda=473\text{ nm}$)

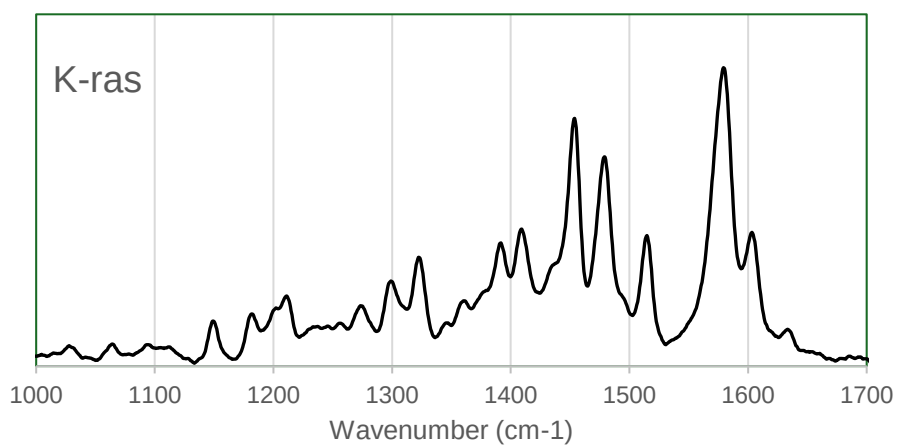


Figure A18: Resonance Raman spectroscopy Ru-PDC3 (100 μM) bound to KRAS 300 μM in KPi buffer pH 7 ($\lambda=473\text{ nm}$)

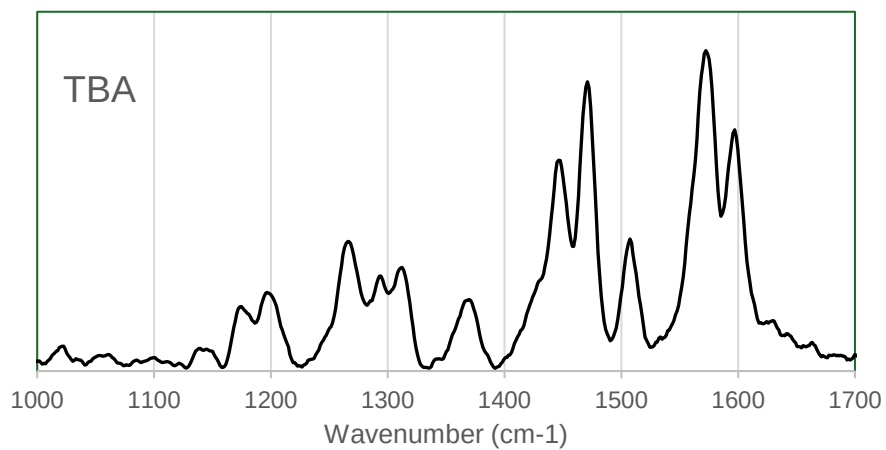


Figure A19: Ru-PDC3 (100 μM) bound to TBA (300 μM) in KPi buffer pH 7 ($\lambda=473$ nm)

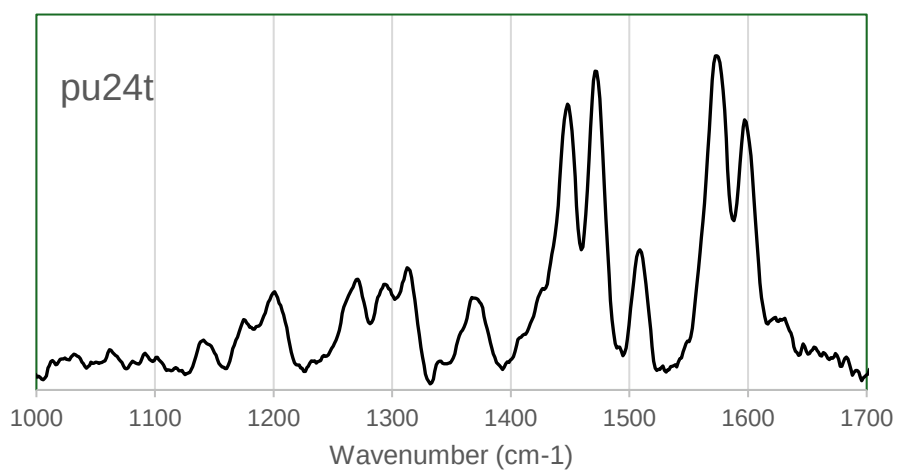


Figure A20: Ru-PDC3 (100 μM) bound to pu24T (300 μM) in KPi buffer pH 7 ($\lambda=473$ nm)

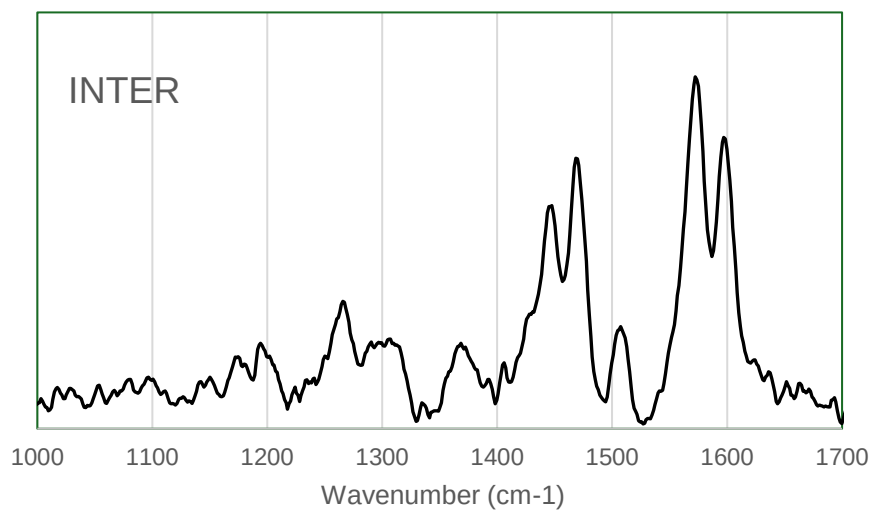


Figure A21: Ru-PDC3 (100 μM) bound to INTER (300 μM) in KPi buffer pH 7 ($\lambda=473$ nm)

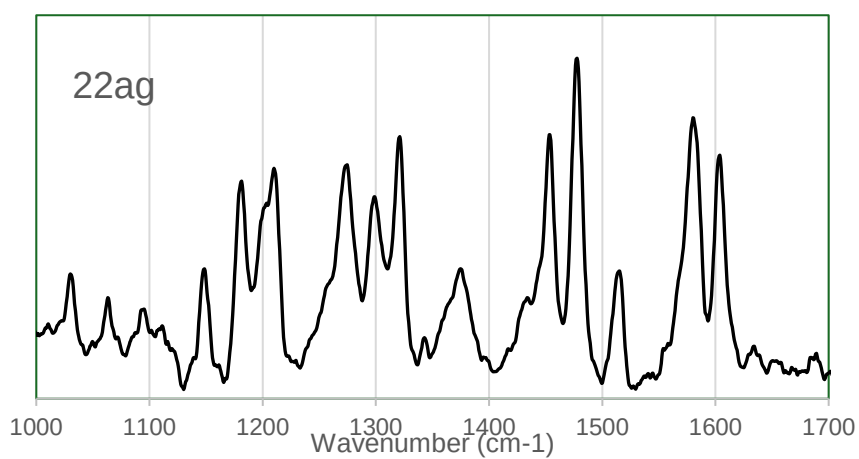


Figure A22: Ru-PDC3 (100 μ M) bound to 22AG (300 μ M) in KPi buffer pH 7 (λ =473 nm)

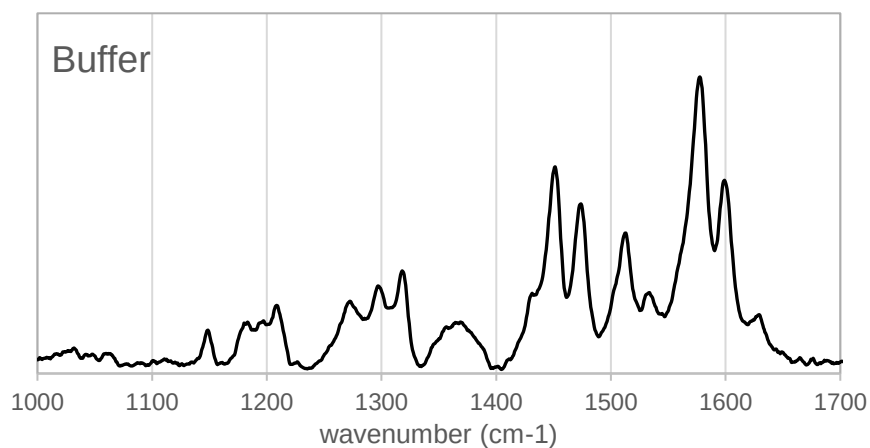


Figure A23: Ru-PDC3 (100 μ M) KPi buffer pH 7 (λ =473 nm)

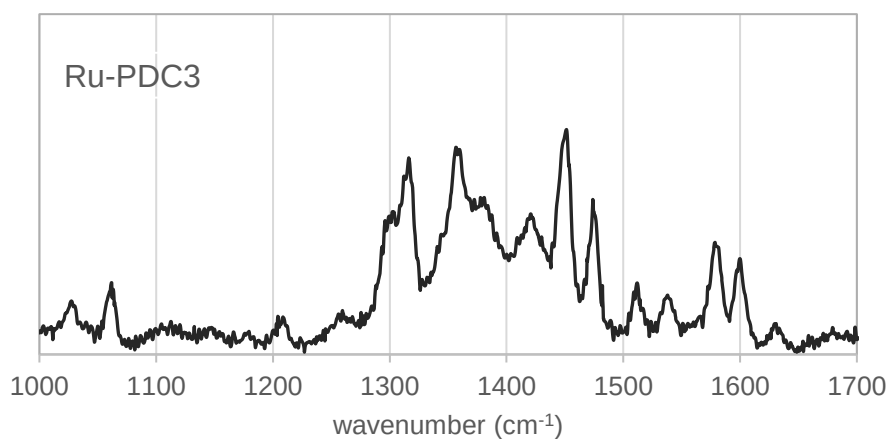


Figure A24: Ru-PDC3(100 μ M) bound to A13 μ 500 M in KPi buffer pH 7 (λ =785 nm)

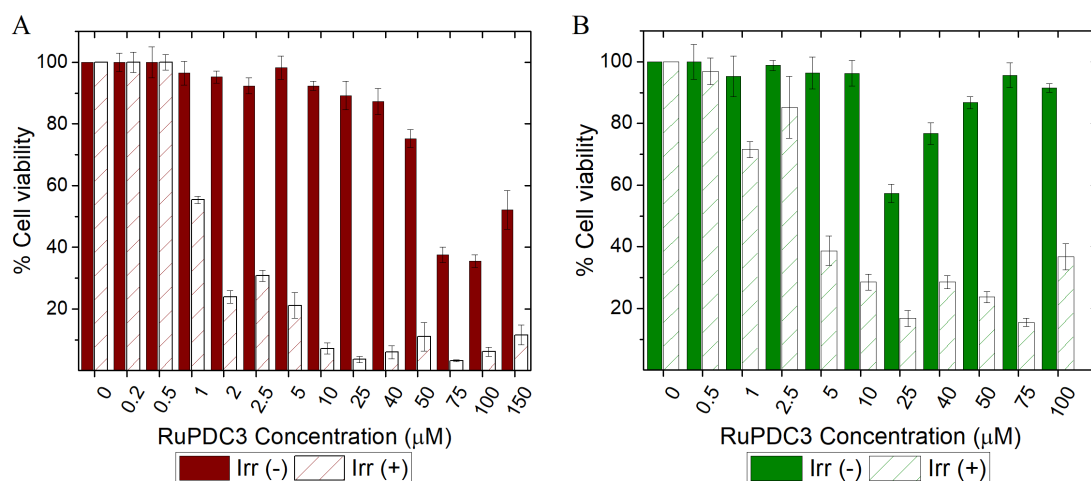


Figure A25. Dark cytotoxicity, Irr (-), and phototoxicity, Irr (+), of RuPDC3 toward adherent A) HeLa cells and B) MCF7 cells. Live cells were treated with the complex over a range of concentrations to determine IC₅₀ in the dark and upon irradiation at 470 nm (total dosage 2.96 ± 0.02 J/cm²). Viability was determined using the MTT assay (n=3) with absorbance measurements performed at 570 nm and 600 nm (corrected for background subtraction). Data were expressed as averaged percentages compared to non-treated cells.

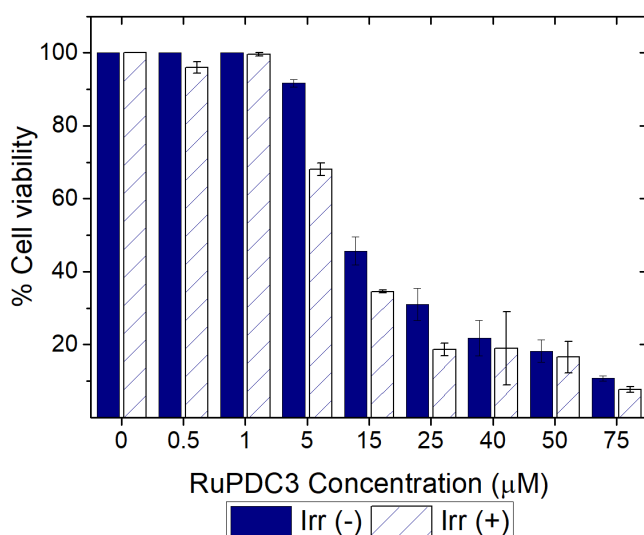


Figure A26 Dark cytotoxicity, Irr (-), and phototoxicity, Irr (+), of RuPDC3 toward three-dimensional HeLa spheroids. HeLa spheroids were treated with the complex over a range of concentrations for 24 h to determine IC_{50} with treatment in the absence of light and upon irradiation at 470 nm (total dosage $5.26 \pm 0.03 \text{ J/cm}^2$). Viability was determined using the CellTiterGlow spheroid Assay (n=3). Data were expressed as averaged percentages compared to non-treated spheroids.

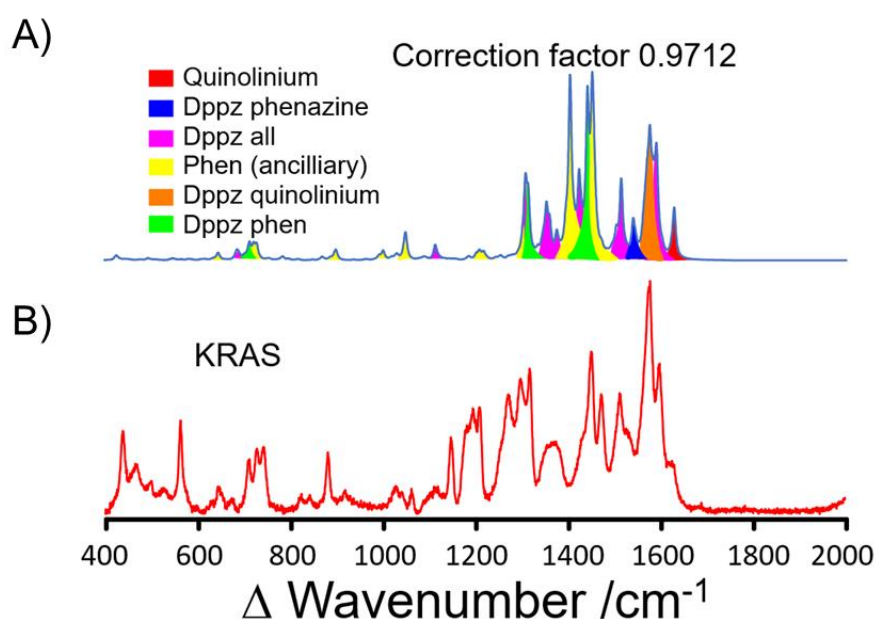


Figure A27. (A) Predicted Raman spectra of RuPDC3. Shaded regions indicate what part of the molecule give rise to their corresponding features. (B) RuPDC3 bound to KRAS DNA ($\lambda=473$).

Appendix B

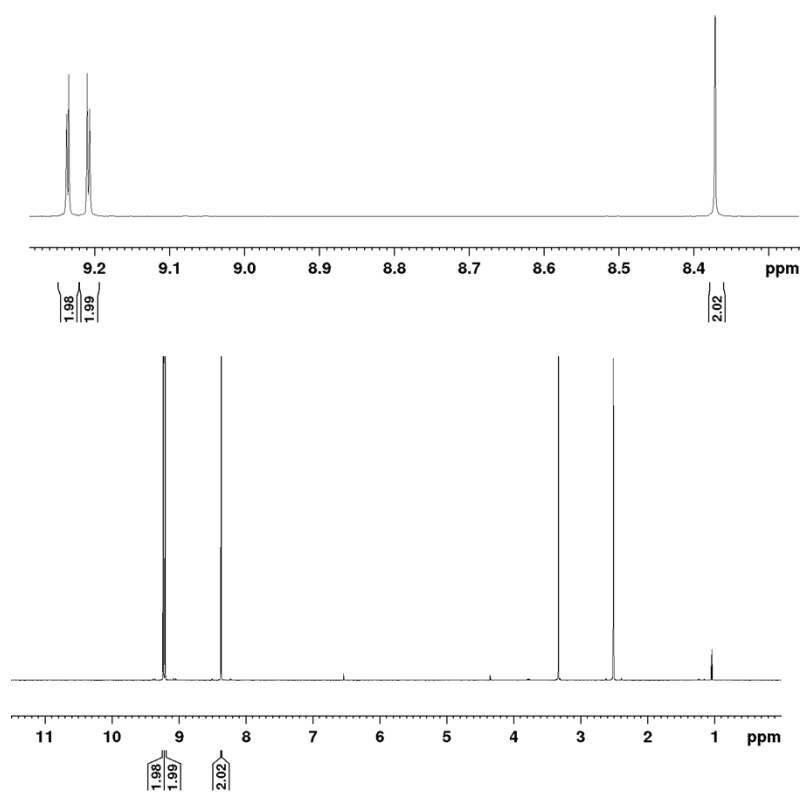


Figure B1. ^1H NMR of TAP (DMSO-d_6)

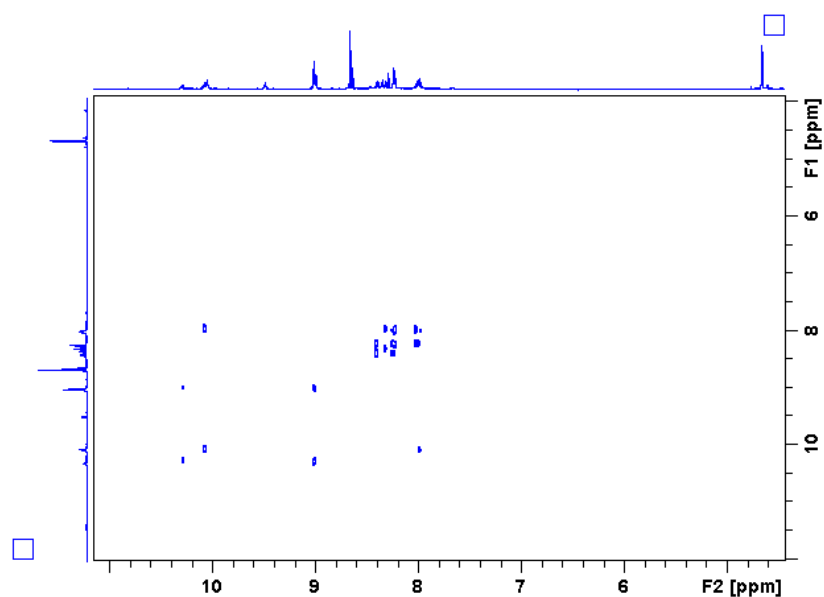


Figure B2. ^1H NMR (600 MHz) COSY spectra of Ru-Tap-PDC3 in CD_3CN .

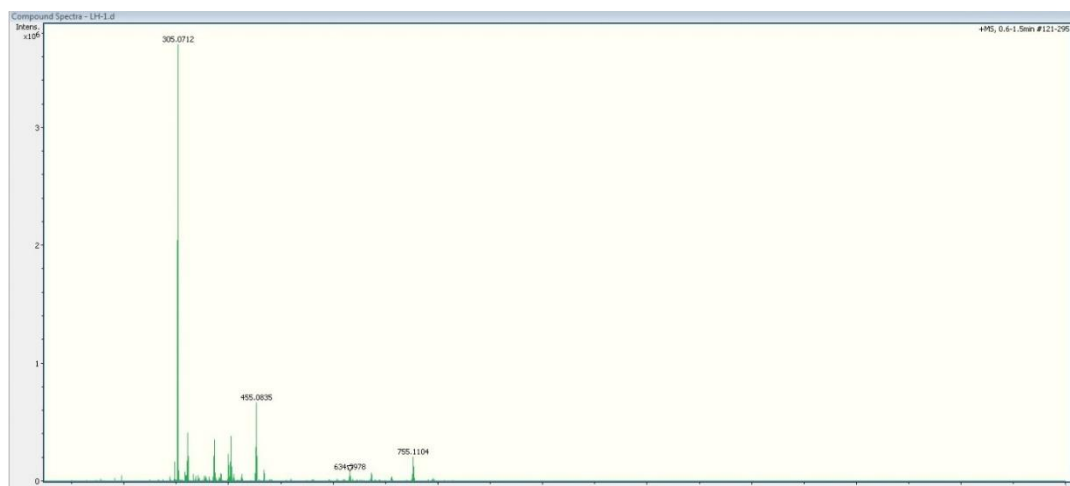


Figure B3. ESI Mass spec of Ru-TAP-PDC3

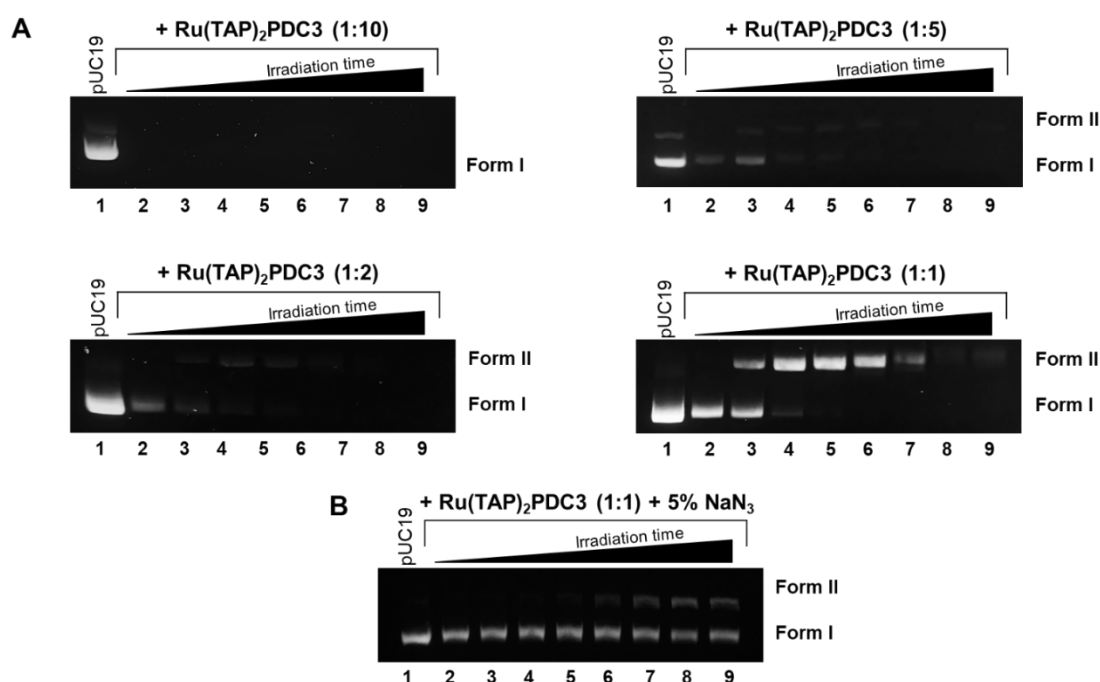


Figure B4. DNA cleavage reactions and ROS scavenger study. Gel electrophoresis of 400 ng of supercoiled pUC19 plasmid exposed to Ru-TAP-PDC3 in a buffer solution of 25 mM NaCl and 80 mM HEPES. **(A)** DNA cleavage reactions with 1:10, 1:5, 1:2 and 1:1 of DNA to Ru-TAP test ratios. **(B)** DNA-PDC3 cleavage reactions in the presence of singlet oxygen scavenger sodium azide (5% NaN_3), with DNA:Ru-TAP-PDC3 at a 1:1 ratio. In both studies labelling is as follows: **1.** pUC19 control. **2.** pUC19+Ru-TAP-PDC3 (in the absence of light), **3 to 9:** pUC19+ Ru-TAP-PDC3 (with irradiation). Irradiated samples were exposed to 470 nm LED irradiation with exposure times from

30 seconds to 30 minutes at a maximum total dose of $7.52 \pm 0.49 \text{ J/cm}^2$. (specifically: **3.** 30 sec, **4.** 1 min, **5.** 2 min, **6.** 5 min, **7.** 10 min, **8.** 20 min, **9.** 30 min).

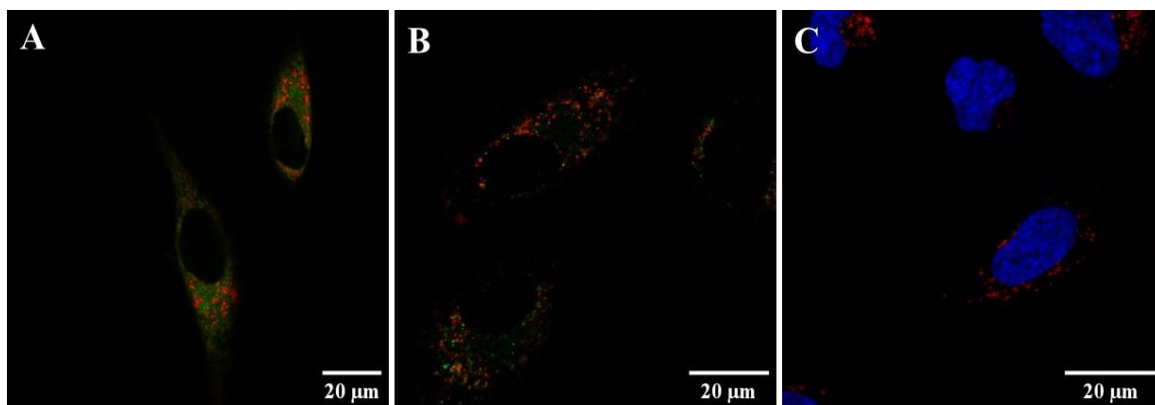


Figure B5. Confocal images of Ru-TAP-PDC3 (50 μM, 24 h) overlay with **(A)** BioTracker 488 ($r = 0.6 \pm 0.11$), **(B)** LysoTracker Green DND-26 ($r = 0.46 \pm 0.08$) and **(C)** Hoechst 33342 ($r = 0.08 \pm 0.01$).

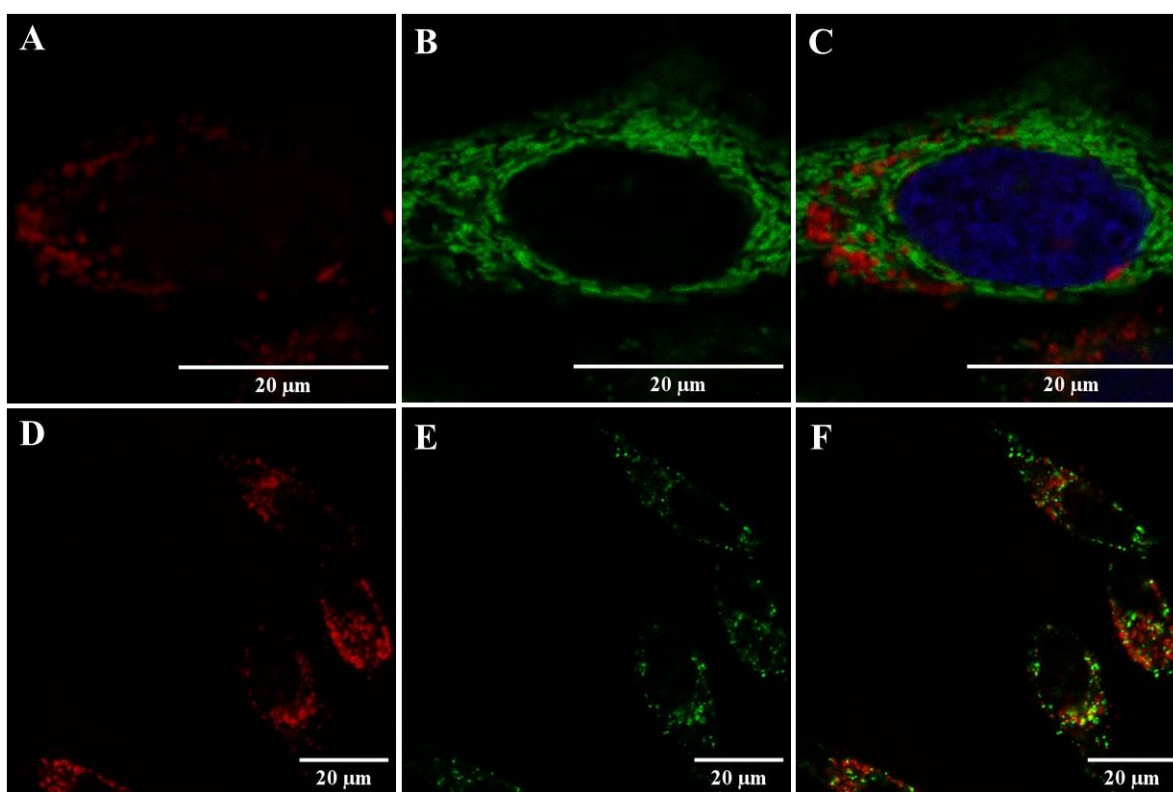


Figure B6. Ru-TAP-PDC3, 50 μM at 48 h incubation with **(A-C)** BioTracker 488 ($r = 0.33 \pm 0.04$) with Hoechst 33342 nuclear staining for contrast and **(D-F)** LysoTracker Green DND-26 ($r = 0.39 \pm 0.06$).

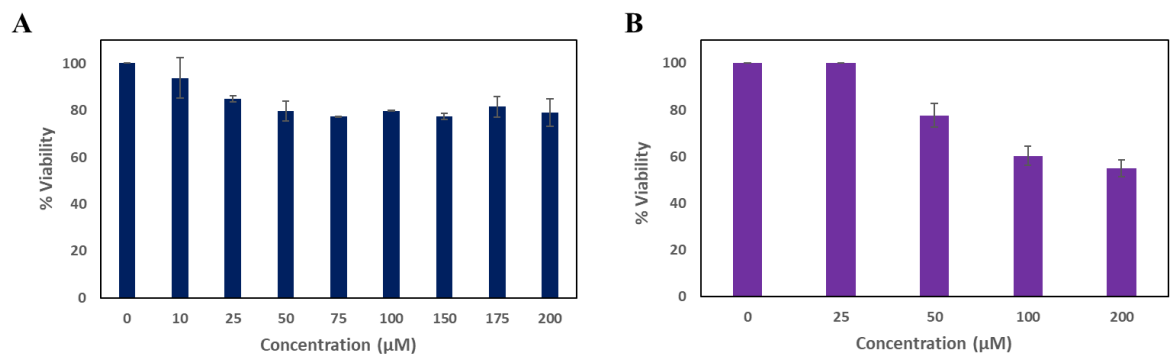


Figure B7. Dark toxicity results of Ru-TAP-PDC3 in **(A)** HeLa and **(B)** HEK-293 cell monolayers at 24 h incubation. A PI value of 52 was determined for HEK-293 cells at 24 h incubation, with a phototoxic IC_{50} of $3.88 \pm 0.14 \mu M$.

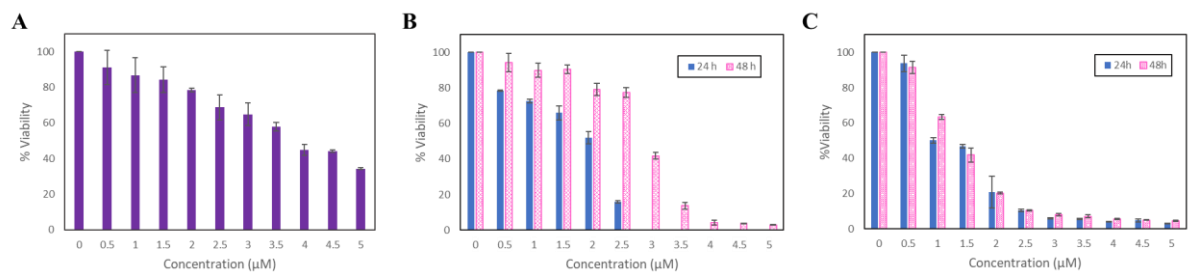


Figure B8. Phototoxicity results of Ru-TAP-PDC3 in **(A)** HEK-293 (24 h incubation, $5 \pm 0.54 J/cm^2$) and HeLa cells at 24 h or 48 h incubation prior to irradiation with a 470 nm LED at a total irradiation dose of **(B)** $5 \pm 0.54 J/cm^2$ or **(C)** $15 \pm 1.6 J/cm^2$.

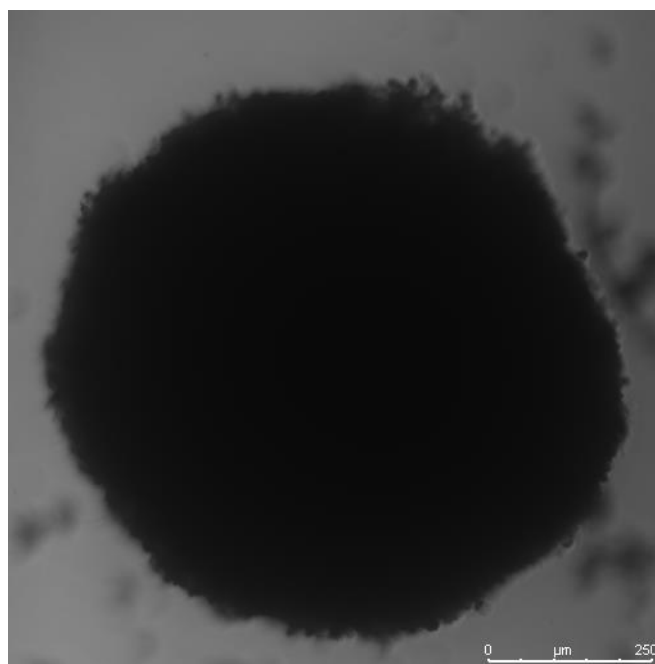


Figure B9. Brightfield image of a HeLa spheroid grown in a round bottom 96 well plate for 96 hours taken using a 10x air objective. This image is indicative of the size of the spheroids prepared for the toxicity studies.



Figure B10. HeLa spheroid (untreated) after 96 hours growth in a u-bottom 96 well plate in DMEM media. The red arrow points to what is thought to be the necrotic centre. This image was obtained using an android camera at 1x zoom and a Nikon (TMS) inverted phase contrast light microscope with a 4x objective.

Appendix C

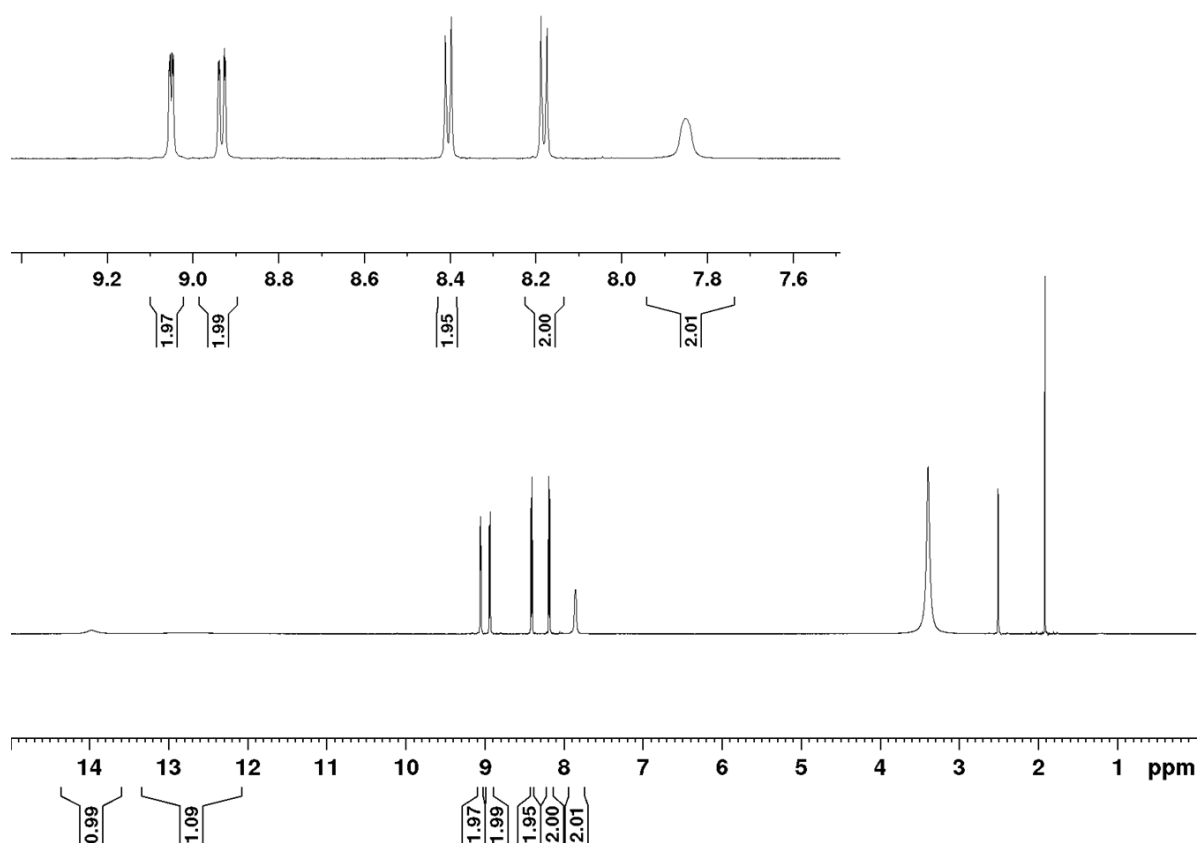


Figure C1. ^1H NMR of PIC-COOH (DMSO-d_6)

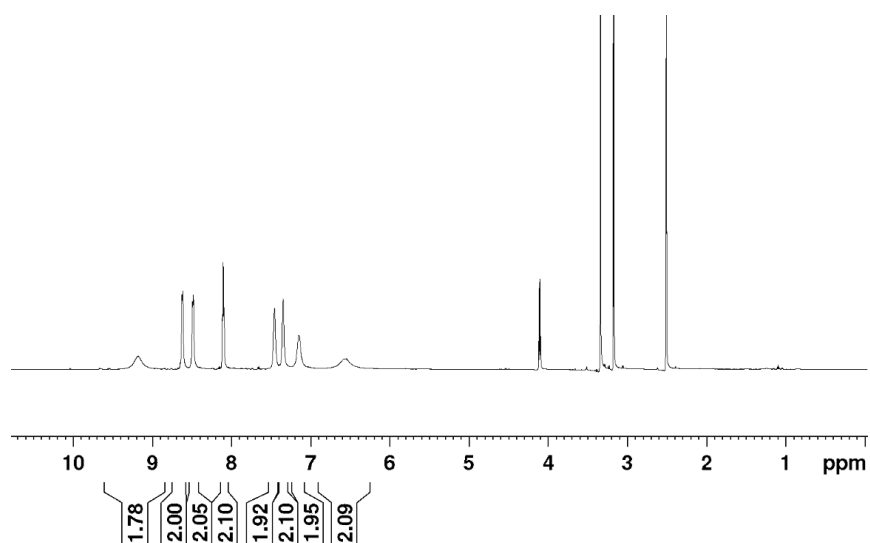


Figure C2. ^1H NMR of $[\text{Ru}(\text{bpy})_2\text{Cl}_2]$

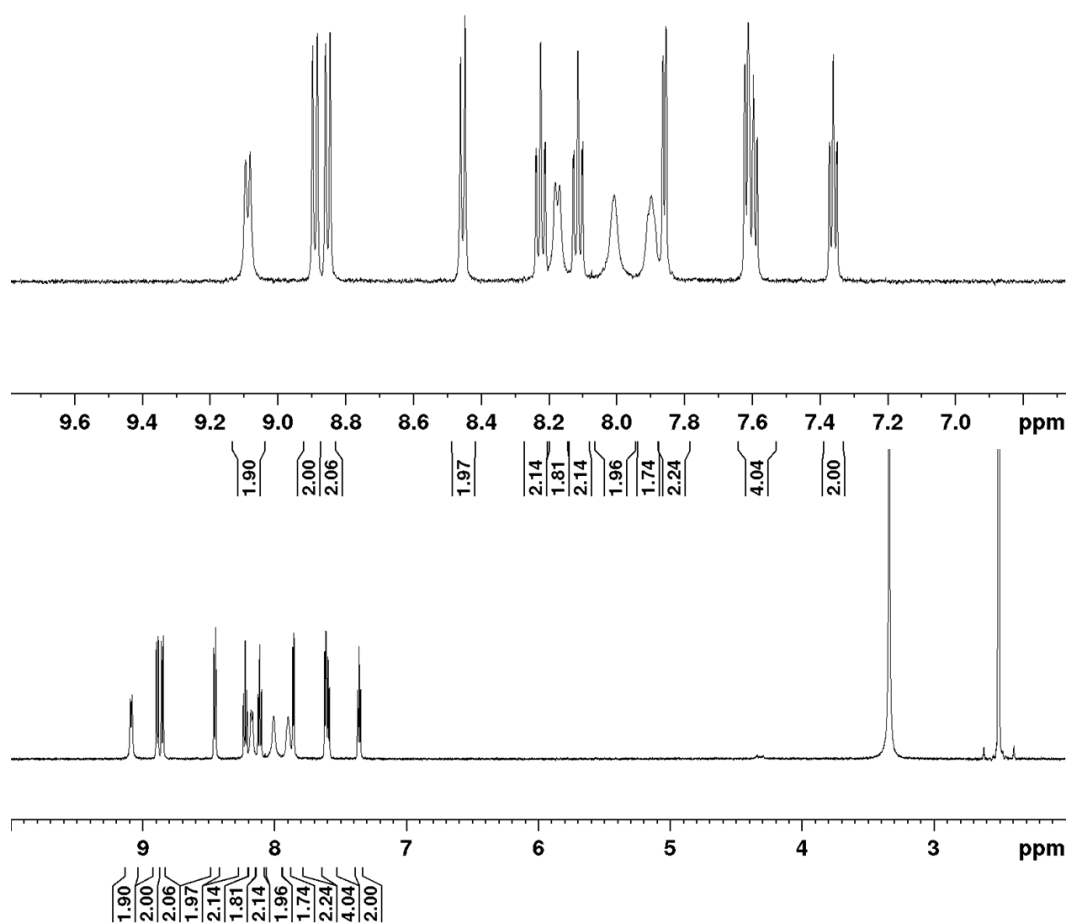


Figure C3. ^1H NMR of $[\text{Ru}(\text{bpy})_2(\text{PIC-COOH})]$ (CD_3CN)

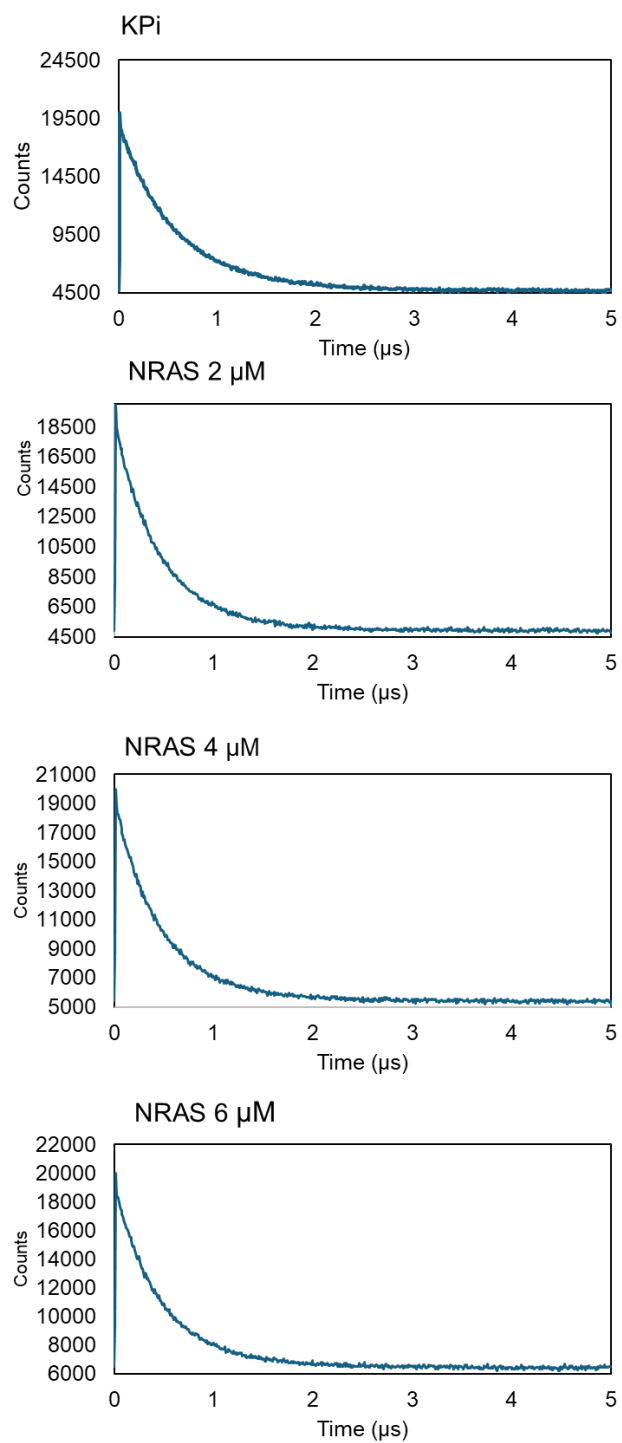


Figure C4. Representative lifetime decays of Ru-RHAU upon association with DNA

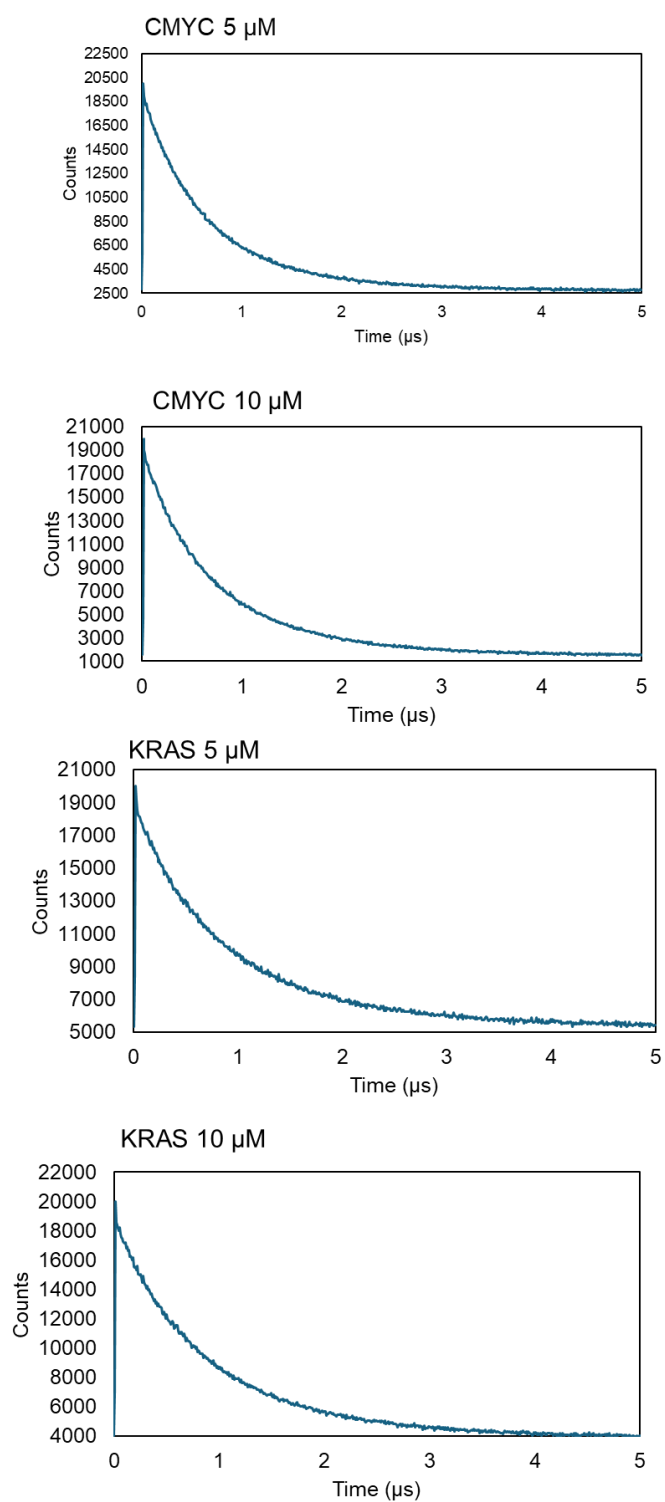


Figure C5. Representative lifetime decays of Ru-RHAU (5 μ M) upon association with DNA.

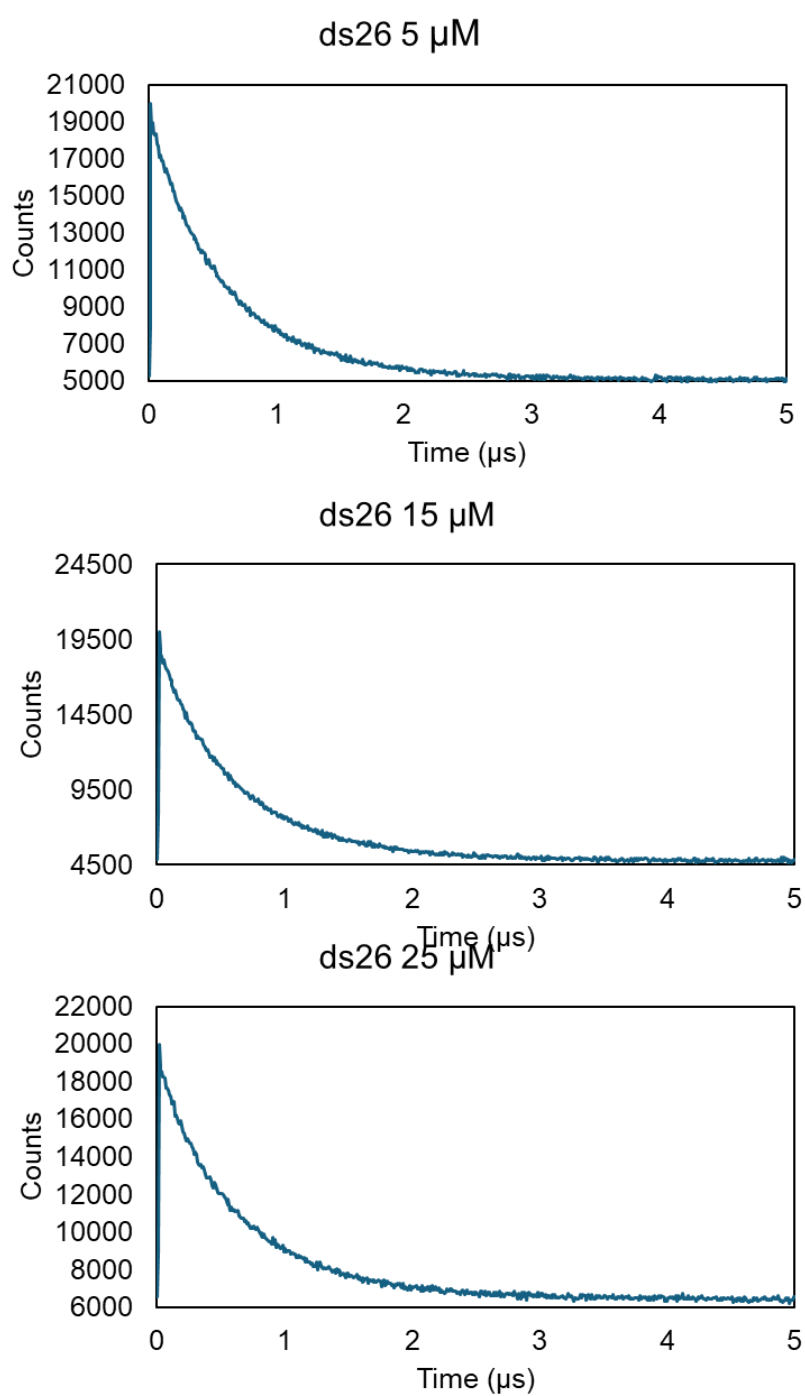


Figure C6. Representative lifetime decays of Ru-RHAU (5 μM) upon association with DNA.

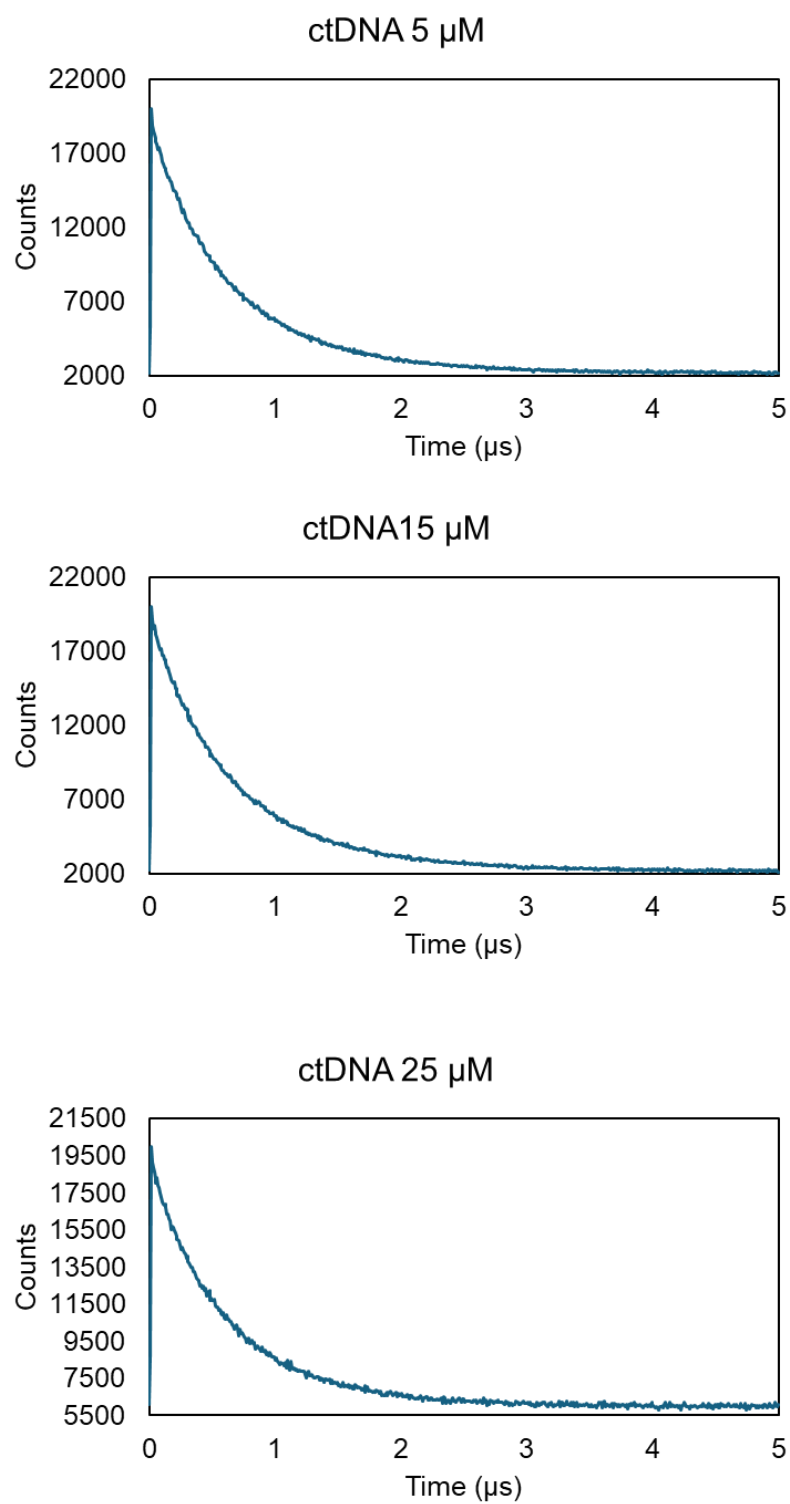


Figure C7. Representative lifetime decays of Ru-RHAU (5 μM) upon association with DNA.

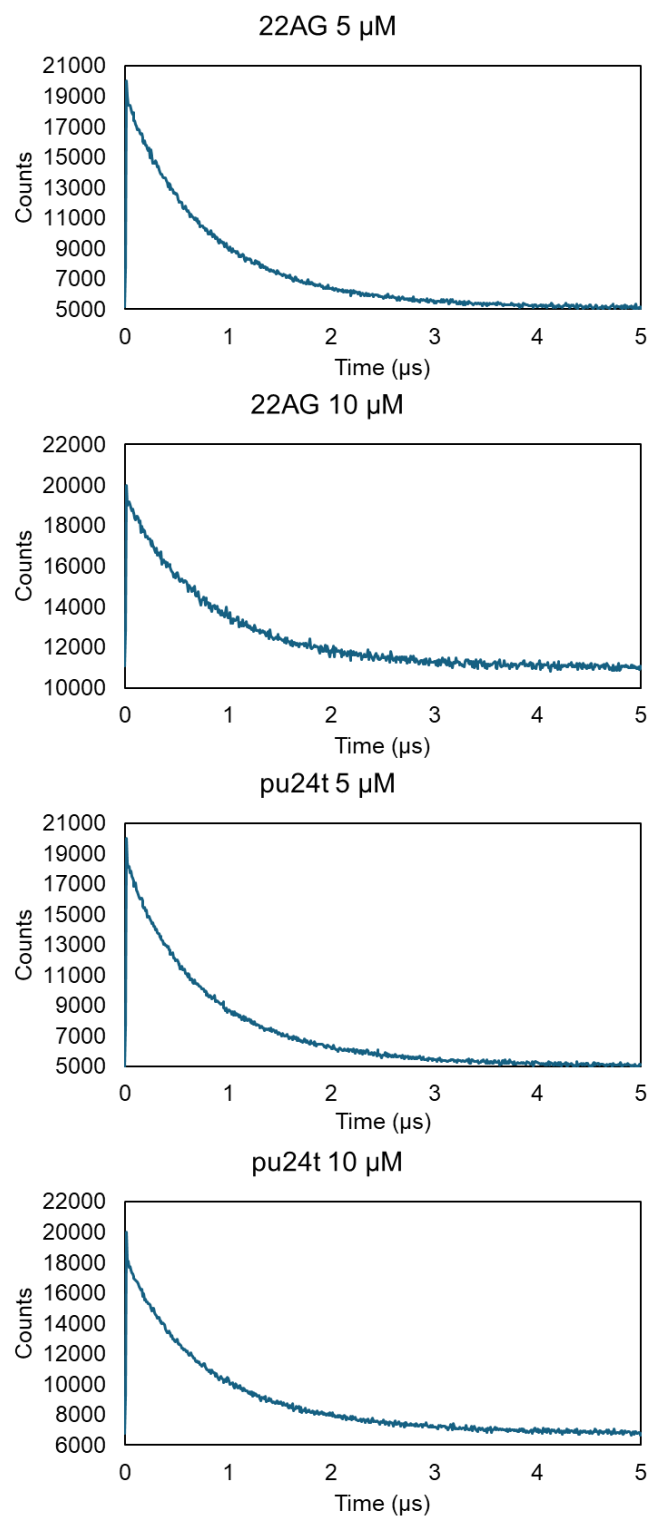


Figure C8. Representative lifetime decays of Ru-RHAU (5 μM) upon association with DNA.

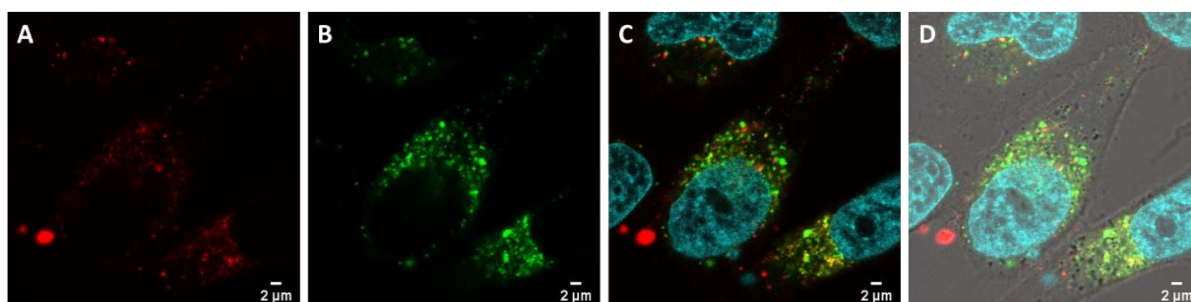


Figure C9. Confocal imaging of **(A)** Ru-RHAU (40 μ M, 6 h), **(B)** LysoTracker Deep Red (100 nM, 45 min) and **(C)** overlay of A and B with Hoechst 33342 nuclear stain (1 μ g/mL, 15 min) and **(D)** phase contrast. 100x oil.

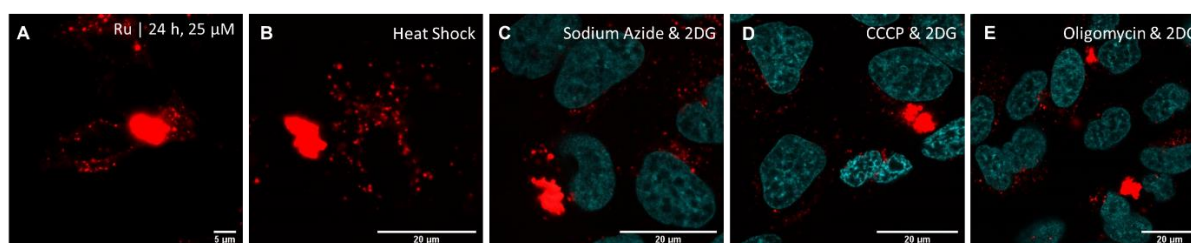


Figure C10. Live HeLa cells with **(A)** Ru-RHAU 25 μ M, 24 h and treated with **(B)** Heat Shock (42 $^{\circ}$ C) or 2DG with **(C)** Sodium Azide, **(D)** CCCP and **(E)** Oligomycin after the 24 h incubation with Ru-RHAU. Hoechst 33342 stains the nucleus of C-E.

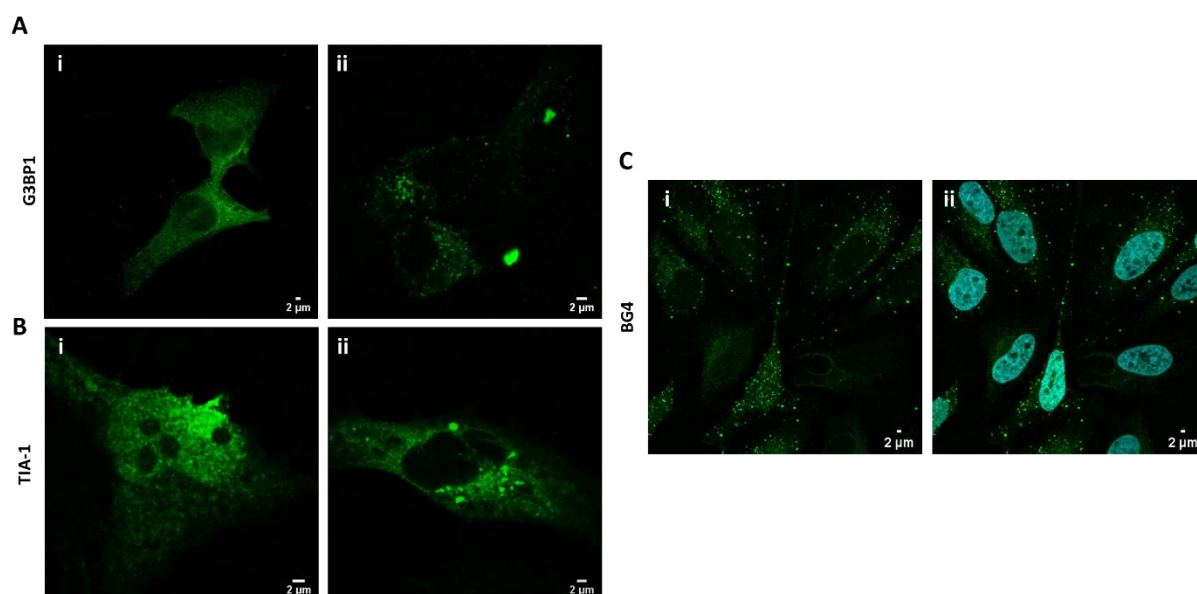


Figure C11. Immunostaining controls in fixed HeLa cells showing (A) G3BP1 and (B) TIA-1 in (i) untreated cells and (ii) cells treated by heat shock (42 °C, 1 h) prior to fixation and (C) untreated control cells with BG4 (with and without Hoechst 33342 nuclear staining).

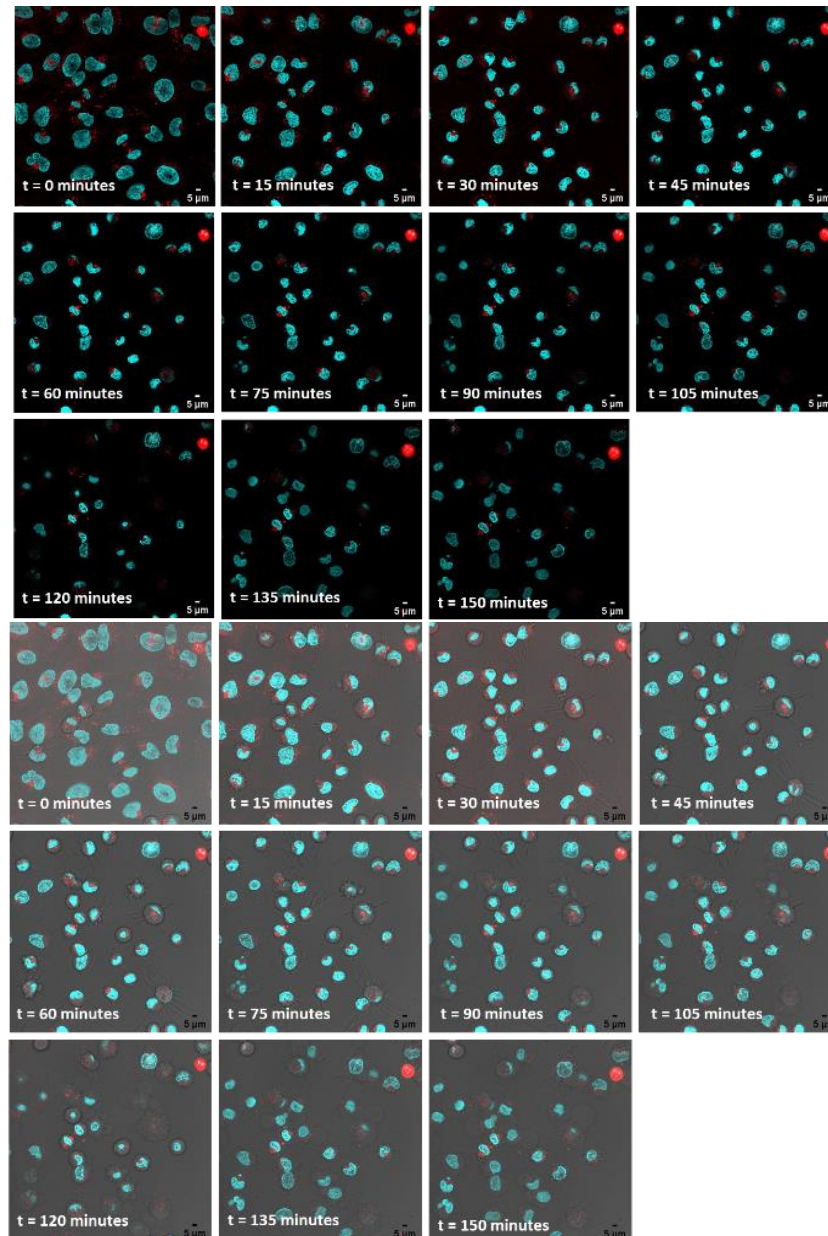


Figure C12. Disassembly over Ru-RHAU (40 μM, 6 h) over time with corresponding phase contrast images in gray, indicating changes in cell morphology over time. Timings are indicated on each image where T = the time since Ru-RHAU has been removed.

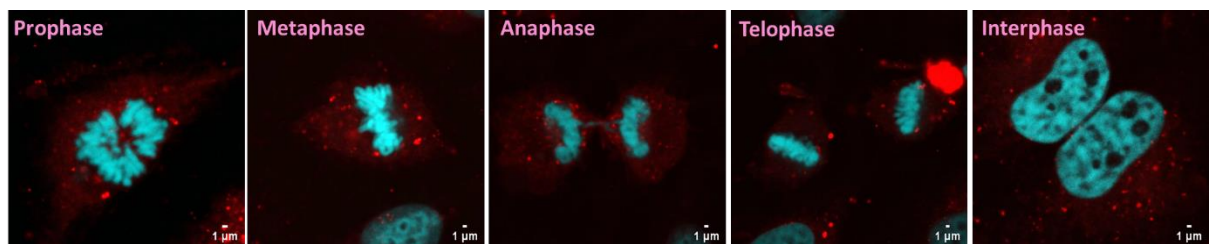


Figure C13. Confocal images of fixed HeLa cells with Ru-RHAU (40 μ M, 6 h) in cells at various cell cycle phases