

Charge-transfer luminophores: theranostic probes of the cellular environment



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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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"Be happy for this moment, this moment is your life" – Omar Khayyam

Research Outputs

Published Manuscripts (+ indicates co-first authorship)

2024 | Curley, R. C.; Arellano-Reyes, R. A.; McPherson, J. N.; McKee, V.; Keyes, T. E. **Enhancing Phototoxicity in BODIPY-perylene Charge-transfer Dyads by Combined Iodination and Mesylation** *Chem. Eur. J.* 2024 (10.1002/chem.202403149)

2024 | Holden, L.⁺; Curley, R. C.⁺; Avella, G.; Long, C.; Keyes, T. E. **Targeting Mitochondrial Guanine Quadruplexes for Photoactivatable Chemotherapy in Normoxic and Hypoxic Environments** *Angew. Chem. Int. Ed.* 2024 (10.1002/anie.202408581)

2023 | Curley, R. C.; Burke, C. S.; Gkika, K. S.; Noorani, S.; Walsh, N.; Keyes, T. E. **Phototoxicity of Tridentate Ru(II) Polypyridyl Complex with Expanded Bite Angle Toward Mammalian Cells and Multicellular Tumour Spheroids.** *Inorg. Chem.* 2023, 62, 32, 13089–13102 (10.1021/acs.inorgchem.3c01982)

2023 | Yang, T.; Arellano-Reyes, R. A.; Curley, R. C.; Jha, K. K.; Chettri, A.; Keyes, T. E.; Dietzek-Ivansic, B. **In cellulose light-induced dynamics in a bodipy-perylene dyad.** *Chem. Eur. J.* 2023 (10.1002/chem.202300224)

Submitted Manuscripts (+ indicates co-first authorship)

2024 | Curley, R. C.⁺; Holden, L.⁺; Keyes, T. E. **Visualising Stress Granule Dynamics with an RNA Guanine Quadruplex Targeted Ruthenium(II) Peptide Conjugate.**

Manuscripts in Preparation

2024 | Gkika, K. S.; Holden, L.; Avella, G.; Curley, R. C.; Sarangi, N. K.; Keyes, T. E. **Phototheranostic probe Ru(phen)₂(dppz-PDC3)]⁴⁺ shows nuclear localization in HeLa cells.**

Awards and Achievements

2024 | 2024 **Postgraduate Gold Medal** by the Irish Biological Inorganic Chemistry Society (IBICS) at **IBICS-8** Conference, 6th December 2024, University College Cork.

2024 | Awarded an **academic** participant **grant** (€1,500) by the European Society of Animal Cell Technology (**ESACT**) to attend the 1st Cell Production and Monitoring practical training course at Institut Universitaire de Technologie (IUT) Lyon, France.

2024 | Best oral **presentation**, 2nd All-Ireland **RNA Club** Meeting, '*Visualising stress granule dynamics using an RNA guanine quadruplex targeted ruthenium(II) peptide conjugate*' 21st June 2024, Trinity Biomedical Sciences Institute (TBSI) Dublin.

2024 | Best [long-form] oral **presentation**, CDT PIADS **9th Annual Conclave**, '*Phototoxicity; using light to activate cancer therapy*' 19th June 2024, Queens University Belfast.

2024 | Awarded an Early Career Researcher (ECR) **registration grant** (€800) to attend the 4th **Frontiers in Photochemistry** conference in Torres Vedras, Portugal

2024 | Colin Barnes **Award for Postgraduate Excellence** (oral presentation prize), 9th Annual **DCU Chemistry Day**, '*Visualising stress granule dynamics with an RNA guanine quadruplex targeted ruthenium(II) peptide conjugate*' 10th May 2024, Dublin City University.

2023 | Awarded an **academic** participant **grant** (€1,250) by the European Society of Animal Cell Technology (**ESACT**) to attend the **13th Animal Cell Technology (ACT)** training course in Llafranc, Spain.

2023 | Awarded a **poster prize**, 8th Annual **DCU Chemistry Day**, '*Uptake of Phototoxic Membrane Permeable Ru(II) Polypyridyl Complexes in Live Mammalian Cells and Multicellular Tumour Spheroids*' 19th May 2023, Dublin City University.

2022 | Awarded a **poster prize**, 7th Annual **DCU Chemistry Day**, '*The uptake and toxicity of Ru(II) polypyridyl peptide conjugates in cancer and non-cancer cell lines*' 29th April 2022, Dublin City University.

Oral and Poster Presentations

2024 | IBICS-8, *'Visualising Stress Granule Dynamics with an RNA Guanine Quadruplex Targeted Ruthenium(II) Peptide Conjugate'* **oral presentation** (long talk), 6th December 2024, University College Cork.

2024 | Centre for Doctoral Training in **FUSE** (Future Ultrasonic Engineering) and **PIADS** (Photonic Integration and Advanced Data Storage) Joint Careers Day, *'Targeting the Mitochondria for Photoactivatable Chemotherapy'* **poster** presentation, 4th September 2024, Advanced Research Centre - University of Glasgow.

2024 | 4th **Frontiers in Photochemistry** Conference, *'Exploring the Efficacy of a Mitochondrial G4 Targeted Ruthenium(II) Complex in Cell Monolayers and Multicellular Tumour Spheroids'* **poster** presentation, 12th July 2024, Dolce CampoReal Lisboa, Portugal.

2024 | 2nd All Ireland **RNA Club** Meeting, *'Visualising stress granule dynamics with an RNA guanine quadruplex targeted ruthenium(II) peptide conjugate'* **oral** and **poster** presentation, 21st June 2024, BDSI Trinity College Dublin.

2024 | Centre for Doctoral Training in Photonic Integration and Advanced Data Storage (**PIADS**) 9th Annual **Conclave**, *'Phototoxicity; Using Light to Improve Anticancer Therapy'* **oral presentation** (long talk), 19th June 2024, Queens University Belfast.

2024 | DCU 9th Annual **Chemistry Day**, *'Visualising stress granule dynamics with an RNA guanine quadruplex targeted ruthenium(II) peptide conjugate'* **oral presentation** (long talk), 10th May 2024, Dublin City University.

2024 | Irish Photonic Integration Centre (**IPIC**) **Industry Day**, *'Metal Complex Probes for Phototherapy'* **poster** presentation, 25th April 2024, hosted virtually via Remo.

2023 | IBICS-7, *'Exploring the Efficacy of a Mitochondrial G4 Targeted Ruthenium(II) Complex in Cell Monolayers and Multicellular Tumour Spheroids'* **oral presentation** (long talk), 15th Dec 2023, University College Dublin.

2023 | DCU Research on Walls *'Lighting up the Future of Cancer Therapy'* **presentation (poster** of a single image to depict research area), 27th September 2023, Dublin City University.

2023 | Centre for Doctoral Training in Photonic Integration and Advanced Data Storage (**PIADS**) 8th Annual **Conclave**, *'Viscosity Sensing in Live Mammalian Cells'* **oral presentation** (flash), 21st June 2023, Queens University Belfast.

2023 | 16th International Symposium on Applied Bioinorganic Chemistry **ISABC-16** *'Uptake of Phototoxic Membrane Permeable Ru(II) Polypyridyl Complexes in Live Mammalian Cells and Multicellular Tumour Spheroids'* **poster** presentation, 12th June 2023, Karolos Papoulias Conference Centre Ioannina, Greece.

2023 | DCU 8th Annual **Chemistry Day**, *'Uptake of Phototoxic Membrane Permeable Ru(II) Polypyridyl Complexes in Live Mammalian Cells and Multicellular Tumour Spheroids'* **poster** presentation, 19th May 2023, Dublin City University.

2023 | Irish Photonic Integration Centre (**IPIC**) **Industry Day**, '*Custom Designed Luminescent Probes for Sensing and Imaging in Live Cells*' **poster** presentation, 14th March 2023, hosted virtually via Remo.

2022 | Irish Biological Inorganic Chemistry Society Symposium, **IBICS-6**, '*Uptake of Phototoxic Membrane Permeable Ru(II) Polypyridyl Complexes in Live Mammalian Cells and Multicellular Tumour Spheroids*' **oral presentation** (flash) & **poster**, 8th December 2022, Dublin City University.

2022 | 8th **European Chemical Society** (EuChemS) Chemistry **Congress** ECC8, '*Phototoxicity of Membrane Permeable Ru(II) Polypyridyl Peptide Conjugates in Cancer and non-Cancer Cell Lines*', **oral presentation** (long talk), 30th August 2022, Lisbon Congress Centre, Portugal.

2022 | Centre for Doctoral Training in Photonic Integration and Advanced Data Storage (**PIADS**) 7th **Conclave**, '*The Uptake and Phototoxicity of Ruthenium(II) Polypyridyl Peptide Conjugates in Cancer and non-Cancer Cell Lines*', **poster** presentation, 24th June 2022, The National Piping Centre, Glasgow.

2022 | Irish Photonic Integration Centre (**IPIC**) **Industry Day - Photonics 4 Disruptive Innovation**, '*Phototherapeutics*' **poster** presentation, 19th May 2022, University College Cork.

2022 | **DCU** 7th Annual **Chemistry Day**, '*The uptake and toxicity of Ru(II) polypyridyl peptide conjugates in cancer and non-cancer cell lines*' **poster** presentation, 29th April 2022, Dublin City University.

2022 | The 2nd Joint Symposium of the **Microscopy Society of Ireland** (MSI) and **Scottish Microscopy Society** (SMS), '*The uptake and toxicity of Ru(II) polypyridyl peptide conjugates in cancer and non-cancer cell lines*' **poster** presentation, 7th April 2022, Galway Bay Hotel.

2021 | 5th Irish Biological Inorganic Chemistry Society Symposium, **IBICS-5**, '*The Uptake and Efflux of Ru(II) Polypyridyl Peptide Conjugates in Cancer and non-Cancer cell lines*' **oral presentation** (flash), 26th November 2021, hosted via MS Teams.

Thesis Outline

Chapter 1: Introduction

The background to the thesis presented is introduced in Chapter 1. Ru(II) complexes and BODIPY-perylene probes, are charge-transfer compounds with favourable photophysical properties and versatile design, showing significant potential as probes for cellular imaging and as therapeutic agents in phototherapy. Cellular uptake and targeting can be achieved using a variety of methods, including conjugation to peptides, leveraging the charge or lipophilicity, and incorporating ligands to enhance uptake. While conventional cell monolayer models are often used to assess the potential of new drugs for phototherapy, increasing evidence to support the importance of incorporating studies using 3D models, such as multicellular tumour spheroids (MCTS), as a more representative model of the tumour microenvironment. To develop charge-transfer compounds for imaging and phototherapy applications, it is essential to strike a balance between the desired photophysical properties of new probes and their biocompatibility, with a particular focus on minimising dark toxicity.

Chapter 2: Phototoxicity of Tridentate Ru(II) Polypyridyl Complex with Expanded Bite Angle toward Mammalian Cells and Multicellular Tumour Spheroids

A ruthenium complex, Ru-bqp-ester and its peptide conjugates Ru-bqp-MPP and Ru-bqp-R8 (synthesised by Dr. Christopher S. Burke) are presented in Chapter 2. This chapter describes the interaction of the ruthenium(II) polypyridyl complexes with live A549 lung cancer, human pancreatic adenocarcinoma (HPAC) cells and Chinese hamster ovary (CHO) cells with respect to their mechanism of uptake, and phototoxicity, in comparison to their behaviour in HPAC MCTS. This family of Ru(II) polypyridyl complexes demonstrated high cell permeability and phototoxicity, showing promise as a PDT agent.

Chapter 3: Visualising Stress Granule Dynamics with an RNA Guanine Quadruplex Targeted Ruthenium(II) Peptide Conjugate

Chapter 3 presents a ruthenium(II) peptide conjugate, Ru-RHAU, synthesised by Dr. Lorcan Holden. This complex binds to RNA G4s (rG4s) and induces stress granules (SGs) in live cells in a concentration and time dependent manner, confirmed through immunostaining studies with anti-G4 antibody BG4 and SG proteins G3BP1 and TIA-1. The potential of SGs to be used as a live cell imaging dye for SGs induced using an external stressor was confirmed at 10 μ M Ru-RHAU in cells treated by heat shock in both HeLa and MCF-7 cells. This complex showed minimal dark toxicity under imaging

conditions and represents a significant step forward in terms of the capability of designing new dyes for imaging SGs in live cells, and there are currently no commercially available dyes to facilitate this.

Chapter 4: Targeting Mitochondrial Guanine Quadruplexes for Photoactivatable Chemotherapy in Normoxic and Hypoxic Environments

A mitochondrial G4-targeting ruthenium complex, Ru-TAP-PDC3 (synthesised by Dr. Lorcan Holden) is presented in Chapter 4. Dark and phototoxicity studies were performed in cell monolayers under hypoxic and normoxic conditions and in 3D spheroids. Irradiation doses of 5 and 15 J/cm² were applied for cell monolayer phototoxicity studies and an increase in irradiation dose was shown to improve the phototoxic capacity of the Ru(II) complex in a hypoxic environment. Studies under normoxic conditions confirmed Ru-TAP-PDC3 induces mitochondrial DNA (mtDNA) damage in HeLa cells when excited by 405 nm light, resulting in a reduction in mitochondrial membrane potential and cell death through apoptosis.

Chapter 5: Enhancing Phototoxicity in BODIPY-perylene Charge-transfer Dyads by Combined Iodination and Mesylation

A family of BODIPY-perylene charge-transfer compounds (synthesised and characterised by Dr. Ruben Arturo Arellano-Reyes) are described in Chapter 5. These compounds were found to be highly phototoxic in MCF-7 and CHO cells, with the presence of an iodine group resulting in higher phototoxicity. Studies to confirm the mechanism of toxicity included ¹O₂ scavenger studies in solution, imaging with ROS dye H₂DCFDA and hypoxic phototoxicity studies. The significant phototoxicity of the BODIPY-perylene compounds, particularly the iodinated derivatives, highlight the potential of combining charge-transfer and iodination to enhance the effectiveness of BODIPY photosensitisers for PDT.

Author Contribution to Publications

Chapter	Title	Publication Status*	Authors	Contribution by Candidate
2	Phototoxicity of Tridentate Ru(II) Polypyridyl Complex with Expanded Bite Angle Toward Mammalian Cells and Multicellular Tumour Spheroids	<i>Inorg. Chem.</i> 2023, 62 , 32, 13089–13102	<u>Curley, R. C.</u> ; Burke, C. S.; Gkika, K. S.; Noorani, S.; Walsh, N.; and Keyes, T. E.	Manuscript primary author. Primary contributor to experimental design and execution.
3	Visualising Stress Granule Dynamics with an RNA Guanine Quadruplex Targeted Ruthenium(II) Peptide Conjugate	Submitted	<u>Curley, R. C.</u> ; Holden, L. ⁺ ; and Keyes, T. E.	Co-first author of manuscript with Dr. Lorcan Holden. Carried out all cellular work including live and fixed cell imaging and bioassays (cytotoxicity and phototoxicity).
4	Targeting Mitochondrial Guanine Quadruplexes for Photoactivatable Chemotherapy in Normoxic and Hypoxic Environments	<i>Angew. Chem. Int. Ed.</i> 2024, e202408581	Holden, L. ⁺ ; <u>Curley, R. C.</u> ; Avella, G.; Long, C; and Keyes, T. E.	Co-first author of manuscript with Dr. Lorcan Holden. Carried out all cellular work including bioassays, confocal & STED imaging and cellular studies using 3D spheroids.
5	Enhancing Phototoxicity in BODIPY-perylene Charge-transfer Dyads by Combined Iodination and Mesylation	<i>Chem. Eur. J.</i> 2024, e202403149	<u>Curley, R. C.</u> ; Arellano-Reyes, R. A.; McPherson, J. N.; McKee, V.; and Keyes, T. E.	Manuscript primary author. Primary contributor to experimental design and execution.

*Publication status: 'in preparation', 'submitted', 're-submitted', 'accepted' or 'published'. + indicates equal authorship.

Signed: Rhianne Curley Tia Keyes
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Abbreviations

χ^2	Goodness of Fit
τ	Luminescence Lifetime
ϵ	Molar Extinction Coefficient
λ	Wavelength
Φ	Quantum Yield
2D	Two Dimensional
2DG	2-Deoxy-D-Glucose
3D	Three Dimensional
A549	Human Lung Carcinoma Cell Line
ALA	5-aminolevulinic acid
ASC	Ascididemin
ATP	Adenosine Triphosphate
BG4	Anti G-quadruplex antibody
bpy	2,2'-bipyridine
BSA	Bovine Serum Albumin
BODIPY	Boron Dipyrromethene
BQP	2,6-bi(quinolin-8-yl)pyridine
CCCP	carbonyl cyanide m-chlorophenyl hydrazone
CHO	Chinese Hamster Ovary cell line
CLSM	Confocal Laser Scanning Microscope
CPP	Cell Penetrating Peptide
DBB	1,2-dibenzoylbenzene
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
DPBF	1,3-diphenylisobenzofuran
DPPZ	dipyrido[3,2-a:2',3'-c]phenazine
ELISA	Enzyme Linked Immunosorbent Assay
ER	Endoplasmic Reticulum

F-12 Ham	Ham's F-12 Nutrient Mixture (F-12) for cell culture
FBS	Fetal Bovine Serum
Flag-tag	Also known as DYKDDDDK-tag. DYKDDDDK is an amino acid sequence, where D=Aspartic acid; K=Lysine and Y=Tyrosine.
FLICA	Fluorochrome Inhibitor of Caspases
FLIM	Fluorescence Lifetime Imaging Microscopy
G	Guanine
G3BP1	ras GTPase-activating protein-binding protein 1
G4	Guanine Quadruplex
GMP	Guanosine Monophosphate
HEK-293	Human embryonic kidney cell line
HeLa	Cervical cancer cell line
HIF	Hypoxia Inducible Factor
HPAC	Human Pancreatic Adenocarcinoma Cells
HL-60	Leukemia Cell Line
HR-MS	High Resolution Mass Spectrometry
HyD	Hybrid Detectors
IC ₅₀	Concentration of a drug causing 50% inhibition <i>in vitro</i>
ISC	Intersystem Crossing
JC-1	Mitochondrial Membrane Potential Probe
LED	Light-Emitting Diode
LD	Lipid Droplet
mAb	Monoclonal Antibody
MCF-7	Human breast cancer cell line
MCTS	Multicellular Tumour Spheroid
MeCN	Acetonitrile
mitoG4	Mitochondrial Guanine Quadruplex
MLCT	Metal-to-Ligand Charge-transfer
MPP	Mitochondrial Penetrating Peptide
mtDNA	Mitochondrial DNA

MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NLS	Nuclear Localising Signal
NMIBC	Non-Muscle-Invasive Bladder Cancer
NMR	Nuclear Magnetic Resonance
$^1\text{O}_2$	Singlet Oxygen
PACT	Photoactivated Chemotherapy / Chemotherapeutics
PBS	Phosphate Buffer Saline
PDT	Photodynamic Therapy
PET	Photoinduced Electron Transfer
PI	Phototoxic Index
PIC-COOH	Phenylimidazo[4,5-f][1,10]phenanthroline carboxylic acid
PLIM	Phosphorescence Lifetime Imaging Microscopy
PMT	Photomultiplier Tube
polyHEMA	Poly(2-hydroxyethyl methacrylate)
PpIX	Protoporphyrin IX
PS	Photosensitiser
R8	Octa-arginine sequence (RRRRRRRR)
rG4	RNA Guanine Quadruplex
RBP	RNA Binding Proteins
RHAU	RNA Helicase Associated with AU-rich Element
RI	Refractive Index
ROS	Reactive Oxygen Species
rpm	Revolutions per Minute
RNA	Ribonucleic Acid
RPMI	Rosewell Park Memorial Institute cell culture medium
RT	Room Temperature
SD	Standard Deviation
SG	Stress Granule
SOCT	Spin Orbit Charge-transfer
STED	Stimulated Emission Depletion (Microscopy)

STORM	Stochastic Optical Reconstruction Microscopy
TAP	1,4,5,8-tetraazaphenanthrene
TATP	1,4,8,9-tetra-aza-triphenylene
TCSPC	Time Correlated Single Photon Counting
TIA-1	T-cell intracellular antigen-1
TNF- α	Tumour Necrosis Factor-alpha
TPPHZ	tetrapyrido[3,2-a:2',3'-c:3'',2''-h:2''',3'''-j]phenazine
WLL	White Light Laser

Units of Measurement

μs	Microsecond
μg	Microgram
μg	Microlitre
μM	Micromolar
g	gram
h	Hour or hours
J/cm^2	Joules per square centimetre
K	Kelvin
L	Litre
M	Molarity
min	Minute
mol	Mole
mL	Millilitre
mM	Millimolar
mW/cm^2	Milliwatts per square centimetre
nm	Nanometre
nM	Nanomolar
ns	Nanosecond
$^{\circ}\text{C}$	Degrees Celsius
v/v	Volume per volume

Rhianne C. Curley

Charge-transfer luminophores: theranostic probes of the cellular environment

Thesis Abstract

Luminescent ruthenium(II) polypyridyl complexes and BODIPY-perylene charge-transfer compounds possess several remarkable properties that make them attractive for a range of applications including cellular imaging and phototherapy. These properties include large Stokes shifts, a crucial characteristic for enhancing the signal-to-noise ratio in fluorescence imaging. Moreover, their versatile synthetic chemistry allows for tailored designs for enhanced cellular imaging or for maximising therapeutic efficacy. For example, the ability of these charge-transfer luminophores to generate cytotoxic reactive oxygen species upon photoexcitation makes them promising candidates for photodynamic therapy.

Presented in this thesis, are experimental findings on the phototherapeutic potential of a Ru(II) parent complex Ru-bqp-ester and its peptide conjugates Ru-bqp-MPP and Ru-bqp-R8, which showed exceptional phototoxicity across a range of cancer and non-cancer cell lines. Conjugation to cell penetrating and signal peptides is a useful strategy for promoting cellular uptake and targeting specific organelles such as the mitochondria or the nucleus, however, guanine quadruplexes (G4s) are gaining increasing interest as therapeutic targets. Two G4 targeted Ru(II) complexes, Ru-TAP-PDC3 and Ru-RHAU, were investigated as probes for imaging and phototherapy. Ru-TAP-PDC3 is the first Ru(II) complex directed toward photodamage of G4s located in the mitochondrial genome and Ru-RHAU is the first reported dye capable of live cell imaging of stress granules (SGs), binding to G4s and inducing SGs in a time and concentration dependent manner. Additionally, a family of iodinated and non-iodinated BODIPY compounds were tested as potential photosensitisers, confirming a significant increase in phototoxicity upon iodination.

A key aim of this thesis was to investigate the phototherapeutic potential of novel Ru(II) or BODIPY-based photosensitisers in live cells and multicellular tumour spheroids, and extend the application of these compounds for cellular imaging and sensing using confocal microscopy.

Chapter 1 : Introduction

Luminescent probes are essential tools to enable researchers to visualise the cellular environment. They are applied across a range of imaging modalities from whole cells and 3D cellular models to subcellular components such as cellular organelles or biomolecules within cells, facilitating high-resolution monitoring of biological processes.

The role of cellular imaging probes in biology has evolved significantly in over the past decade, shifting from simple fluorescent markers for contrast imaging to multifunctional sensors. More recently, these probes have been designed not only for imaging, but also to serve additional diagnostic or therapeutic functions, for example to perform intracellular sensing such as viscosity or oxygen sensing with phototoxic ROS generation.^[1,2] This concept can be referred to as ‘theranostics’ i.e. combining ‘therapy’ and ‘diagnostics’.^[3]

The ideal photophysical properties of a luminescent imaging probe can vary depending on its specific application. However, in general, luminescent imaging probes possess a number of desirable photophysical characteristics that can be exploited for different imaging techniques. These include long luminescent lifetimes, large Stokes shift, emission in the near-infrared (NIR) spectral region, excellent photostability and high quantum yields.^[4] Additionally, low cytotoxicity and good aqueous solubility are essential to ensure compatibility with live-cell imaging.

Traditional imaging probes based on organic dyes such as fluorescein have limitations such as short excited state lifetimes (typically <10 ns), self-quenching and susceptibility to photobleaching, which limit their use in monitoring long-lived biomolecular processes. The rapid advancement of microscopy in recent years, for example super resolution microscopy methods such as Stimulated Emission Depletion (STED), structured illumination microscopy (SIM) and Stochastic Optical Reconstruction Microscopy (STORM) has significantly increased the demand for new and improved imaging probes to allow for multi-modal imaging.^[5] Bright organic dyes such as the Alexa Fluor, ATTO or cyanine (Cy) dyes have received significant attention for conventional and super-resolution cell imaging due to their well-established performance and commercial availability. While luminescent transition metals offer a promising avenue for the development of specialised imaging probes, they are often overlooked.

Luminescent transition metal complexes like ruthenium, osmium, iridium, platinum or rhenium as well as other systems such as BODIPY-perylene moieties have received significant attention for biomedical applications, including live cell imaging. The presence

of heavy metal atoms induce significant spin–orbit coupling (SOC) and promote intersystem crossing (ISC) to reach triplet excited states, which play a key role in the unique photophysical properties of many charge-transfer compounds that can be tuned to suit diagnostic and imaging demands.^[6] In the case of BODIPY dyads, although they do not inherently contain heavy metals, halogenation (e.g. with iodine or bromine) has been effectively used to enhance SOC and ISC, thereby optimising their photophysical properties for similar advanced applications.^[7]

Charge-transfer is an important process in photochemistry, photobiology, and materials science that involves the movement of an electron or electrons from one part of a molecular structure to another resulting in a charge-separated state in the electronically excited state with polarisation of charge density on the molecule while it persists in the excited state. This confers a number of properties on the molecule such as broad absorbance and emission bands, large Stokes shift, solvent-dependent optical characteristics particularly emission spectroscopy and frequently high extinction coefficients that can be useful in an imaging or sensing context.

Traditional fluorophores used in contrast imaging form delocalised excited states that can have the benefits of very high quantum yields but tend to have narrow Stokes shift and low environmental sensitivity. Another advantage of charge-transfer states is that they can promote the formation of triplet states through SOCT-ISC, as discussed in Chapter 5, that may offer the prospect of theranostic applications. Understanding charge-transfer mechanisms allows for the design of compounds with tailored properties for specific applications.

Charge-transfer excited states are often categorised as one of the following: metal-to-ligand charge-transfer (MLCT), ligand-to-metal charge-transfer (LMCT), intraligand charge-transfer (ILCT) and ligand-to-ligand charge-transfer (LLCT).^[6,8] In metal free systems like the BODIPY-perylene dyads described in Chapter 5, the use of a donor and acceptor moiety in a supramolecular system or in push-pull molecules also leads to charge-transfer states and in these systems the extent of charge-transfer can be controlled by parameters such as the relative orientation of donor and acceptor around a rotational bond which can have applications in e.g. viscosity sensing.

For metal complexes, a challenge that has limited their application in bioimaging and biosensing probes is their poor cellular permeability and organelle targeting.^[9] However, this issue has been largely addressed in recent years through various strategies, such as modifying the physicochemical properties of the complexes or by bioconjugation, for example conjugating these complexes to cell-penetrating peptides (CPPs) or signal

peptides.^[4] This strategy is particularly valuable for metal complex luminophores, as it allows for precise targeting to organelles like the mitochondria, the nucleus or to nucleic acids of interest. This is generally less of an issue in metal-free charge-transfer compounds including BODIPY-based compounds like the BODIPY-perylene dyads discussed here and those reported by other groups such as the BODIPY-pyridine compounds designed by Harriman *et al.*^[10] and the *meso*-phenyl-BODIPY type compounds by Hu *et al.*^[11] Organic charge-transfer compounds often exhibit favourable cellular uptake properties due to their size and lipophilicity, depending on their specific substituents. Despite this, it must be noted that BODIPY compounds are often poorly water-soluble, therefore care must be taken in assigning them membrane permeable as solvents used to solubilise them in aqueous media may permeate the membrane. While this is generally acceptable in 2D cell cultures (provided the solvent is not cytotoxic) it may not translate well to 3D cell cultures or *in vivo* environments, where solvent use could potentially impact compound uptake, distribution, or therapeutic efficacy. It must also be noted that examples of organic charge-transfer compounds are limited, and the literature is dominated by organics with conventional delocalised excited states that have applications in cellular imaging and sensing.

This chapter examines the advantages and applications of luminescent compounds as imaging and phototheranostic probes, with examples of both charge-transfer compounds and other probes which can be used as alternatives to traditional organic fluorophores. The focus is mainly confined to ruthenium(II) complexes and BODIPY-based systems, because of the breadth of the literature, and I highlight their roles in cellular imaging, sensing, and therapy. Additionally, this chapter touches on probes based on other transition metal complexes, such as iridium and platinum, to provide a broader context. This thesis explores the potential of ruthenium and BODIPY-perylene charge-transfer compounds for high-resolution cell imaging, and for both oxygen-dependent and oxygen-independent phototherapy.

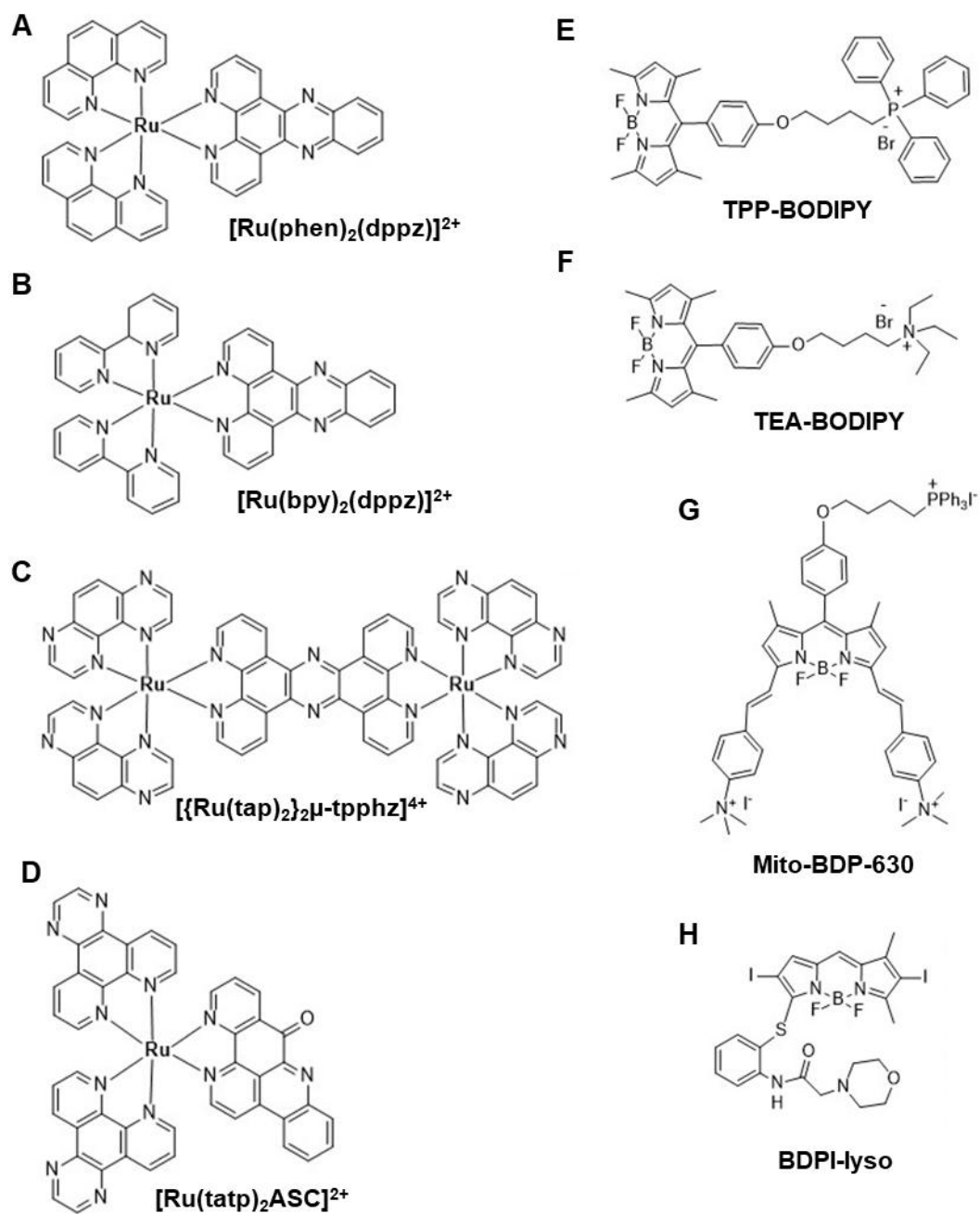


Figure 1.1 Molecular structures of the ruthenium complexes and BODIPY derivatives discussed throughout Chapter 1

1.2 Cellular Imaging

Imaging techniques such as fluorescence, confocal and two-photon excitation microscopy are extensively used for the study of biological systems. These methods allow researchers to track the localisation and dynamics of new probes at a subcellular level. Additionally, more specialised time-resolved microscopy techniques such as fluorescence lifetime imaging microscopy (FLIM), or phosphorescence lifetime imaging microscopy (PLIM) in the case of many metal luminophores, are essential for examining

the excited-state lifetimes of luminescent compounds. FLIM and PLIM can provide a quantitative understanding of the photophysical behaviour of a charge-transfer compound in a live cellular environment, including information on the environmental sensitivity of the probe in real time.^[12,13] This section includes a brief introduction on molecular photophysics and origin of emission, followed by a description of the microscopy techniques employed in this thesis and a discussion of cellular imaging probes.

1.2.1 Principles of Photophysics

The photophysical processes that occur upon excitation of a molecule can be illustrated using a Jablonski diagram as shown in Figure 1.2. When a molecule absorbs a photon of light, it transitions from its ground state (S_0) to a higher energy excited state (S_n). As this excited molecule is generally unstable, deactivation will occur through various deactivation pathways, illustrated in Figure 1.2.

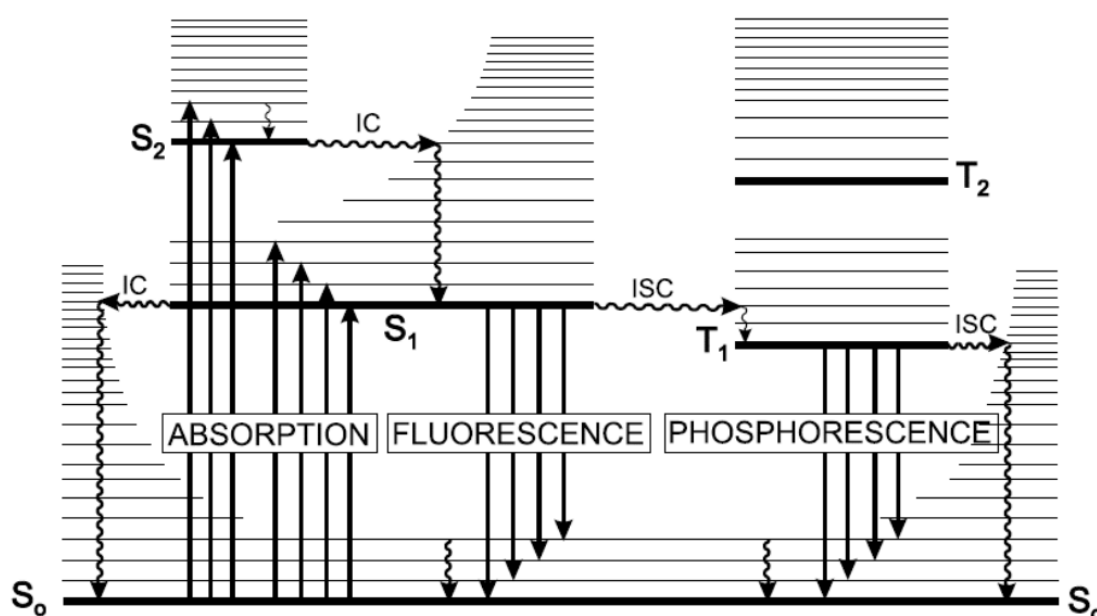


Figure 1.2 General Jablonski diagram illustrating key photophysical mechanisms. A straight line indicates absorption when directed upwards and an emissive process when directed downwards. A waved arrow represents a non-radiative process. IC = internal conversion and ISC = intersystem crossing. Reproduced from Reference 14.^[14]

Luminescence is a general term that describes radiative decay pathways, which can occur as either fluorescence or phosphorescence. Fluorescence is a radiative transition that occurs for most organic fluorophores, where the excited molecule deactivates from the first singlet excited state (S_1) to the ground state (S_0) i.e. $S_1 \rightarrow S_0$. Fluorescence occurs when a molecule transitions between states of the same multiplicity (usually between singlet states) resulting in emission at lower energy (longer wavelengths) than

the absorption spectrum, where the gap between the absorption and emission maximums is known as the Stokes shift. Fluorescence exhibits short luminescence lifetimes, of the order of 10^{-11} - 10^{-9} seconds, reflecting the transition between states of the same multiplicity is allowed.

In contrast, phosphorescence involves photon emission as the molecule transitions between states of different multiplicity, e.g. triplet excited state (T_1) back to the ground state (S_0) i.e. $T_1 \rightarrow S_0$. This transition, particularly in organic molecules, is generally forbidden and occurs through the de-excitation process known as intersystem crossing (ISC). In molecules such as luminescent metal complexes, the presence of heavy atoms can promote spin-orbit coupling (SOC) which causes singlet and triplet mixing and allows phosphorescence to occur. The forbidden nature of this transition results in slower emission decay rates, leading to longer phosphorescent lifetimes, typically from 10^{-6} seconds up to the order of seconds. Charge-transfer compounds can exhibit photophysical properties similar to both fluorescence and phosphorescence, for example Ru(II) complexes tend to have excited states where singlet and triplet character is mixed, so they have long emission lifetimes in the order of microseconds, but not as long as phosphorescent organic molecules with large Stokes shifts. These properties can make them valuable in biological imaging applications, but their photophysical processes often involve more complex mechanisms related to electron transfer and solvent interactions.

1.2.2 Confocal Laser Scanning Microscopy (CLSM)

In conventional light microscopy, illuminating light from a high intensity arc or filament lamp is passed through the entire sample simultaneously. This method can cause issues such as photobleaching and for thicker samples, its z resolution is limited as light from both above and below the focal plane will be detected simultaneously, resulting in an image with in-focus and out-of-focus light (Figure 1.3). The out-of-focus light reduces the resolution of the image, making it difficult to produce detailed images of cellular structures.^[15]

Confocal microscopy can be used to overcome the challenges associated with conventional light microscopy. Confocal laser scanning microscopy (CLSM) is the most widely used confocal technique, however spinning disk and hybrid confocal microscopes are also available.^[16] Confocal microscopy enhances the optical resolution of samples by exciting the sample using a laser that can be focused on a specific (small) volume of the sample and incorporating a detection pinhole to block out-of-focus light from the

detector, meaning only the fluorescence from the focal plane will reach the detector during image formation.^[15]

Photomultiplier tube (PMT) or hybrid detectors (HyD) are often used for confocal microscopy. The dichroic mirror, also known as a dual-band mirror or dual-wavelength mirror, is used to reflect the light below a set wavelength and the emission filter ensures only specific fluorescence is transmitted to the detector. Typically, in CLSM, the laser beam is swept over the sample, meaning only a single spot of the sample is illuminated at any time. To produce a 2D image, the laser is directed onto a pair of scanning galvanometer mirrors, which rotate to move the laser beam in the x and y directions across the sample. CLSM can also be used to produce a 3D reconstruction of a sample by scanning in the z direction as well as the x and y. Scanning in the z direction allows for the acquisition of a 'z-stack' which is a collection of several slices obtained at different focal points and provides a complete cross-sectional view of a 3D sample, increasing the accuracy of uptake and toxicity assessments.

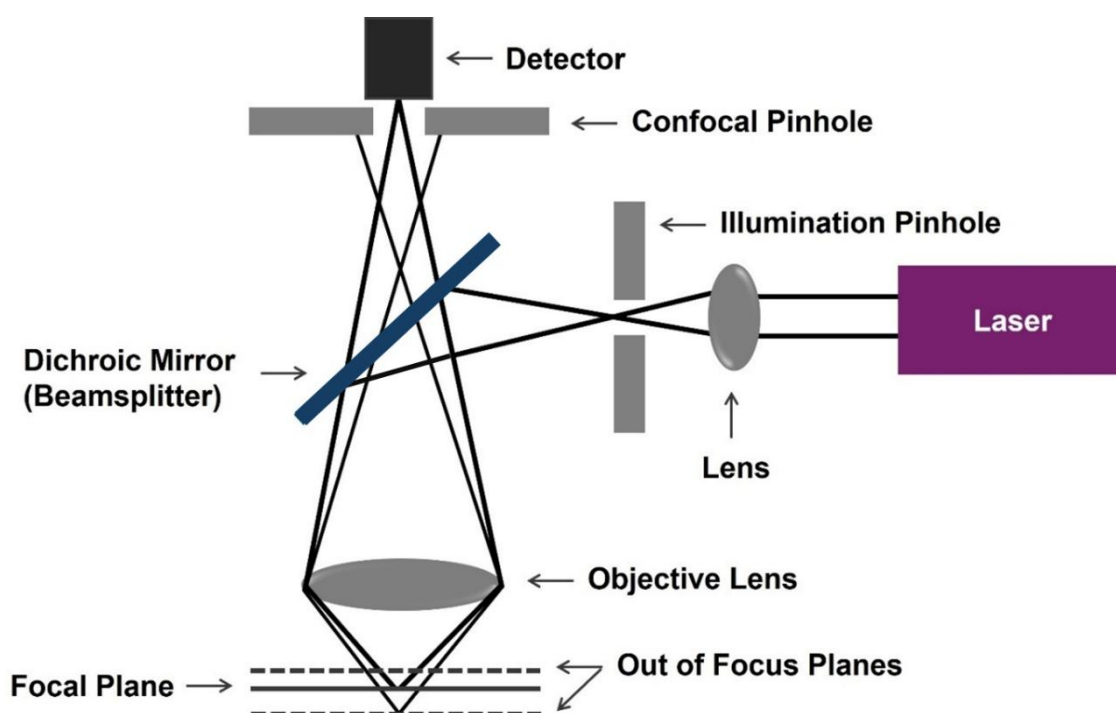


Figure 1.3 Illustration of confocal microscope setup, highlighting the key components and their arrangement.

Photobleaching is a challenge associated with CLSM, particularly during longer imaging sessions. This is a more common issue with organic fluorophores however and with many charge-transfer compounds employed as cellular imaging probes photobleaching is less of a problem. It is important to acknowledge that due to their triplet excited states

the use of luminescent metal complexes introduces the potential challenge of phototoxicity in live cells due to the generation of cytotoxic singlet oxygen ($^1\text{O}_2$).^[17] Therefore, it is necessary to design these complexes with careful consideration and optimise imaging conditions to minimise any phototoxic affects. To address the limitations associated with CLSM, new imaging dyes are being developed which exhibit characteristics such as good photostability, large Stokes shift and long-lived excited states, effectively overcoming the common challenges associated with confocal microscopy using organic fluorophores.

1.2.3 Super-Resolution Microscopy

Super-resolution microscopy was a groundbreaking advancement in imaging technology, surpassing the traditional diffraction limit of light and therefore overcoming the resolution restrictions associated with conventional optical methods. Confocal microscopy has a resolution limit of ~250 nm, whereas super-resolution techniques like STED, STORM and SIM surpass this limit and enable the resolution of details down to the nm level. Super-resolution microscopy has enabled real-time tracking of organelle dynamics, enhanced understanding of the organisation of molecular assemblies *in cellulo* and allows for the visualisation of fine structures within cell organelles such as the nucleus or mitochondria and the cytoskeleton.^[18] The first super-resolution technique to be reported was STED, which led to the award of the Nobel Prize in Chemistry to Stefan W. Hell, William E. Moerner, and Eric Betzig, and inspired the generation of newer super-resolution techniques.^[19]

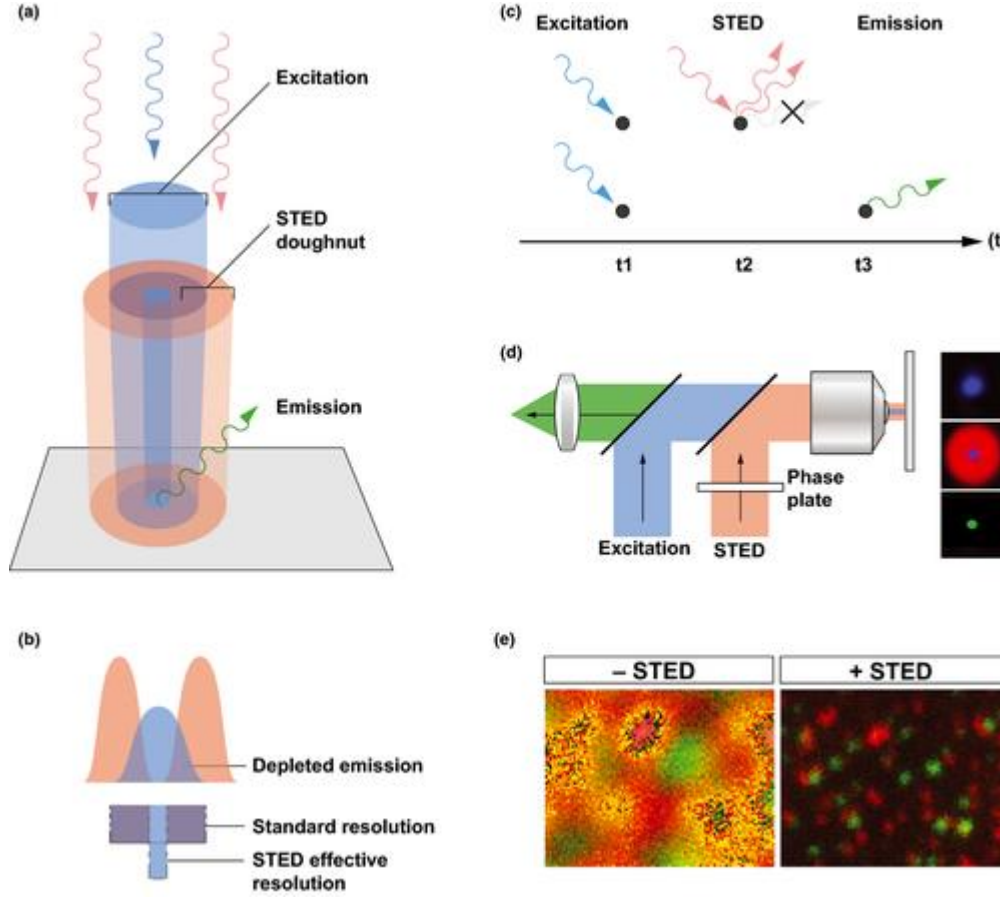


Figure 1.4 The principles of STED, where **(a)** a laser is used to excite (blue) the fluorophore, and the STED depletion laser (red) is used to for de-excitation in the outer periphery. The concept of using emission depletion by the STED laser to generate sharper nanoscale images is further illustrated in **(b)** and **(c)**. A diagram of a STED microscope is depicted in **(d)**, where the ‘doughnut-shape’ of the emission depletion is clear, and the detected fluorescence emission is shown in green. A standard fluorescence microscopy image (-STED) of a mixture of 40 nm fluorescent beads, color-coded in red and green is shown in **(e)** along with a super-resolution image (+STED) revealing nanoscale separation. This diagram is adapted from Reference 20.^[20]

In STED microscopy, a luminophore (dye) is first excited by a standard laser pulse, similar to routine confocal imaging, which raises it to an excited state where it would typically emit fluorescence. However, before the luminophore can emit this fluorescence, it is de-excited by a second, doughnut-shaped STED laser pulse, so that the only fluorescence emitted comes from the small central region of the doughnut shape (Figure 1.4).^[21] The improved resolution of STED can be explained by Equation 1.1:

$$d = \frac{\lambda}{2NA\sqrt{1 + \frac{I_{max}}{I_{sat}}}}$$

Where λ is the emission wavelength, NA is the numerical aperture of the objective lens, I_{max} is the peak intensity of the STED laser within the depletion zone, and I_{sat} is the applied STED laser threshold intensity, i.e. the intensity that depletes 50% of the luminophore’s emission intensity.^[22] The intensity of the laser is proportional to the

effective depletion, thus the STED process depends heavily on the characteristics of the luminophore and there are stringent requirements when designing a STED dye. The luminophore must have excellent photostability to withstand the high-intensity depletion laser and its emission spectrum must sufficiently overlap with the STED laser to enable effective depletion yet have a large enough Stokes shift to prevent the STED laser from unintentionally re-exciting the luminophore or populating a long-lived dark state which could reduce the resolution.^[23]

Moreover, the pulse width of the STED laser must be narrower than the excited state lifetime of the luminophore to ensure the depletion occurs while the luminophore is still in its excited state. Hence, STED is a particularly effective technique when working with dyes that have longer luminescent lifetimes such as luminescent transition metals. A longer lifetime also facilitates the use of time-gating to improve sensitivity. Time-gating can be combined with STED imaging to eliminate undesired fluorescence based on the difference in lifetimes of a STED dye and autofluorescence (~2 ns) or the difference in lifetimes between two dyes being used in the same sample.^[24] SIM and STORM are popular super-resolution alternatives to STED which operate on different principles.^[21,25]

The choice of immersion medium is crucial for achieving the best resolution in STED microscopy. The refractive index (RI) of an immersion medium must be considered when planning imaging studies as there can be significant differences between the options available; for example the RI of air is 1, water is 1.33 and the immersion oil used in this thesis, Leica Type-F, is 1.52.^[26] To achieve the best resolution possible, the RI of all materials in the imaging setup must match as closely as possible. For live cell imaging, the immersion oil should match the RI of the imaging dish or slide and for fixed cell imaging, the immersion oil should match the RI of the glass slide, coverslip and mounting medium (e.g. ProLong Gold RI = 1.47). An RI close to 1.5 is widely selected for live and fixed cell imaging, thus most imaging materials are produced to suit this requirement. Matching the RI of each component minimises light refraction and aberration, ensuring that the maximum amount of light is collected from the sample which results in clearer, sharper images with higher resolution.^[27]

1.2.4 Cellular Imaging Probes

In the field of cellular imaging, both traditional organic dyes and newer dyes based on luminescent metal complexes have played pivotal roles. A wide array of commercial dyes is available, several of which are used in this thesis, targeting key cellular organelles such as the nucleus, lysosomes, mitochondria, late endosomes, lipid droplets, endoplasmic reticulum, among others. These commercial dyes operate using

different targeting mechanisms. For example, LysoTracker targets the lysosomes due to their acidic pH,^[28] while Hoechst 33342 stains DNA in the nucleus by binding to the minor groove of DNA (Figure 1.5).^[29] By utilising these diverse dyes, precise cellular targeting can be achieved which allows for co-localisation studies which were used to determine where the Ru(II) or BODIPY-based probes presented in this thesis accumulate in live cells. The ideal characteristics of an imaging probe can vary depending on the methodology; however, all imaging methods share desirable probe characteristics, including photostability and high molecular brightness.

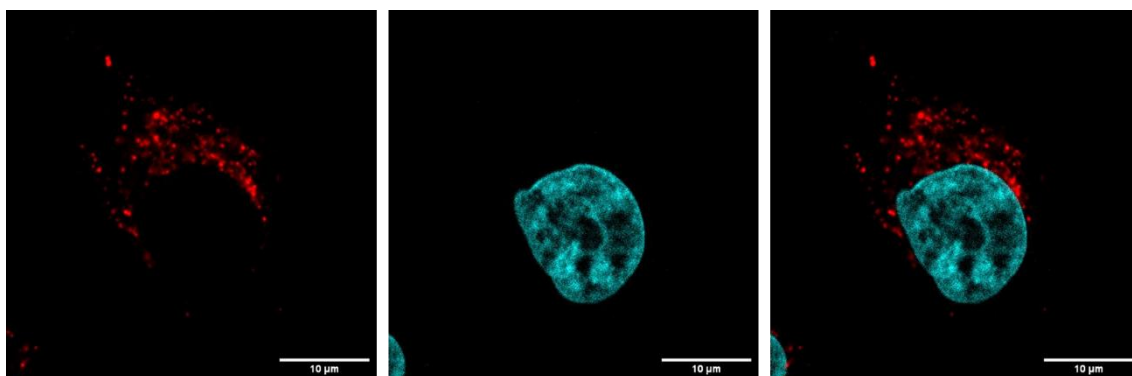


Figure 1.5 Confocal images of live CHO cells with LysoTracker Deep Red (red), Hoechst 33342 (cyan) and the overlay of both dyes in a single cell.

BODIPY dyes are a class of fluorescent dyes that are known for their high chemical stability, brightness and photostability. They are composed of a central boron atom that is co-ordinated to two nitrogen atoms, known as dipyrromethene units. BODIPY dyes have absorption and emission properties in the visible and near-infrared regions, making them useful for cellular imaging.^[30] Among the most popular BODIPY-based dyes are BODIPY 505/515 and BODIPY 493/503, commonly used for staining lipid droplets in live cells.^[31,32] However, these dyes share the limitations common to many organic fluorophores including short Stokes shift, which increases the likelihood of self-quenching. For example, both BODIPY 505/515 and BODIPY 493/503 exhibit a Stokes shift of only 10 nm.^[33,35-36] In contrast, organic charge-transfer compounds such as BODIPY-perylene dyads can exhibit desirable photophysical properties due to the charge-transfer interactions between the BODIPY and perylene moieties including large Stokes shift (~110 nm) and extended lifetimes.^[34]

Similarly, the photophysical properties of metal complexes mentioned previously, including long luminescent lifetimes linked to their long-lived triplet MLCT (³MLCT) excited states, excellent photostability, and large Stokes shifts, make them attractive imaging agents.^[4,37] Additionally, luminescent transition metal complexes tend to have long lifetimes and high photostability, making them ideal for in-cell sensing.^[38,39]

The photophysics of the prototype metal complex $[\text{Ru}(\text{bpy})_3]^{2+}$ have been reported extensively and are often used as a standard to reflect the photophysics of Ru(II) complexes. A benefit to designing imaging probes from Ru(II) complexes is the tunability of the ligands. For example, the photophysical properties of Ru(II) can easily be tuned by altering the nature and quantity of polypyridyl ligands surrounding the Ru(II) centre.^[4] These complexes have strong emission properties in the visible and near-infrared regions of the electromagnetic spectrum which makes them useful for cellular imaging as they can be used to visualise cells and other biological structures with high resolution and sensitivity. For example, the Keyes group have previously published an endoplasmic reticulum (ER) targeted ruthenium complex, $[\text{Ru}(\text{bpy})_2\text{-phen-Ar-ER}]^{9+}$ that could be used for STED imaging to achieve clearer images for visualising the tubular structure of the ER.^[23] Recently, Huang *et al.* demonstrated the use of $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$ (Figure 1.1A) as a probe to visualise nuclear and mitochondrial DNA using both STED and SIM.^[40] An example of the enhanced resolution of chromosomes using STED vs CLSM is shown in Figure 1.6. Additionally, a wide range of other luminescent metal complexes have been reported for super-resolution imaging.^[41–43]

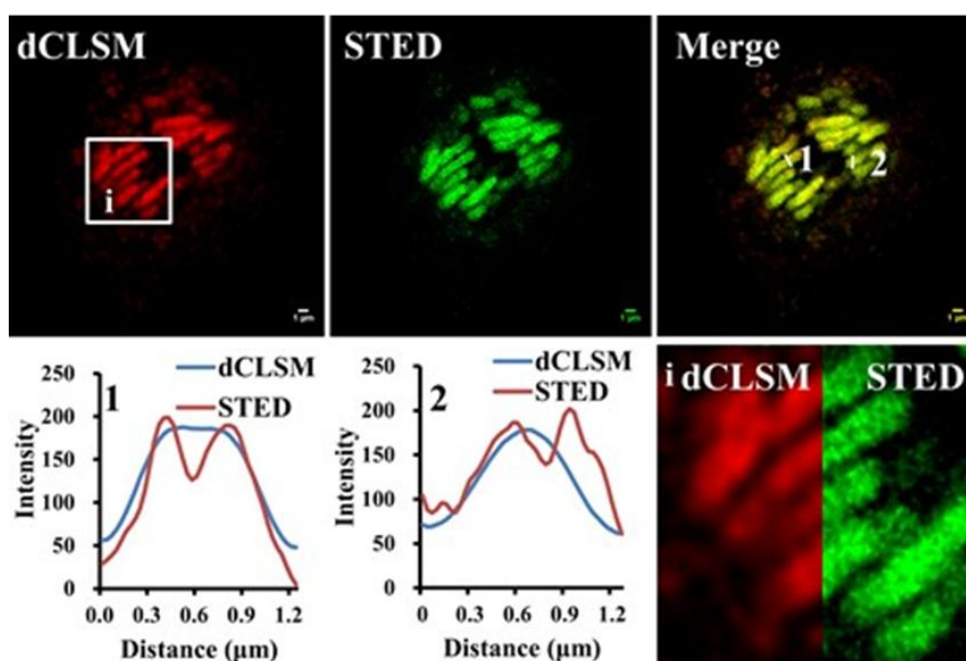


Figure 1.6 Confocal and STED images of $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$ staining chromosomes in HeLa cells and corresponding plot profiles at ROI 1 and 2 indicated on the merged image, highlighting the improved resolution with STED in comparison to confocal microscopy. 775 nm STED depletion laser. Adapted from Reference 40.^[40]

1.3 Transport Across the Cell Membrane

1.3.1 Mechanisms of Cellular Uptake

For a luminophore to enter the cytosol of a cell from the extracellular environment it needs to cross the cell membrane, a semi-permeable 2D fluidic assembly of phospholipids and protein. Luminescent complexes may enter cells through various uptake mechanisms including passive or facilitated diffusion, active transport, and endocytosis.^[44] These mechanisms are typically categorised as either energy-dependent or energy-independent (Figure 1.7). Energy dependence can be deduced by switching off metabolic pathways either by incubating the cells at 4 °C or by ATP-depletion using metabolic inhibitors such as treating the cells with a combination of 2-deoxy-D-glucose (2DG) and oligomycin and monitoring the uptake of the complex, a method first reported for ruthenium complexes by the Barton group.^[45]

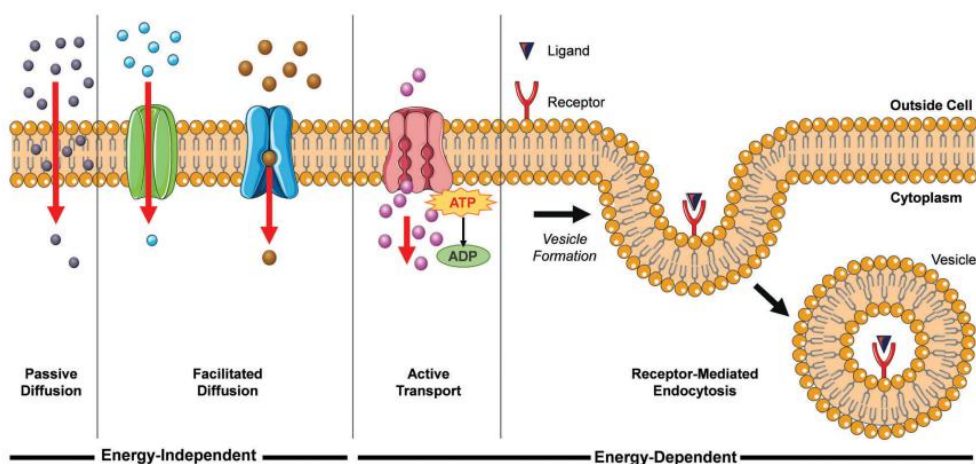


Figure 1.7 Schematic of the potential mechanisms by which molecules can enter cells, reproduced from Reference 46.^[46]

Passive uptake is energy independent and operates via diffusion along a concentration gradient.^[47] Facilitated diffusion utilises a similar mechanism to passive diffusion but requires the assistance of membrane transport proteins to permeate into the cell. Passive mechanisms of uptake could be exploited depending on the physicochemical properties of the molecule, such as size, lipophilicity, and charge. In general, small, hydrophobic, and non-charged molecules are taken up into cells by passive diffusion.^[48]

In contrast, active uptake mechanisms such as active transport and endocytosis are energy dependent. Active transport utilises energy provided by membrane ATPase enzymes and internal ATP to overcome the uptake barrier.^[45] Endocytosis is an energy-dependent process where incoming molecules are enveloped within membrane-bound vesicles, facilitating their transport across the lipid bilayer.^[49] Following transport across

the membrane, escape from endocytic vesicles can be facilitated through interactions with endosomes and lysosomes.^[50]

1.3.2 Strategies to Achieve Cellular Uptake and Targeting

1.3.2.1 BODIPY-based Compounds

BODIPY-based compounds are generally more membrane-permeable than luminescent transition metal complexes due to their size, lipophilicity and lower molecular weight. Nevertheless, various chemical modifications have been explored to further enhance their cellular uptake and improve organelle targeting. BODIPY compounds can be conjugated to biomolecules such as lipids, sugars, or peptides to enhance cellular uptake and target specificity. For example, Gao *et al.* reported a pair of mitochondrial targeted fluorophores, where the BODIPY is attached to either triphenylphosphonium (TPP-BODIPY, Figure 1.1E) or tetraethylammonium (TEA-BODIPY, Figure 1.1F)^[51] and Wang *et al.* reported a BODIPY-based derivative, Mito-BDP-630 (Figure 1.1G), with electron donor–acceptor structure for mitochondrial imaging.^[52] Additionally, many BODIPY dyes naturally target lipid droplets within cells due to their high lipophilicity.^[33] Despite the advantages of using BODIPY-based compounds for cellular imaging, their poor aqueous solubility remains a key limitation, and there is a fine balance between improving water-solubility and maintaining effective cellular uptake.^[53]

1.3.2.2 Luminescent Metal Complexes

As a result of great efforts over the past two decades, a number of successful approaches have emerged to tune Ru(II) complexes and related organometallic complexes for enhanced cell uptake and targeting. One approach that has been explored to address the poor cell membrane permeability of luminescent metal complexes involves the use of agents such as DMSO, or detergents such as digitonin and Triton-X to enhance cellular uptake by permeabilising the membrane.^[9,54,55] However, these methods are associated with cell damage and can irreversibly alter the cell membrane. Furthermore, the complexes cannot be said to be cell permeable if they require the use of permeabilisation agents to achieve cellular uptake. The recommended concentration of DMSO is <5% v/v, though control studies completed by the Keyes group in collaboration with the Dietzek-Ivanšić group have indicated a reduction in MCF-7 cell viability at only 3.8% DMSO (24 h).^[56]

Tuning the lipophilicity and charge of Ru(II) complexes is another approach that has shown success for enhancing cellular uptake without the requirement of permeabilisation agents. Common strategies to modify the lipophilicity of metal complexes include attaching alkyl chains, poly-ethylene glycol (PEG), triphenylphosphonium (TPP), or other

lipophilic organic groups, as well as directly altering the lipophilicity of the ligands themselves.^[4] The Barton group pioneered investigations into the importance of lipophilicity, reporting a series of ruthenium(II) dppz complexes with varying auxiliary ligands to alter lipophilicity. Cell uptake of the lipophilic $[\text{Ru}(\text{DIP})_2(\text{dppz})]^{2+}$ complex was observed at only 0.5 μM , a significantly lower concentration than was required for the less lipophilic complexes of the series, for example $[\text{Ru}(\text{bpy})_2(\text{dppz})]^{2+}$ required 5 μM indicating that increasing lipophilicity directly correlates with increased cell uptake.^[57]

To render ruthenium complexes cell permeable and improve targeting, they have been modified to include specific functional groups or ligands. The addition of these functional groups allows researchers to both enhance the cellular uptake of a complex and target cellular organelles with greater precision. Among the strategies aimed at enhancing the performance of these luminescent complexes in cellular imaging and theranostic applications, bioconjugation to peptides has been a promising avenue of exploration.^[4] Peptides are composed of up to 50 amino acid residues and hold potential for highly specific targeting.^[58,59] Cell penetrating peptides (CPPs) have proven highly effective in enhancing the cellular uptake of ruthenium complexes. They facilitate not only improved intracellular delivery but also enable precise targeting to specific organelles within the cell using specific organelle targeting sequences, which has the potential to enhance the therapeutic or diagnostic efficacy of a ruthenium complex. The first generation of CPPs consisted of poly-arginine sequences, such as octa-arginine (R8) sequences, which initially improved cell uptake but exhibited unpredictable localisation. Subsequent advancements include mitochondrial penetrating peptides (MPP) and nuclear localising peptides (NLS), which have shown considerable success for targeted cellular uptake.^[60] Another class of peptides gaining popularity are those that target guanine quadruplex (G4) DNA or RNA, inspired by the G4 binding domains of G4 binding proteins.^[61]

1.4 DNA and RNA

1.4.1 General Structural and Biological Aspects of DNA and RNA

Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) play essential roles in the storage, transmission, and expression of genetic information in living organisms. DNA is a double-stranded molecule composed of nucleotide building blocks, each containing a sugar-phosphate backbone and one of four nitrogenous bases: adenine (A), cytosine (C), guanine (G), and thymine (T). The sequence of these bases along the DNA strand encodes the genetic instructions necessary for the development, functioning, and reproduction of an organism. Mitochondrial DNA (mtDNA) is distinct from nuclear DNA in several key aspects. Unlike the linear chromosomes of nuclear DNA, mtDNA exists

as small circular chromosomes within the mitochondria. Each mitochondrion contains multiple copies of mtDNA, which encode essential genes for mitochondrial function, including those involved in oxidative phosphorylation. Unlike nuclear DNA, mtDNA lacks protective histones and has a more compact genome structure, making it more susceptible to damage from reactive oxygen species. This vulnerability is particularly relevant in the context of phototherapeutic strategies targeting the mitochondria and will be discussed further in Chapter 4.

RNA is structurally similar to DNA, but is usually single stranded with a ribose sugar instead of deoxyribose, and the uracil (U) base is found in place of T. The first discovered and most common form of DNA is B form (Figure 1.8), which was reported by Watson and Crick in 1953.^[62] It was later understood that DNA can adopt many forms, including A-DNA, Z-DNA, and the less common triplex, i-motif, four-way junctions, and guanine quadruplexes (G4s).^[63]

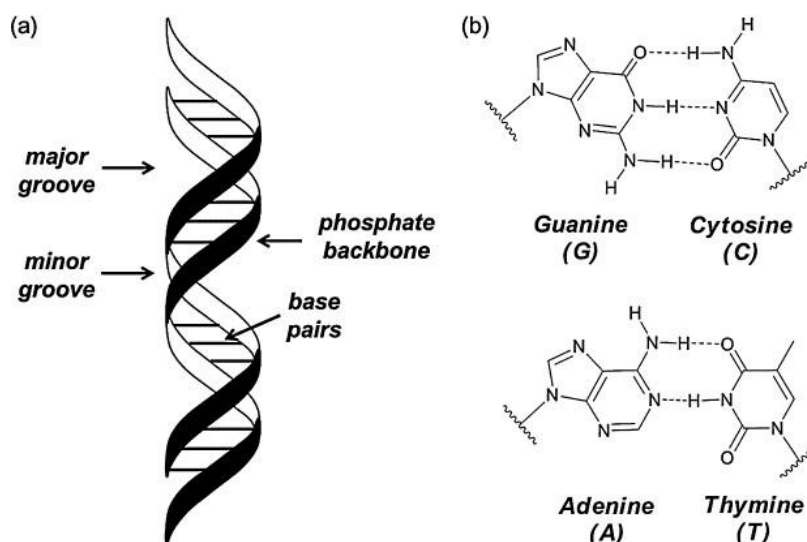


Figure 1.8 (A) depicts the DNA double helix with grooves, phosphate backbone and base pairs and **(B)** base pairs of guanine-cytosine and adenine-thymine formed by hydrogen bonding. Reproduced from Reference 64.^[64]

1.4.2 Guanine Quadruplexes

G4s are non-canonical DNA or RNA structures which form from G-rich sequences under physiological conditions and are stabilised by Hoogsteen hydrogen bonding.^[65] The term non-canonical refers to forms of DNA or RNA that do not conform to typical Watson-Crick base pairs.^[66] The guanine tetrads are further stabilised by the presence of monovalent cation (Figure 1.9) such as sodium, potassium or lithium with a preference order of $K^+ > Na^+ > Li^+$, where G4s formed in the presence of K^+ are thought to be more biologically relevant as there is a high intracellular K^+ concentration (~140 mM) compared to Na^+ (5-15 mM).^[67–70] Due to their physiological significance Na^+ and K^+ are

the most extensively characterised cations for G4 stabilisation, however there are a number of other cations that can promote G4 stabilisation, including other monovalent cations such as NH_4^+ and Cs^+ as well as divalent cations Ba^{2+} and Pb^{2+} , to name a few.^[69,71–73] Upon stabilisation by the cation, guanine tetrads stack together to form a G4, which is further stabilised through Van der Waals forces.^[74] Though G4s were initially recognised in DNA, these structures were later identified in RNA, and the role of G4s can differ between the two nucleic acids. RNA guanine quadruplexes (rG4s) differ from their DNA counterparts due to the presence of a 2'-hydroxyl group in the structure of their ribose sugar, which has been linked to the increased stability of rG4s due to the increased intramolecular interactions it can introduce. 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 greater variety of structures.

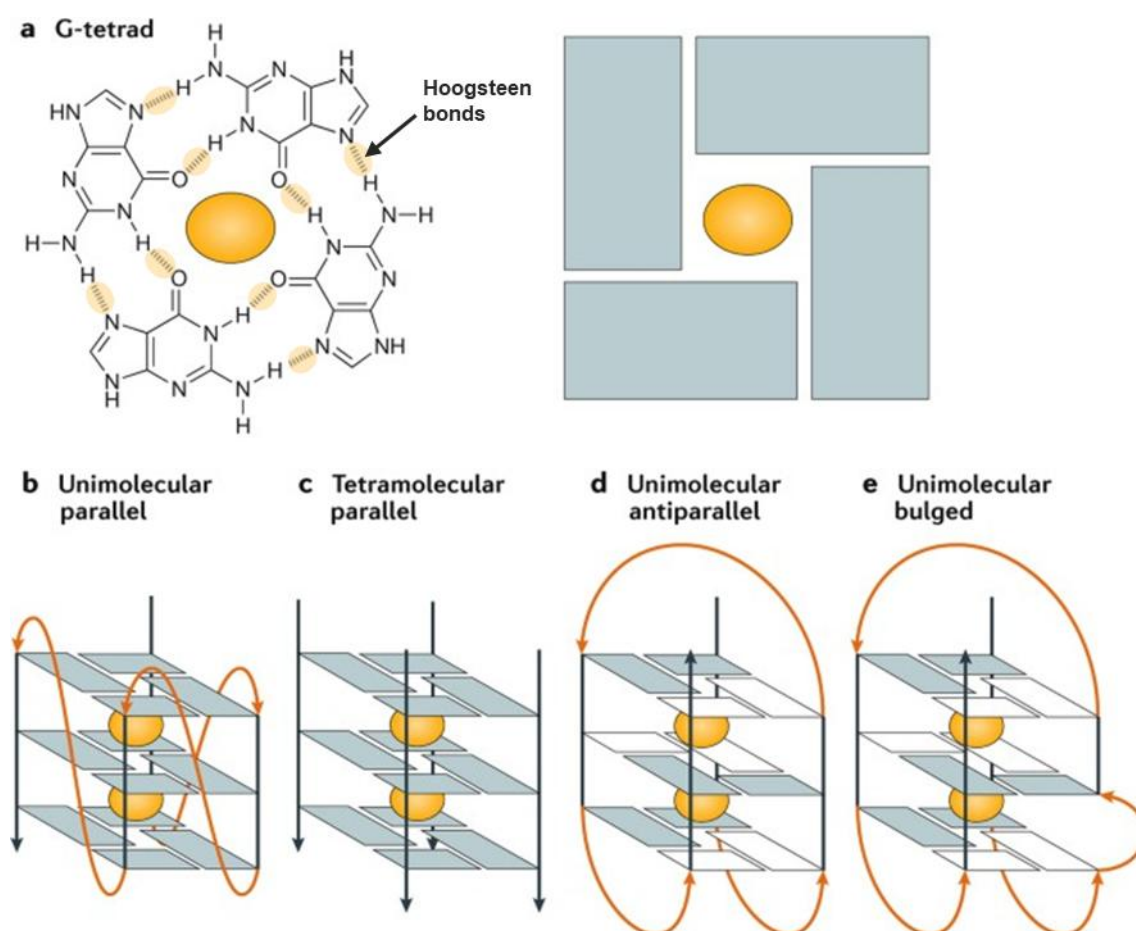


Figure 1.9 Guanine quadruplex structures adapted from Reference 75.^[75]

In DNA, G4s play an important role in numerous biological processes, including gene regulation, genome stability, and telomere maintenance, which has been extensively studied.^[76,77] Their dynamic nature allows them to serve as potential therapeutic targets, as stabilising or disrupting G4s can modulate gene expression and impact cellular

processes. Furthermore, their involvement in telomere maintenance highlights their significance in cellular aging and tumorigenesis, making G4s attractive targets for anticancer therapies.^[78,79]

The design and development of charge-transfer compounds for DNA binding is an active area of biomedical research, especially the development of probes for targeting non-canonical DNA structures. Duplex DNA is tightly packed within nucleosomes, limiting the accessibility for charge-transfer complexes or other DNA-binders.^[80] The partial unfolding of DNA during cellular processes like replication and transcription exposes single strands that can form non-canonical DNA structures, like G4s, which provide unique binding sites for charge-transfer compounds. Another important type of G4 are mitochondrial G4s (mitoG4s).^[81] Recently, Kuang *et al.* have reported a G4-binding tolyl-terpyridin-platinum complex (Pt-ttpy) that disrupts mitochondrial function, which they suggest is caused by nuclear G4 binding and likely mitoG4 binding.^[82] Chapter 4 of this thesis describes a novel mitoG4 targeted Ru(II) complex for imaging and phototherapy.^[83] Though there are over 200 potential G4 forming sequences in the mitochondria, they have not been extensively studied to date, making this an interesting area of research.^[84,85]

In comparison, RNA G4s (rG4s), have emerged as key regulators of gene expression and mRNA processing.^[86] Moreover, a link between rG4s and the formation of stress granules (SGs) has been reported, though this link is not yet fully elucidated.^[87,88] SGs are membraneless condensates that form when a cell experiences stress (e.g. heat shock or high arsenic salt concentrations) and are comprised of RNA and proteins held together through liquid-liquid phase separation.^[89] At present there are no commercially available imaging agents for visualising SGs in a live cell environment, therefore it is of great interest to researchers to develop a dye that can achieve this. A charge-transfer compound such as ruthenium(II) is a great candidate for this application due to its desirable photophysical characteristics mentioned previously.^[90]

Overall, the structural and functional complexity of DNA and RNA G4s make them intriguing targets for studies aimed at better understanding their biological significance and exploiting their potential for biomedical applications. In recent decades, G4s have grown from a 'structural curiosity' to an important biological target, and charge-transfer probes show great promise for theranostic targeting of these structures.

1.4.2.1 Validation of G4-Binding in Cells

To determine if a probe is binding to G4s in cells, a number of techniques can be employed including BG4 immunostaining, treating cells with rG4 probe QUMA-1 or

transfecting the cells with the SG4 nanobody. G4-specific probes are vital for enhanced understanding of the structure and function of G4s. BG4, shown in Figure 1.10A, is a popular G4-binding probe for visualising G4s and can be used for co-localisation studies. In this case, cells treated with the test compound are fixed, permeabilised, blocked using an appropriate blocking buffer and incubated with the BG4 antibody. A blocking buffer is added to the cells prior to the antibody incubation step to decrease non-specific antibody binding and can be performed with serum from the same species as the secondary antibody or with a universal blocking agent such as BSA (bovine serum albumin). The cells can be incubated with BG4 (the primary antibody) in serum for several hours at room temperature or overnight at 4 °C. The ratio of BG4:buffer should be optimised in untreated control cells to ensure adequate brightness for imaging prior to any co-localisation studies. Following the incubation with BG4, a secondary anti-FLAG rabbit monoclonal antibody (mAb) can be added to the cells, which recognises a FLAG tag epitope that is present in the BG4 antibody. Finally, a fluorophore must be added to enable visualisation of the G4 structures and should be selected to ensure minimal spectral overlap of BG4 and the new G4-targeting probe being analysed. In this thesis, an AlexaFluor antibody 'goat anti-rabbit IgG' was used to visualise BG4. This antibody is goat-derived and designed to bind specifically to the anti-FLAG rabbit mAb. The co-localisation of the new probe and BG4 can be determined by overlaying the channels and determining the overlap. Imaging software, such as ImageJ, is often used to display a graph of the spectral overlap between two probes to confirm co-localisation or to calculate Pearson's Correlation Coefficient, or similar correlation values which correspond to the overlap in pixel intensities between the two probes.^[91]

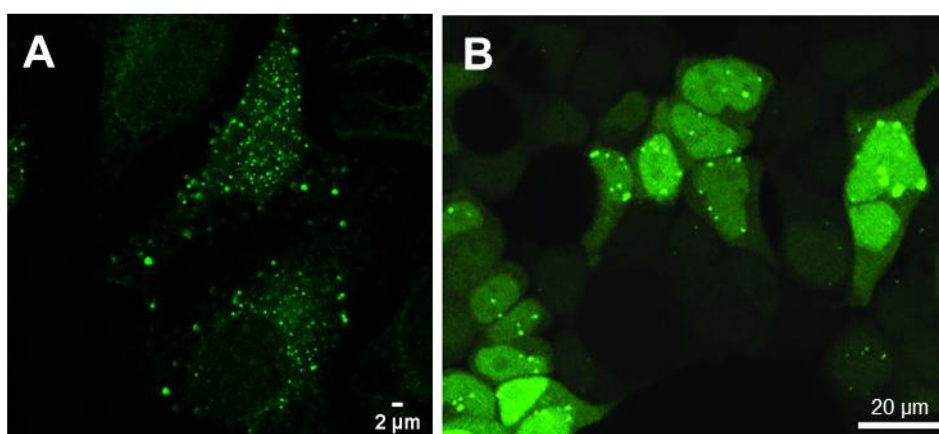


Figure 1.10 Confocal images of **(A)** BG4 in a fixed HeLa cell and **(B)** SG4 in live HEK293T cells expressing SG4-GFP. Figure **(B)** reproduced from Reference 92.^[92]

Though BG4 has been used successfully as a G4 probe, it is limited by its inability to bind to G4s in live cells. As a result, the Balasubramanian group developed the SG4 nanobody (Figure 1.10B), which exhibits high affinity for a range of folded G4 structures

in vitro and can be transfected into cells for live cell imaging and analysis.^[92] Additionally, BioTracker QUMA-1 dye can be used as a live or fixed cell dye for studying rG4 behaviour.^[93] Nonetheless, there remains a need for the development of more G4-targeted probes which are targeted to specific G4 structures like mitoG4s, to further elucidate the dynamics of these non-canonical DNA and RNA structures *in vivo*.

1.4.2.2 G4-Binding Probes

Small molecule G4 ligands have traditionally focused on polycyclic planar compounds, such as Phen-DC3, Braco-19, and telomestatin, which stabilise G4 structures by stacking on the G-tetrads.^[94,95] These ligands can inhibit telomerase activity and induce a DNA damage response, thus providing anticancer effects. In recent decades there has been increased interest in transition metal-based complexes as probes for DNA and G4s. Notably, in 1990, Barton and collaborators reported $[\text{Ru}(\text{bpy})_2(\text{dppz})]^{2+}$ for use as a luminescent DNA probe (where dppz is dipyrdo[3,2-a:2',3'-c]phenazine).^[96] To date, this complex remains the most well-known ruthenium(II) polypyridyl complex for studying DNA, alongside $[\text{Ru}(\text{phen})_2(\text{dppz})]^{2+}$.^[90,97] These complexes are often referred to as molecular 'light-switch' probes, as they exhibit almost no luminescence in aqueous solvents but fluoresce when bound to DNA.

Shi and co-workers studied both $[\text{Ru}(\text{phen})_2(\text{dppz})]^{2+}$ and $[\text{Ru}(\text{bpy})_2(\text{dppz})]^{2+}$ (Figure 1.1A and 1.1B) binding to G4 and i-motif DNA and found that $[\text{Ru}(\text{phen})_2(\text{dppz})]^{2+}$ luminescence was five times higher when bound to G4 DNA than when bound to i-motif DNA.^[98] They also observed differential binding of $[\text{Ru}(\text{bpy})_2(\text{dppz})]^{2+}$ with high affinity for 22AG, human telomeric G4 DNA. Ruthenium complexes offer strong and specific binding to G4s with excellent photostability, and more versatility as they rely on MLCT for their photophysical properties like efficient generation of reactive oxygen species (ROS), which enables both imaging and therapeutic applications.^[90] This versatility is exemplified by the wide array of ruthenium complexes that have been developed to enhance binding affinity and specificity toward G4s. For example, the Shi group reported the complex $[\text{Ru}(\text{tatp})_2\text{ASC}]^{2+}$ (tatp = 1,4,8,9-tetra-aza-triphenylene, ASC = ascididemin) which demonstrates significant binding and stabilisation properties (Figure 1.1C). $[\text{Ru}(\text{tatp})_2\text{ASC}]^{2+}$ binding to G4s htelo and c-kit resulted in an increase in melting temperature (ΔT_m) values by 11.5 °C and 11.0 °C respectively, indicating robust stabilisation of the G4 structures.^[99] Similarly, the Thomas group reported a nuclear-localising ruthenium tap complex (tap = 1,4,5,8-tetraazaphenanthrene) $[\{\text{Ru}(\text{tap})_2\}_2\mu\text{-tpphz}]^{4+}$ which exhibited binding affinity to a human telomere G4 sequence, though it showed a stronger affinity for duplex DNA. This complex was investigated for its potential in both cell imaging and phototherapy using C8161 melanoma cells (Figure 1.1D).^[100]

These studies, along with the rest of the literature, highlight the strong G4 binding capabilities of ruthenium complexes and their potential as phototherapeutic agents.

Though luminescent transition metal complexes have received more attention as G4 probes, BODIPY-based probes have been developed for G4 targeting and even cellular sensing.^[101,102] Chen and co-workers designed a push-pull solvatochromic BODIPY dye, BSK for ratiometric sensing of G4s.^[103] BSK is based on a D- π -A structure and, when excited, exhibits intramolecular charge-transfer (ICT) from the donor to the acceptor. Upon G4 binding, the local environment around BSK changes, affecting the ICT process and altering the fluorescence signal. Probes designed for ICT are highly sensitive to environmental changes, however they can also be susceptible to non-specific interactions with surrounding biomolecules and cellular components. Charge-transfer compounds that utilise distinct donor-acceptor pairs may be designed to mitigate some of these non-specific interactions. This approach has the potential to enable the development of more robust and selective probes, enhancing their effectiveness in G4 imaging and sensing applications.

Despite the advantages of ruthenium complexes as G4 probes they are not without their own challenges, for example cytotoxicity and higher synthesis costs in comparison to organic compounds. Hence, when designing probes for targeting G4s the choice of compounds, both charge-transfer or otherwise, depends heavily on the desired application. The focus may be precise imaging and sensing of G4s in a live cellular environment or combining imaging with therapeutic capabilities.

1.5 Phototherapy

The term 'phototherapy' refers to a range of treatments that utilise light to activate photosensitising compounds, leading to the targeted destruction of diseased cells. Central to the efficacy of these treatments are the underlying photophysical processes that control energy transfer and charge separation within photosensitisers (PSs). Charge-transfer compounds are particularly significant as phototherapeutics due to their ability to engage in various energy transfer mechanisms, including Intramolecular Charge-transfer (ICT), Photoinduced Electron Transfer (PET), and Resonance Energy Transfer (RET), which encompasses both Förster Resonance Energy Transfer (FRET) and Dexter Energy Transfer (DET).

FRET is a coulombic, collision-free, radiationless energy transfer mechanism that occurs from an excited donor (fluorophore) to a suitable acceptor (a protein or fluorophore) when they are in close proximity (1-10 nm).^[104] In contrast, DET (Figure 1.11) is an energy transfer process that occurs by short-range electron exchange mechanism it

requires intimate contact between donor and acceptor and efficiency is dependent on donor-acceptor orbital overlap and spin state.^[105] DET is responsible for singlet oxygen generation and plays a key role in oxygen quenching. This process is particularly important in the design of luminescent transition metal-based probes for photodynamic therapy (PDT), where efficient energy transfer can enhance the production of ROS.

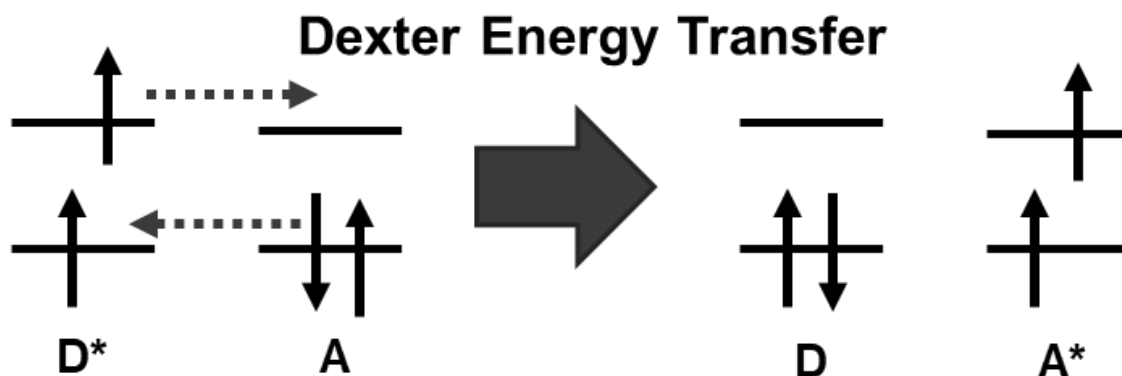


Figure 1.11 Triplet-triplet DET mechanism. The horizontal lines indicate HOMO and LUMO energy levels and the dashed arrows represent simultaneous rearrangement of the electronic configuration. D = donor, A = acceptor, * = excited state. Inspired by Reference 106.^[106]

Additionally, ICT is common in BODIPY-based systems and involves the movement of charge within a molecule upon excitation, often resulting in significant changes in fluorescence properties and enhanced ROS generation upon light activation for PDT. PET is another crucial process, especially in transition metal-based complexes. In PET, the absorption of light leads to the excitation of the electron, which is transferred from the photoexcited donor molecule to an acceptor molecule. This process is essential in generating ROS for PDT, leading to oxidative stress and subsequent cell death.

Each of the energy transfer mechanisms are critical in determining the photophysical behaviour of PSs including charge-transfer compounds, ultimately influencing their effectiveness in phototherapy. Understanding and optimising these energy transfer processes are essential for advancing both PDT and photoactivated chemotherapy, where precise control over the photosensitisation process is key to achieving therapeutic specificity and efficacy.

1.5.1 Absorbance and Fluorescence-Based Assays for Assessing Therapeutic Potential

The primary step in drug discovery often involves absorbance and fluorescence-based assays performed using conventional plate readers to determine the potential of new compounds to become therapeutic agents and to gather more information regarding their mechanisms of toxicity. To measure cell viability cytotoxicity assays such as alamar blue

or 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) are often employed. In the case of the alamar blue assay, a 10% resazurin-media solution is added to the cells, where the dark blue resazurin solution is reduced to pink resorufin in the presence of metabolically active cells. The results can be determined using either the absorbance or fluorescence intensity of the solutions. The MTT assay is based on the transformation of a water-soluble (yellow) tetrazolium salt, which is reduced to purple formazan crystals by mitochondrial succinic dehydrogenase in the mitochondria of viable cells.^[107]

CellTiter-Glo is a luminescent cell viability assay that uses ATP, a required co-factor of the luciferase reaction, as an indicator of metabolically active cells. The enzyme luciferase acts on luciferin in the presence of ATP to produce oxyluciferin and to release energy in the form of luminescence. Because the luciferase reaction relies on ATP, only living cells in the sample can produce a signal.

There are advantages and disadvantages to all viability assays. Assays which measure ATP for determining cell viability, such as CellTiter-Glo, are the most sensitive and convenient assays in comparison to redox assays like alamar blue and MTT, however it is also a more expensive method. To assess cell viability in cell monolayers, redox assays offer adequate performance, however they are not as effective in 3D cell models where the more expensive CellTiter-Glo 3D assay may be required.

When assessing the potential of new therapeutics, it is not only important to determine the effect on cell viability, the overall effect of the compound on biological systems is of interest. For example, the effect of a new compound on the mitochondrial membrane potential of test cells can be important, particularly in cells that are specifically targeting the mitochondria to induce cell death. Photodamage-induced cell death occurs through several key mechanisms, primarily necrosis, apoptosis, and autophagy. Necrosis is the dominant form of tumour cell death when a PS localises in the plasma membrane and lactate dehydrogenase (LDH) release assays can be employed to detect this mechanism of cell death. In contrast, apoptosis is often triggered by mitochondrial damage, which initiates a cascade of caspase activation leading to programmed cell death. Mitochondrial membrane potential assays are a key indicator of mitochondrial health and can be employed as an early marker of apoptosis. To further probe the mechanism of cell death, caspase assays can be performed. The FLICA polycaspase assay provides a broad-spectrum approach and detects multiple active caspases simultaneously. Autophagy, on the other hand, acts as a protective mechanism, attempting to repair cell

damage, though the extent of damage can overwhelm autophagic capacity and lead to cell death.

For reproducible results, regardless of the assay, a number of criteria are essential including: optimising the reagent incubation time and conditions for positive or negative controls for each test cell line and performing biological replicates using a consistent source of cells that are carefully maintained and counted accurately to ensure the correct seeding concentration depending on assay type and cell line.

1.5.2 Photodynamic Therapy

It is widely recognised that cell death can be induced by the interaction of light with phototoxic agents, a concept that was initially reported in 1900.^[108] Photodynamic therapy (PDT), a term coined by Hermann von Tappeiner in 1904,^[109] is a medical treatment that uses a photosensitiser (PS) in combination with visible or near-infrared light (linked to the absorption of the PS) to produce cytotoxic ROS and destroy abnormal cells. Molecular oxygen availability is essential for successful PDT as the PS directly interacts with oxygen to generate $^1\text{O}_2$ (Type II PDT) or form radicals such as superoxide anions, hydrogen peroxides and hydroxyl radicals (Type I PDT).^[110] These mechanisms are illustrated in Figure 1.12.

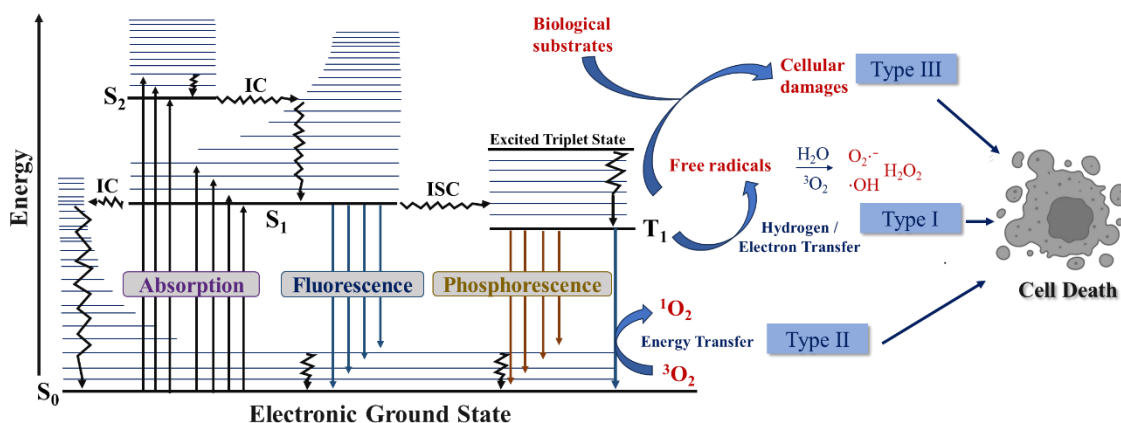


Figure 1.12 Illustration of the mechanisms of phototherapy. Inspired by References 14 and 111.^[111]

The ideal characteristics of a PS include minimal dark cytotoxicity, high photocytotoxicity, low skin photosensitivity, tumour-specific accumulation, and ease of administration.^[112] Furthermore, strong absorption with a high extinction coefficient is advantageous.^[113] Additionally, a low light flux requirement allows for effective treatment with reduced light exposure and the use of low fluence rates has been shown to increase apoptosis, the preferred pathway for inducing cell death.^[114,115] While necrosis can stimulate an immune response, apoptosis is more desirable due to its controlled nature.

Although Photofrin, the first clinically approved PDT agent, has shown success in a clinical setting since its approval in 1993, its potential has been challenged by the side effects associated with its use including prolonged skin photosensitivity and its weak absorption in the near-infrared region (activated at 630 nm).^[110,116] This is important as PSs that can be activated at wavelengths as close to the near-infrared (NIR) as possible are required for treating deeper or larger tumours (Figure 1.13).

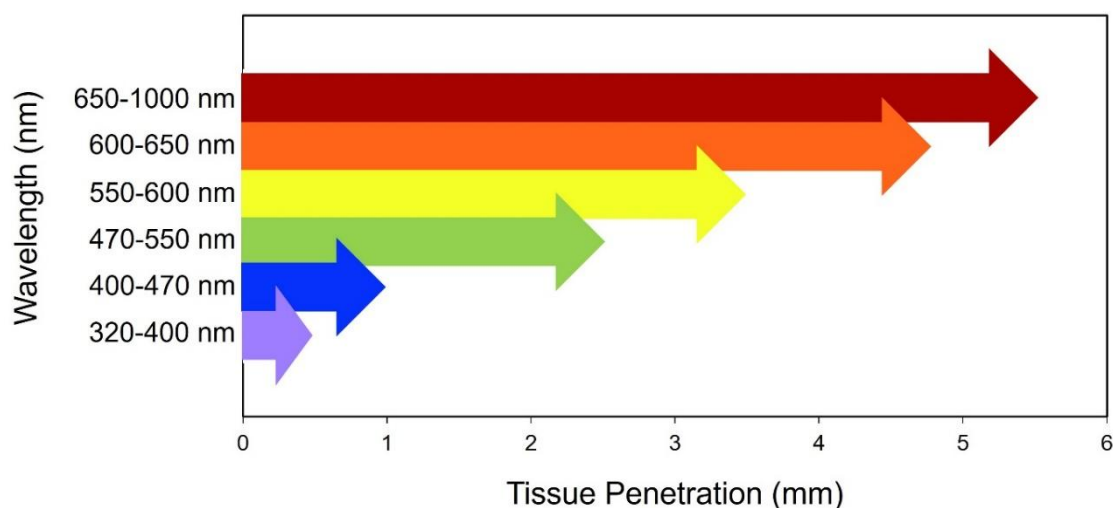


Figure 1.13 Penetration depth of light into tissue.

Second-generation PSs were developed to overcome these limitations, including mesotetra hydroxyphenyl chlorin (also known as mTHPC or Foscan), which received clinical approval for the treatment of advanced head and neck cancer in 2001.^[117] Second-generation PSs are said to have a stronger ability to produce cytotoxic $^1\text{O}_2$ and exhibit a red-shifted absorption. To further improve the efficacy of PDT agents, a third generation are being developed which utilise targeting and delivery moieties, for example ligands for targeting specific subcellular locations or peptides in combination with liposomes.^[118]

Table 1.1 Phototoxicity Values of Ruthenium Complexes and Reference Agents (Photofrin / Cisplatin)

<i>Photosensitiser</i>	<i>Cell Line</i>	<i>Dark IC₅₀ (μM)</i>	<i>Light IC₅₀ (μM)</i>	<i>PI^a</i>	<i>Light Dose (J/cm²)</i>	<i>λ (nm)</i>	<i>Ref</i>
[Ru(bpy)₂dppz]²⁺ Compound 2	HeLa	>100	2.12 ± 0.11	>47	9.27	420	[119]
[Ru(tap)₂(pdppz)]²⁺	HeLa	70 ± 6.3	8.8 ± 2.9	12.5	18	≥ 400	[120]
[Ru(phen)₂pdppz]²⁺	HeLa	>100	>100	n.d.	18	≥ 400	[120]
[Ru(biq)₂(phen)]²⁺	HL-60	47.3	2.3	20	14	>600	[121]
RuL4 Ru(II) polypyridyl complex	HeLa	>100	3.5 ± 1.1	>28	12	450	[122]
TLD-1433	CRMM2	1	0.0048	210	19	520	[123]
Photofrin	HeLa	2.6 ± 0.12	>25	>10	5	400-650	[124]
Cisplatin^b	HeLa	9.0 ± 0.8	8.8 ± 0.8	1	12	450	[122]

^aPI = phototoxic index, the ratio of dark IC₅₀ to light IC₅₀, ^bCisplatin is a well-established cytotoxic agent that does not exhibit phototoxicity and is often used to compare IC₅₀ values, n.d. = not determinable.

Despite their success, there are still some drawbacks associated with the clinically approved PSs, including but not limited to, poor water solubility, complicated synthesis, photosensitivity caused by low excretion metabolism and low cancer cell selectivity.^[125] Thus, there remains a need for new and improved PSs with fewer limitations. Furthermore, successful phototherapy relies on the efficient delivery and activation of the therapeutic agent within the target cells or tissues and therapeutic efficacy can be limited significantly by poor uptake or uneven distribution of a PS.^[126] Inductively coupled plasma-mass spectrometry (ICP-MS) is an analytical technique that can be used to determine uptake or localisation of a PS, but is expensive and time-consuming. In contrast, designing probes that combine phototherapeutic functions with imaging capabilities offers a more straightforward and cost-effective alternative for assessing drug uptake and localisation.

Luminescent transition metal complexes have been studied extensively for their potential in PDT. Ruthenium complexes in particular, featuring a variety of ligands have garnered

significant attention as phototherapeutics. Among these, polypyridyl ligands, are particularly noteworthy for their ability to enhance MLCT properties of the complex.^[127] A selection of ruthenium-based PSs reported in the literature are shown in Table 1.1 along with clinically approved Photofrin (light activated) and Cisplatin (dark toxic). The fluence rates selected for the phototoxicity studies reported in this thesis (5–17 J/cm²) balanced clinical relevance, literature precedent, and the need for rates low enough to demonstrate phototoxicity without introducing cell damage from excessive irradiation. Individual control studies on each cell line used for both cell monolayers and spheroids confirmed no toxicity from irradiation alone prior to any phototoxicity studies.

BODIPY-based dyes have not been studied as extensively for use in PDT in comparison to porphyrins or transition metal complexes as conventional structures tend to have limited triplet state yield. However, in recent years, BODIPY-based dyes have gained increasing interest as metal-free PSs due to their versatile nature, allowing for simple structural modifications that can lead to increased ¹O₂ yield or enhanced bioavailability.^[7,128] Specific targeting moieties can be attached to BODIPY dyes to enhance cellular uptake and localisation, therefore resulting in targeted localisation for PDT, for example Wang and Qian reported a lysosome targeting BODIPY (BDPI-Lyso, Figure 1.1H) with high ¹O₂ quantum yield, which showed good phototoxic potential with an IC₅₀ of 0.4 μM (30 minutes at 4 mW/cm², 525 nm).^[129]

The photophysical characteristics of BODIPY-based dyes that make them excellent imaging agents, such as their high photostability and tuneable absorption and emission wavelengths are amongst those that make them excellent candidates for PDT. Additionally, being metal-free, BODIPY compounds are less prone to issues related to metal accumulation in biological systems. Hence, current research in this area is focused on harnessing the unique characteristics of BODIPY-based dyes to develop more effective PSs for PDT with reduced side effects.

1.5.3 The Impact of Hypoxia on Phototherapeutic Efficacy

Hypoxia is a key feature of most solid tumours characterised by a low oxygen concentration of tissues and reportedly promotes tumour metastasis. Cancerous cells in tumours are reported to proliferate uncontrollably, leading to excessive consumption of oxygen and nutrients, contributing to hypoxia.^[130,131] The hypoxic nature of tumours significantly limits the effectiveness of many anticancer therapies including chemotherapy, radiotherapy and PDT. For example, the Bonnet group reported a ruthenium complex, [Ru(tpy)(biq)(STF31)]²⁺ (tpy = 2,2':6',2''-terpyridine; biq = 2,2'-biquinoline; STF31 = 4-(((4-(tert-butyl)phenyl)sulfonamido)methyl)-N-(pyridin-3-

yl)benzamide) with a PI value of 2.86 under normoxic conditions, reduced to 1.53 under hypoxia (630 nm, 21 J/cm²). Additionally, the Ruiz group reported a series of ruthenium complexes with general formula [Ru(phen)₂(N $\wedge$ N')]²⁺, where N $\wedge$ N' represents a bidentate ligand based on the 1-aryl-4-benzothiazolyl-1,2,3-triazole (BTAT) scaffold. In the case of Ru1, this ligand featured a methyl group (CH₃) substituent and treatment with the complex resulted in a normoxic PI of >24.4 and a hypoxic PI of >7.5 (465 nm, 14 J/cm²) in A375 melanoma cells.^[132,133]

Oxygen is required to generate the ROS required for PDT, therefore the low oxygen concentration of tumours reduces the efficacy of this treatment and the consumption of molecular oxygen for PDT can further increase tumour hypoxia, potentially leading to treatment failure.^[134] Additionally, tumour cells adapt to the anaerobic environment through the production of hypoxia-inducible factors (HIF), further contributing to treatment resistance.^[135]

There are a few key strategies for determining tumour hypoxia. An oxygen electrode can be used to evaluate the local oxygen concentration, the degree of hypoxia can be evaluated by monitoring endogenous markers like HIF-1 α or HIF-2 α and finally, exogenous probes such as pimonidazole and etanidazole pentafluoride (EF5) can be used to establish oxygen concentration.^[136] Furthermore, the development of probes that can determine intracellular oxygen levels based on their luminescent lifetimes is an active area of research.^[137,138]

1.5.4 Photoactivated Chemotherapy

Photoactivated chemotherapy (PACT), a term introduced by Sadler *et al.* in 2009 refers to a targeted cancer treatment that can overcome the oxygen-dependency limitation associated with PDT.^[139] Unlike PDT, PACT does not rely on the presence of oxygen to generate a toxic effect, rather it relies on the activation of a prodrug through photoirradiation which often involves charge-transfer processes that are critical to its function.^[140] For ruthenium polypyridyl complexes, light irradiation can populate various excited states including but not limited to ³MLCT, ³MC, ³ILCT, leading to different photochemical pathways.^[140] These excited states can lead to the generation of cytotoxic species that are crucial for the therapeutic effect in both PDT and PACT and ruthenium complexes can be fine-tuned to achieve specific therapeutic outcomes within this realm.^[141] The Glazer group first explored the potential of ruthenium polypyridyl complexes for PACT in 2012,^[142] and their work over the past decade has made a significant contribution to the field, particularly in addressing the challenge of achieving

effective phototoxicity under hypoxic conditions. More recently, the Glazer group studied a family of bis-heteroleptic strained Ru(II) complexes for their potential as PACT agents, though only one complex, the naphthalene-ligand-containing Compound 4, displayed promising phototoxicity under hypoxia with a normoxic phototoxic index (PI) of 22 and hypoxic PI of 15 (where PI is the ratio of dark IC₅₀ to light IC₅₀ values).^[143] The success of this compound, while noteworthy, emphasises the inherent difficulty in designing ruthenium probes that function effectively under hypoxic conditions, pointing to the potential need for alternative approaches or further structural modifications to improve the efficacy of such compounds. The Bonnet group has also made significant contributions to the field of PACT by investigating various ruthenium-based agents. For example, they studied a known PACT complex [Ru(bpy)₂(dmbpy)]²⁺ and a novel complex [Ru(Ph₂phen)₂(mtmp)]²⁺ in A549 lung cancer cells. PI values of 19 and 6 were observed for the complexes respectively after blue light irradiation at 6.4 J/cm².^[144] By examining the ¹O₂ generation, they confirmed the cytotoxicity stems from a PACT mechanism. Phototoxicity was attributed to the release of the dmbpy ligand for [Ru(bpy)₂(dmbpy)]²⁺, whereas in [Ru(Ph₂phen)₂(mtmp)]²⁺ the ruthenium photoproduct itself bears the toxic load, rather than the ligand.³

It is important to note that PACT agents require higher doses than PDT agents as they do not regenerate after producing cytotoxic species, whereas PDT agents act in a catalytic manner.^[145] This limitation poses a significant challenge in clinical applications, where higher doses may lead to increased side effects, therefore negligible dark toxicity is of utmost importance. Nonetheless, promising PACT agents have been developed to effectively target and destroy cancer cells in low-oxygen environments.^[139,146] For example, ruthenium-based complexes, such as TAP (1,4,5,8-tetraazaphenanthrene) derivatives like the Ru-TAP-PDC3 complex reported in Chapter 4, have emerged as potent PACT agents. Unlike prodrugs, TAP complexes directly interact with biomolecules and induce damage even under hypoxic conditions, offering a versatile approach for cancer therapy.

While many multimodal PDT/PACT agents have been reported, including the Sadler group's platinum-iridium complex for both PDT and PACT, where charge-transfer occurs between the donor Ir(III) and acceptor Pt(IV),^[147] the dual functionality of these probes raises questions about the complexity of optimising both PDT/PACT activity to be effective within a single agent.

1.6 3D Cell Models

Traditional studies using cell monolayers offer a simplified platform to obtain essential insights into cellular responses of various charge-transfer compounds, including new PSs, in any number of cell lines. These monolayers consist of adherent cells cultured on a flat surface and allow for a more direct examination of drug interactions with individual cells, offering a controlled setting to gain a broader understanding of drug behaviour before moving to more complex cellular models.^[148]

Despite the benefits of traditional cellular models in drug discovery, they cannot mimic the tumour environment accurately. For example, in a cell monolayer, all cells have the same access to nutrients, oxygen, and waste in the overlay medium, which is not the case in a tumour *in vivo*.^[149] Numerous 3D culture systems are available including organoids, organ(s)-on-a-chip, 3D bioprinting and spheroids (also known as multicellular tumour spheroids or MCTS).^[150] Spheroids present a simple 3D culture system and are being used increasingly in cancer research as an alternative to conventional 2D cell culture models or as an additional step between studies in cell culture and *in vivo*. Although 3D cell models cannot replicate tumours *in vivo* exactly, they have several vital characteristics in common with *in vivo* tumours that make them useful for evaluating prospective treatments and therapeutics.

The 3D arrangement of the cells in spheroids (Figure 1.14) enables cell-cell and cell-extracellular matrix interactions that are more similar to those observed *in vivo*, more accurately reflecting the uptake of a drug into a tumour.^[151] The increased cell-cell and cell-extracellular matrix interactions create a barrier, making drug or dye penetration more difficult in spheroids, in comparison to cells in a monolayer.

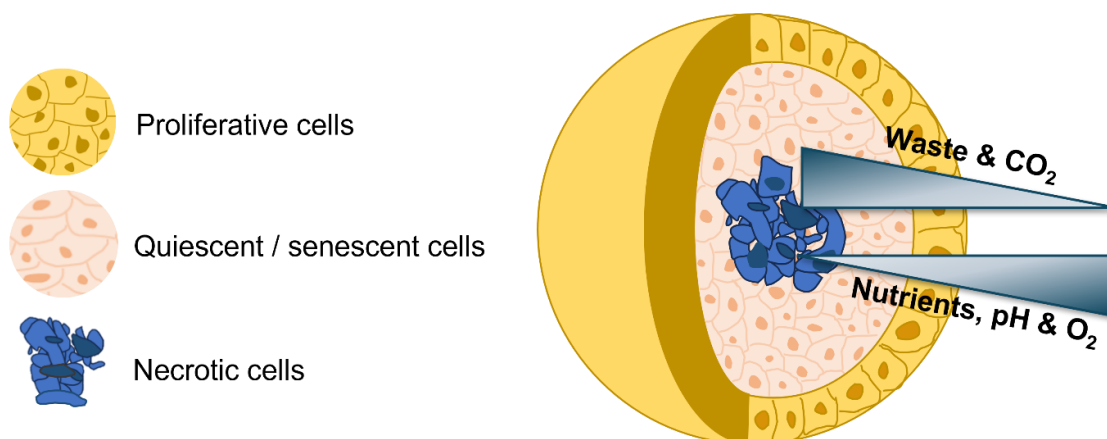


Figure 1.14 Graphic of a tumour spheroid indicating its layered structure.

Thus, spheroids and other 3D cell models enable researchers to gain a deeper understanding of how a test drug, for example, the Ru(II) and BODIPY-perylene charge-

transfer compounds described in this thesis, interact with cells and tumour models, shedding a light on their potential therapeutic properties. Nevertheless, despite the benefits of 3D cell models in drug discovery, there is still a need to establish routine 3D assays for analysing the efficacy of a potential imaging or therapeutic agent. At present, there is a number of methods which can be used to determine how spheroids react to a novel drug including; cell viability assays which mimic those performed in cell monolayers,^[17,83,152–154] performing live/dead cell staining and imaging the spheroids^[155] and many groups have studied the effects of novel compounds on spheroid growth over time in comparison to untreated control spheroids.^[156] The Gasser group utilised HeLa spheroid growth as a measure of phototherapeutic potential of a group of 7 Ru complexes.^[157] The spheroids were incubated with the family of ruthenium complexes and exposed to both 1P irradiation (500 nm, 10 J/cm²) and 2P irradiation (800 nm, 10 J/cm²) and both cell viability and growth inhibition were monitored. A significant decrease in tumour size was observed for irradiated spheroids compared to non-irradiated controls treated with the Ru complexes reported. These results demonstrate the value of monitoring the effect of novel PSs on spheroid growth to determine therapeutic potential.

While assessing spheroid cell growth offers crucial insights into the phototoxic potential of charge-transfer compounds, integrating this method with cell viability assays and advanced imaging techniques could significantly enhance the evaluation process of novel charge-transfer compounds. Furthermore, time-resolved fluorescence or phosphorescence methods such as FLIM and PLIM have been reported for 3D structures and could shed light on the dynamics of excited states and ROS generation in a tumour-like environment. PLIM has been employed successfully in HT-29 human colorectal adenocarcinoma spheroids by Mizukami *et al.*, using O₂ sensitive Ir(III) complexes. In this case, cells closer to the spheroid core that have access to lower levels of O₂ exhibited longer lifetimes than those in the outer layers.^[158]

1.7 Ruthenium Complexes in a Clinical Setting

The consistent interest in metal complexes for anti-cancer therapy can be attributed to the success of the first metal-based anticancer agent, cisplatin, which was approved in 1978.^[159] A number of ruthenium complexes have entered clinical trials including NAMI-A, KP1019/KP1339 and TLD-1433 (chemical structures in Figure 1.15).^[160–162] NAMI-A [ImH][trans-RuCl₄(dmsO-S)(Im)] (dmsO-S = sulfur-bonded dimethyl sulfoxide, Im = imidazole) was the first ruthenium drug to be tested in humans.^[163] Unfortunately it showed limited efficacy with only partial response in phase I and II clinical trials resulting in the end of the clinical investigations.^[159,164] BOLD-100 (sodium trans-[tetrachloridobis

therapeutics. Additionally, McFarland and co-workers have suggested that the future of transition metal complexes and PDT/PCT (photochemotherapy) will involve the developing regimens to stimulate antitumour immunity.^[171]

1.8 BODIPY-Transition Metal Complexes in Imaging, Sensing and Therapy

Recent advances in the design of luminescent probes have seen the incorporation of BODIPY dyes with transition metal centres. These complexes combine the strong fluorescence of BODIPY and therapeutic functions of the metal centre, creating versatile compounds with potential applications in both imaging, sensing and therapy.

While BODIPY-perylene systems rely solely on organic components to achieve charge-transfer, the inclusion of transition metals in BODIPY-transition metal complexes introduces metal-specific therapeutic effects.^[174] Metal complexes display unique redox chemistry, slow ligand exchange rates and are known to have multiple oxidation states.^[175,176] The choice between designing probes based on luminescent transition metal complexes, BODIPY-based compounds, or a combined BODIPY-transition metal complex approach may ultimately depend on the specific application of the probe. For example, when designing a probe for cellular sensing, negligible toxicity and high fluorescence are essential and, in this case, BODIPY-based compounds might be more desirable. In contrast, for a probe with both imaging and therapeutic capabilities, BODIPY-transition metal complexes could be more effective and in applications where high photostability, long luminescent lifetimes and a large Stokes shift are required, luminescent metal complexes alone may offer superior performance.

It is also important to consider organelle targeting, due to their lipophilic nature BODIPY-based compounds often localise in lipid droplets and other lipid-rich environments whereas transition metal complexes may be more desirable for specific organelle targeting through bioconjugation to peptides. Moreover, iridium complexes often target the mitochondria without the need for peptide conjugation.^[177] Other factors to consider include the optimum photophysical properties such as quantum yield, photostability and the cost and complexity of the synthesis. Overall, numerous factors must be considered when designing a new theranostic, each influencing the choice of charge-transfer system. Computational studies can be used to assist in the design of these drugs, for example the Mazzone group have reported computational work assessing the photosensitising potential of a Ru(II)-BODIPY assembly.^[178]

There have been a number of successful compounds combining both BODIPY and transition metal complexes reported in the literature for cellular imaging, sensing and therapy.^[180–182] The Keyes group have reported the use of a solvent switchable

bichromophoric ruthenium(II) polypyridyl-BODIPY dyad which showed potential as self-referenced oxygen probes and also as imaging agents.^[183] Building on this, Ru(II)/BODIPY nanoparticles (where Ru(II) is $[\text{Ru}(\text{dpp})(\text{phen-NH}_2)(\text{bpybenzCOOEt})]^{2+}$) were successfully designed for ratiometric oxygen sensing in a live cell environment.^[179] In this case, the BODIPY acts as a reference which does not respond to oxygen levels. This is essential for ratiometric sensing as the intensity of emission is often influenced by more than just the target analyte. Therefore, using BODIPY as the reference signal, any change in Ru(II) emission can be attributed to its oxygen sensitivity. Another important requirement for ratiometric probes is the ability of the sensor and reference probe to be excited simultaneously.^[184] Given that BODIPY molecular rotors are known to respond to changes in viscosity,^[185] and can be excited at the same wavelength,^[34] BODIPY-perylene compounds have the potential to be used as ratiometric sensors to study fluctuations in viscosity in a live cellular environment, however more research is necessary to fully establish this concept.

Novel compounds have been developed for both cellular imaging and PDT, including a series of BODIPY-ruthenium(II) bis-terpyridine complexes, $[\text{Ru}(\text{tpy-BODIPY})(\text{tpy-R})]\text{Cl}_2$ (tpy = terpyridine; 1–4) reported by Paul *et al.*, where complex 4 containing a biotin moiety displayed an impressive PI value of >1400 in HeLa cells at a very low irradiation dose of 2.2 J/cm² (400–700 nm).^[186] A BODIPY-Ir(III) conjugate was recently reported by Chao *et al.* which localises in the mitochondria and induces apoptosis in triple-negative breast cancer cells after photoactivation, showing effective phototherapeutic potential in both normoxic and hypoxic (2.5% O₂) conditions.^[187] This work highlights the promise of designing targeted BODIPY-transition metal conjugates for both imaging and phototherapy where bright uptake of the conjugate can be seen in MDA-MB-231 breast cancer cells at just 100 nM, however the high dark toxicity of this complex across a number of test cell lines (<6.5 μM) poses a significant limitation. The ongoing evolution of these materials highlights the importance of developing new hybrid approaches that employ the desirable properties of both organic and metallic compounds.

1.9 Conclusions

While transition metal complexes have been the focus of extensive research both as imaging probes, sensors and in PDT in recent decades, studies on organic charge-transfer compounds remain comparatively less explored. As the demand for multifunctional probes grows, these compounds are poised to play a crucial role, offering applications not only in imaging but also in cellular sensing or therapeutics. Moreover, the incorporation of targeting ligands and peptides may allow these probes to be highly

targeted and tailored to specific applications, whether it be targeting specific organelles for phototherapy or visualising G4s and other cellular structures such as stress granules to better understand their biological relevance. To date, most studies assessing these compounds have been completed in cell models, with limited studies *in vivo*. Future development of multifunctional probes that harness the benefits of charge-transfer, including next generation organometallic probes, may enhance progress in advancing these probes from lab to clinical settings.

Chapter 2 explores a Ru(II) polypyridyl complex, Ru-bqp-Ester and its peptide conjugates Ru-bqp-R8 and Ru-bqp-MPP synthesised by Dr. Christopher Burke, which exhibited remarkable phototoxicity, particularly in pancreatic cancer cells. These complexes were studied in a number of cell models including CHO non-cancer, A549 lung cancer and HPAC pancreatic cancer cells and 3D HPAC tumour spheroids. The results of this study highlight the need to assess the efficacy of a potential photosensitiser in both adherent cell monolayers and 3D cell models such as tumour spheroids, where a distinct decrease in phototoxic index was observed between HPAC cell monolayers and spheroids.

Chapter 3 describes a study on Ru-RHAU, a Ru(II) polypyridyl peptide conjugate synthesised by Dr. Lorcan Holden that strongly stabilises the RNA quadruplex, NRAS. Ru-RHAU was studied in both HeLa cervical cancer and MCF-7 breast cancer cells and is the first reported probe that can induce and image stress granules in a live cell environment, confirmed by co-localisation studies with stress granule markers G3BP1 and TIA-1. This complex can also be used as a live cell stain for stress granules induced through external stress stimuli, as exemplified by cells treated with heat shock prior to Ru-RHAU staining.

A novel mitochondrial G4 (mitoG4) targeted ruthenium(II) complex Ru-TAP-PDC3, synthesised by Dr. Lorcan Holden is studied in Chapter 4. Ru-TAP-PDC3 was shown to localise in the mitochondria of HeLa cells and showed high affinity for mitoG4s in solution. Immunostaining studies with the commercially available G4 probe BG4 confirmed Ru-TAP-PDC3 localises in G4s of HeLa cells. This complex was found to be highly phototoxic, inducing mitochondrial DNA damage and dysfunction and ultimately leading to cell death by apoptosis under both normoxic and hypoxic conditions.

Chapter 5 focuses on the combination of charge-transfer and iodination to enhance the efficacy of BODIPY-perylene dyads (synthesised by Dr. Ruben Arturo Arellano-Reyes) for phototherapy, where a distinct increase in phototoxicity was observed in the iodinated analogues. Studies were performed in both CHO non-cancer and MCF-7 breast cancer

cell monolayers and MCF-7 spheroids to determine the phototoxicity of the BODIPY-
perylene dyads.

Chapter 6 discusses the conclusions from this work and considers future directions.

1.10 References

- [1] G.-G. Yang, X.-J. Hu, W. Liu, X. Xu, *Sens. Actuators B Chem.* **2022**, 370, 132403.
- [2] H. Yu, B. Yu, Y. Song, P. Hai, *Inorganica Chim. Acta* **2023**, 550, 121435.
- [3] C.-P. Tan, Y.-M. Zhong, L.-N. Ji, Z.-W. Mao, *Chem. Sci.* **2021**, 12, 2357–2367.
- [4] L. Holden, C. S. Burke, D. Cullinane, T. E. Keyes, *RSC Chem. Biol.* **2021**, 2, 1021–1049.
- [5] C. G. Galbraith, J. A. Galbraith, *J. Cell Sci.* **2011**, 124, 1607–1611.
- [6] L. C.-C. Lee, K. K.-W. Lo, *Chem. Rev.* **2024**, DOI 10.1021/acs.chemrev.3c00629.
- [7] E. Bassan, A. Gualandi, P. Giorgio Cozzi, P. Ceroni, *Chem. Sci.* **2021**, 12, 6607–6628.
- [8] A. M. May, J. L. Dempsey, *Chem. Sci.* **2024**, 15, 6661–6678.
- [9] K. S. Gkika, D. Cullinane, T. E. Keyes, *Top. Curr. Chem. Cham* **2022**, 380, 30.
- [10] A. Harriman, L. J. Mallon, G. Ulrich, R. Ziessel, *ChemPhysChem* **2007**, 8, 1207–1214.
- [11] W. Hu, Y. Lin, X.-F. Zhang, M. Feng, S. Zhao, J. Zhang, *Dyes Pigments* **2019**, 164, 139–147.
- [12] R. Datta, T. M. Heaster, J. T. Sharick, A. A. Gillette, M. C. Skala, *J. Biomed. Opt.* **2020**, 25, 071203.
- [13] S. Kalinina, J. Breymayer, P. Schäfer, E. Calzia, V. Shcheslavskiy, W. Becker, A. Rück, *J. Biophotonics* **2016**, 9, 800–811.
- [14] In *Mol. Fluoresc.*, John Wiley & Sons, Ltd, **2012**, pp. 1–30.
- [15] A. D. Elliott, *Curr. Protoc. Cytom.* **2020**, 92, e68.
- [16] J. Jonkman, C. M. Brown, *J. Biomol. Tech. JBT* **2015**, 26, 54–65.
- [17] V. Ramu, L. S. Wijaya, N. Beztsinna, C. V. de Griend, B. van de Water, S. Bonnet, S. E. L. Dévédec, *Chem. Commun.* **2024**, 60, 6308–6311.
- [18] N. Sun, Y. Jia, S. Bai, Q. Li, L. Dai, J. Li, *Adv. Colloid Interface Sci.* **2023**, 314, 102880.
- [19] L. Möckl, D. C. Lamb, C. Bräuchle, *Angew. Chem. Int. Ed Engl.* **2014**, 53, 13972–13977.
- [20] H. Blom, H. Brismar, *J. Intern. Med.* **2014**, 276, 560–578.
- [21] H. Blom, J. Widengren, *Chem. Rev.* **2017**, 117, 7377–7427.
- [22] G. Moneron, R. Medda, B. Hein, A. Giske, W. Westphal, S. Hell, *Opt. Express* 2010, 18, DOI 10.1364/OE.18.001302.

- [23] A. Byrne, C. S. Burke, T. E. Keyes, *Chem. Sci.* **2016**, 7, 6551–6562.
- [24] K. König, P. T. So, W. W. Mantulin, B. J. Tromberg, E. Gratton, *J. Microsc.* **1996**, 183, 197–204.
- [25] Y. Badawi, H. Nishimune, *Neurosci. Lett.* **2020**, 715, 134644.
- [26] M.-T. Ke, T. Imai, *Bio-Protoc.* **2018**, 8, e3046.
- [27] T. Boothe, L. Hilbert, M. Heide, L. Berninger, W. B. Huttner, V. Zaburdaev, N. L. Vastenhouw, E. W. Myers, D. N. Drechsel, J. C. Rink, *eLife* **2017**, 6, e27240.
- [28] L. V. Albrecht, N. Tejeda-Muñoz, E. M. De Robertis, *STAR Protoc.* **2020**, 1, 100132.
- [29] B. Chazotte, *Cold Spring Harb. Protoc.* **2011**, pdb.prot5557.
- [30] T. Papalia, G. Siracusano, I. Colao, A. Barattucci, M. C. Aversa, S. Serroni, G. Zappalà, S. Campagna, M. T. Sciortino, F. Puntoriero, P. Bonaccorsi, *Dyes Pigments* **2014**, 110, 67–71.
- [31] G. Li, J. Li, Y. Otsuka, S. Zhang, M. Takahashi, K. Yamada, *Materials* **2020**, 13, 677.
- [32] J. Rumin, H. Bonnefond, B. Saint-Jean, C. Rouxel, A. Sciandra, O. Bernard, J.-P. Cadoret, G. Bougaran, *Biotechnol. Biofuels* **2015**, 8, 42.
- [33] J. Zhu, N. K. Tan, K. Kikuchi, A. Kaur, E. J. New, *Anal. Sens.* **2024**, 4, e202300049.
- [34] R. A. Arellano-Reyes, A. Prabhakaran, R. C. E. Sia, J. Guthmuller, K. K. Jha, T. Yang, B. Dietzek-Ivanšić, V. McKee, T. E. Keyes, *Chem. – Eur. J.* **2023**, 29, e202300239.
- [35] X. Pan, C. Dalm, R. H. Wijffels, D. E. Martens, *Appl. Microbiol. Biotechnol.* **2017**, 101, 8101–8113.
- [36] T. K. Fam, A. S. Klymchenko, M. Collot, *Materials* **2018**, 11, 1768.
- [37] N. Sinha, O. S. Wenger, *J. Am. Chem. Soc.* **2023**, 145, 4903–4920.
- [38] A. K. Vidanapathirana, B. J. Pullen, R. Zhang, M. Duong, J. M. Goyne, X. Zhang, C. S. Bonder, A. D. Abell, C. A. Bursill, S. J. Nicholls, P. J. Psaltis, *Sci. Rep.* **2019**, 9, 1720.
- [39] D.-L. Ma, V. P.-Y. Ma, D. S.-H. Chan, K.-H. Leung, H.-Z. He, C.-H. Leung, *Coord. Chem. Rev.* **2012**, 256, 3087–3113.
- [40] R. Huang, C.-H. Huang, J. Chen, Z.-Y. Yan, M. Tang, J. Shao, K. Cai, B.-Z. Zhu, *Nucleic Acids Res.* **2023**, 51, 11981–11998.
- [41] Q. Chen, C. Jin, X. Shao, R. Guan, Z. Tian, C. Wang, F. Liu, P. Ling, J.-L. Guan, L. Ji, F. Wang, H. Chao, J. Diao, *Small* **2018**, 14, 1802166.
- [42] K. L. Smitten, E. J. Thick, H. M. Southam, J. B. de la Serna, S. J. Foster, J. A. Thomas, *Chem. Sci.* **2020**, 11, 8828–8838.
- [43] L.-Y. Liu, H. Fang, Q. Chen, M. H.-Y. Chan, M. Ng, K.-N. Wang, W. Liu, Z. Tian, J. Diao, Z.-W. Mao, V. W.-W. Yam, *Angew. Chem. Int. Ed.* **2020**, 59, 19229–19236.

- [44] C. A. Puckett, R. J. Ernst, J. K. Barton, *Dalton Trans. Camb. Engl.* **2003** **2010**, 39, 1159–1170.
- [45] C. A. Puckett, J. K. Barton, *Biochemistry* **2008**, *47*, 11711–11716.
- [46] F. E. Poynton, S. A. Bright, S. Blasco, D. C. Williams, J. M. Kelly, T. Gunnlaugsson, *Chem. Soc. Rev.* **2017**, *46*, 7706–7756.
- [47] K. J. Dix, in *Handb. Pestic. Toxicol. Second Ed.* (Eds.: R.I. Krieger, W.C. Krieger), Academic Press, San Diego, **2001**, pp. 563–582.
- [48] M. Dickerson, Y. Sun, B. Howerton, E. C. Glazer, *Inorg. Chem.* **2014**, *53*, 10370–10377.
- [49] B. Alberts, A. Johnson, J. Lewis, M. Raff, K. Roberts, P. Walter, in *Mol. Biol. Cell 4th Ed.*, Garland Science, **2002**.
- [50] C. Qiu, F. Xia, J. Zhang, Q. Shi, Y. Meng, C. Wang, H. Pang, L. Gu, C. Xu, Q. Guo, J. Wang, *Research* **2023**, *6*, 0148.
- [51] T. Gao, H. He, R. Huang, M. Zheng, F.-F. Wang, Y.-J. Hu, F.-L. Jiang, Y. Liu, *Dyes Pigments* **2017**, *141*, 530–535.
- [52] J.-L. Wang, L. Zhang, L.-X. Gao, J.-L. Chen, T. Zhou, Y. Liu, F.-L. Jiang, *J. Mater. Chem. B* **2021**, *9*, 8639–8645.
- [53] F.-Z. Li, Z. Wu, C. Lin, Q. Wang, G.-C. Kuang, *Results Chem.* **2022**, *4*, 100384.
- [54] S. K. Fung, T. Zou, B. Cao, T. Chen, W.-P. To, C. Yang, C.-N. Lok, C.-M. Che, *Nat. Commun.* **2016**, *7*, 10655.
- [55] U. Neugebauer, Y. Pellegrin, M. Devocelle, R. J. Forster, W. Signac, N. Moran, T. E. Keyes, *Chem. Commun.* **2008**, 5307.
- [56] T. Yang, R. A. Arellano-Reyes, R. C. Curley, K. K. Jha, A. Chettri, T. E. Keyes, B. Dietzek-Ivanšić, *Chem. – Eur. J.* **2023**, *29*, e202300224.
- [57] C. A. Puckett, J. K. Barton, *J. Am. Chem. Soc.* **2007**, *129*, 46–47.
- [58] B. Todaro, E. Ottalagana, S. Luin, M. Santi, *Pharmaceutics* **2023**, *15*, 1648.
- [59] C. Lamers, *Future Drug Discov.* **2022**, *4*, FDD75.
- [60] A. Borrelli, A. L. Tornosello, M. L. Tornosello, F. M. Buonaguro, *Mol. J. Synth. Chem. Nat. Prod. Chem.* **2018**, *23*, 295.
- [61] V. Meier-Stephenson, *Biophys. Rev.* **2022**, *14*, 635–654.
- [62] A. Klug, *J. Mol. Biol.* **2004**, *335*, 3–26.
- [63] N. Pandya, S. R. Bhagwat, A. Kumar, *Biochim. Biophys. Acta BBA - Rev. Cancer* **2021**, 1876, 188594.
- [64] J. D. Knoll, C. Turro, *Coord. Chem. Rev.* **2015**, 282–283, 110–126.
- [65] J. Spiegel, S. Adhikari, S. Balasubramanian, *Trends Chem.* **2020**, *2*, 123–136.
- [66] A. Bansal, S. Kaushik, S. Kukreti, *Front. Genet.* **2022**, *13*, 959258.

- [67] S. Burge, G. N. Parkinson, P. Hazel, A. K. Todd, S. Neidle, *Nucleic Acids Res.* **2006**, *34*, 5402–5415.
- [68] E. Palma, J. Carvalho, C. Cruz, A. Paulo, *Pharmaceuticals* **2021**, *14*, 605.
- [69] D. Bhattacharyya, G. Mirihana Arachchilage, S. Basu, *Front. Chem.* **2016**, *4*, DOI 10.3389/fchem.2016.00038.
- [70] A. K. Grover, in *Adv. Pharmacol.* (Ed.: R.A. Khalil), Academic Press, **2017**, pp. 145–170.
- [71] N. Nagesh, D. Chatterji, *J. Biochem. Biophys. Methods* **1995**, *30*, 1–8.
- [72] F. W. Kotch, J. C. Fettinger, J. T. Davis, *Org. Lett.* **2000**, *2*, 3277–3280.
- [73] E. A. Venczel, D. Sen, *Biochemistry* **1993**, *32*, 6220–6228.
- [74] C. J. Lech, B. Heddi, A. T. Phan, *Nucleic Acids Res.* **2013**, *41*, 2034–2046.
- [75] R. Hänsel-Hertsch, M. Di Antonio, S. Balasubramanian, *Nat. Rev. Mol. Cell Biol.* **2017**, *18*, 279–284.
- [76] K. Sato, P. Knipscheer, *DNA Repair* **2023**, *130*, 103552.
- [77] I. Frasson, V. Pirota, S. N. Richter, F. Doria, *Int. J. Biol. Macromol.* **2022**, *204*, 89–102.
- [78] F.-C. Huang, C.-C. Chang, J.-M. Wang, T.-C. Chang, J.-J. Lin, *Br. J. Pharmacol.* **2012**, *167*, 393–406.
- [79] S. G. Fernandes, R. Dsouza, G. Pandya, A. Kirtonia, V. Tergaonkar, S. Y. Lee, M. Garg, E. Khattar, *Cancers* **2020**, *12*, 1901.
- [80] A. K. Michael, N. H. Thomä, *Cell* **2021**, *184*, 3599–3611.
- [81] V. J. Sahayasheela, Z. Yu, T. Hidaka, G. N. Pandian, H. Sugiyama, *Trends Genet.* **2023**, *39*, 15–30.
- [82] K. Kuang, C. Li, F. Maksut, D. Ghosh, R. Vinck, M. Wang, J. Poupon, R. Xiang, W. Li, F. Li, Z. Wang, J. Du, M.-P. Teulade-Fichou, G. Gasser, S. Bombard, T. Jia, *J. Biomed. Sci.* **2024**, *31*, 50.
- [83] L. Holden, R. C. Curley, G. Avella, C. Long, Keyes, Tia, *Angew. Chem. Int. Ed.* **2024**, e202408581.
- [84] A. Bedrat, L. Lacroix, J.-L. Mergny, *Nucleic Acids Res.* **2016**, *44*, 1746–1759.
- [85] G.-X. Tang, M.-L. Li, C. Zhou, Z.-S. Huang, S.-B. Chen, X.-C. Chen, J.-H. Tan, *Cell Chem. Biol.* **2024**, DOI 10.1016/j.chembiol.2024.05.003.
- [86] K. Lyu, E. Y.-C. Chow, X. Mou, T.-F. Chan, C. K. Kwok, *Nucleic Acids Res.* **2021**, *49*, 5426–5450.
- [87] B. Van Treeck, D. S. W. Protter, T. Matheny, A. Khong, C. D. Link, R. Parker, *Proc. Natl. Acad. Sci. U. S. A.* **2018**, *115*, 2734–2739.
- [88] P. Kharel, M. Fay, E. V. Manasova, P. J. Anderson, A. V. Kurkin, J. U. Guo, P. Ivanov, *Nat. Commun.* **2023**, *14*, 205.

- [89] S. R. Millar, J. Q. Huang, K. J. Schreiber, Y.-C. Tsai, J. Won, J. Zhang, A. M. Moses, J.-Y. Youn, *Chem. Rev.* **2023**, *123*, 9036–9064.
- [90] J. Jiang, T. Teunens, J. Tisaun, L. Denuit, C. Moucheron, *Molecules* **2022**, *27*, 1541.
- [91] A. D. McCall, *BMC Bioinformatics* **2024**, *25*, 55.
- [92] S. Galli, L. Melidis, S. M. Flynn, D. Varshney, A. Simeone, J. Spiegel, S. K. Madden, D. Tannahill, S. Balasubramanian, *J. Am. Chem. Soc.* **2022**, *144*, 23096–23103.
- [93] X.-C. Chen, S.-B. Chen, J. Dai, J.-H. Yuan, T.-M. Ou, Z.-S. Huang, J.-H. Tan, *Angew. Chem. Int. Ed.* **2018**, *57*, 4702–4706.
- [94] S. Neidle, in *Annu. Rep. Med. Chem.* (Ed.: S. Neidle), Academic Press, **2020**, pp. 517–546.
- [95] A. Awadasseid, X. Ma, Y. Wu, W. Zhang, *Biomed. Pharmacother.* **2021**, *139*, 111550.
- [96] A. E. Friedman, J. C. Chambron, J. P. Sauvage, N. J. Turro, J. K. Barton, *J. Am. Chem. Soc.* **1990**, *112*, 4960–4962.
- [97] M. L. Di Pietro, G. La Ganga, F. Nastasi, F. Puntoriero, *Appl. Sci.* **2021**, *11*, 3038.
- [98] S. Shi, J. Zhao, X. Geng, T. Yao, H. Huang, T. Liu, L. Zheng, Z. Li, D. Yang, L. Ji, *Dalton Trans.* **2010**, *39*, 2490–2493.
- [99] M. Wumaier, J.-J. Shi, T.-M. Yao, X.-C. Hu, R.-R. Gao, S. Shi, *J. Inorg. Biochem.* **2019**, *196*, 110681.
- [100] S. A. Archer, A. Raza, F. Dröge, C. Robertson, A. J. Auty, D. Chekulaev, J. A. Weinstein, T. Keane, A. J. H. M. Meijer, J. W. Haycock, S. MacNeil, J. A. Thomas, *Chem. Sci.* **2019**, *10*, 3502–3513.
- [101] G.-F. Liu, Y.-S. Chen, Z.-L. Wang, D. Gu, M.-Q. Wang, *Dyes Pigments* **2024**, *225*, 112107.
- [102] L. Zhang, J. C. Er, K. K. Ghosh, W. J. Chung, J. Yoo, W. Xu, W. Zhao, A. T. Phan, Y.-T. Chang, *Sci. Rep.* **2014**, *4*, 3776.
- [103] Y.-S. Chen, H.-W. Cao, J.-Z. Li, H.-W. Chen, L.-Y. Li, Z.-K. Li, H.-J. Wang, M.-Q. Wang, *Tetrahedron Lett.* **2024**, *140*, 155033.
- [104] D. Shrestha, A. Jenei, P. Nagy, G. Vereb, J. Szöllösi, *Int. J. Mol. Sci.* **2015**, *16*, 6718–6756.
- [105] F. H. Quina, G. T. M. Silva, *J. Photochem. Photobiol.* **2021**, *7*, 100042.
- [106] O. Mikhnenko, P. Blom, T.-Q. Nguyen, *Energy Env. Sci* **2015**, *8*, DOI 10.1039/C5EE00925A.
- [107] R. Hamid, Y. Rotshteyn, L. Rabadi, R. Parikh, P. Bullock, *Toxicol. In Vitro* **2004**, *18*, 703–710.
- [108] H. Abrahamse, M. R. Hamblin, *Biochem. J.* **2016**, *473*, 347–364.

- [109] R.-M. Szeimies, J. Dräger, C. Abels, M. Landthaler, in *Compr. Ser. Photosciences* (Eds.: P. Calzavara-Pinton, R.-M. Szeimies, B. Ortel), Elsevier, **2001**, pp. 3–15.
- [110] G. Gunaydin, M. E. Gedik, S. Ayan, *Front. Chem.* **2021**, 9, 691697.
- [111] Y. Wu, S. Li, Y. Chen, W. He, Z. Guo, *Chem. Sci.* **2022**, 13, 5085–5106.
- [112] C. Imberti, P. Zhang, H. Huang, P. J. Sadler, *Angew. Chem. Int. Ed.* **2020**, 59, 61–73.
- [113] J. A. Rodrigues, J. H. Correia, *Cells* **2022**, 11, 3995.
- [114] J. H. Correia, J. A. Rodrigues, S. Pimenta, T. Dong, Z. Yang, *Pharmaceutics* **2021**, 13, 1332.
- [115] B. A. Hartl, H. Hirschberg, L. Marcu, S. R. Cherry, *Biomed. Opt. Express* **2015**, 6, 770–779.
- [116] Y. Miyamoto, D. Nishikiori, F. Hagino, M. Wakita, I. Tanabe, M. Toida, *Laser Ther.* **2011**, 20, 135–138.
- [117] M. O. Senge, J. C. Brandt, *Photochem. Photobiol.* **2011**, 87, 1240–1296.
- [118] S. Chen, X. Zhao, J. Chen, J. Chen, L. Kuznetsova, S. S. Wong, I. Ojima, *Bioconjug. Chem.* **2010**, 21, 979–987.
- [119] S. Roy, E. Colombo, R. Vinck, C. Mari, R. Rubbiani, M. Patra, G. Gasser, *ChemBioChem* **2020**, 21, 2966–2973.
- [120] S. M. Cloonan, R. B. P. Elmes, M. Erby, S. A. Bright, F. E. Poynton, D. E. Nolan, S. J. Quinn, T. Gunnlaugsson, D. C. Williams, *J. Med. Chem.* **2015**, 58, 4494–4505.
- [121] E. Wachter, D. K. Heidary, B. S. Howerton, S. Parkin, E. C. Glazer, *Chem. Commun.* **2012**, 48, 9649.
- [122] J. Liu, Y. Chen, G. Li, P. Zhang, C. Jin, L. Zeng, L. Ji, H. Chao, *Biomaterials* **2015**, 56, 140–153.
- [123] Q. Chen, V. Ramu, Y. Aydar, A. Groenewoud, X.-Q. Zhou, M. J. Jager, H. Cole, C. G. Cameron, S. A. McFarland, S. Bonnet, B. E. Snaar-Jagalska, *Cancers* **2020**, 12, 587.
- [124] E. Delaey, F. van Laar, D. De Vos, A. Kamuhabwa, P. Jacobs, P. de Witte, J. *Photochem. Photobiol. B* **2000**, 55, 27–36.
- [125] Y. Sun, X. Geng, Y. Wang, X. Su, R. Han, J. Wang, X. Li, P. Wang, K. Zhang, X. Wang, *ACS Pharmacol. Transl. Sci.* **2021**, 4, 802–812.
- [126] J. Li, Q. Wang, G. Xia, N. Adilijiang, Y. Li, Z. Hou, Z. Fan, J. Li, *Pharmaceutics* **2023**, 15, 2233.
- [127] J. Shum, P. K.-K. Leung, K. K.-W. Lo, *Inorg. Chem.* **2019**, 58, 2231–2247.
- [128] M. C. Malacarne, M. B. Gariboldi, E. Caruso, *Int. J. Mol. Sci.* **2022**, 23, 10198.
- [129] C. Wang, Y. Qian, *Org. Biomol. Chem.* **2019**, 17, 8001–8007.
- [130] B. Muz, P. de la Puente, F. Azab, A. K. Azab, *Hypoxia* **2015**, 3, 83–92.

- [131] P. Vaupel, L. Harrison, *The Oncologist* **2004**, 9 Suppl 5, 4–9.
- [132] S. Abyar, L. Huang, Y. Husiev, L. Bretin, B. Chau, V. Ramu, J. H. Wildeman, K. Belfor, L. S. Wijaya, V. E. van der Noord, A. C. Harms, M. A. Siegler, S. E. Le Dévédec, S. Bonnet, *J. Med. Chem.* **2024**, 67, 11086–11102.
- [133] F. J. Ballester, A. Hernández-García, M. D. Santana, D. Bautista, P. Ashoo, E. Ortega-Forte, G. Barone, J. Ruiz, *Inorg. Chem.* **2024**, 63, 6202–6216.
- [134] L. Hong, J. Li, Y. Luo, T. Guo, C. Zhang, S. Ou, Y. Long, Z. Hu, *Biomolecules* **2022**, 12, 81.
- [135] Z. Chen, F. Han, Y. Du, H. Shi, W. Zhou, *Signal Transduct. Target. Ther.* **2023**, 8, 70.
- [136] Y. Sun, D. Zhao, G. Wang, Y. Wang, L. Cao, J. Sun, Q. Jiang, Z. He, *Acta Pharm. Sin. B* **2020**, 10, 1382–1396.
- [137] R. I. Dmitriev, D. B. Papkovsky, *Cell. Mol. Life Sci. CMLS* **2012**, 69, 2025–2039.
- [138] T. Yoshihara, Y. Hirakawa, M. Hosaka, M. Nangaku, S. Tobita, *J. Photochem. Photobiol. C Photochem. Rev.* **2017**, 30, 71–95.
- [139] N. J. Farrer, L. Salassa, P. J. Sadler, *Dalton Trans. Camb. Engl. 2003* **2009**, 10690–10701.
- [140] S. Bonnet, *J. Am. Chem. Soc.* **2023**, 145, 23397–23415.
- [141] C. Mari, V. Pierroz, S. Ferrari, G. Gasser, *Chem. Sci.* **2015**, 6, 2660–2686.
- [142] B. S. Howerton, D. K. Heidary, E. C. Glazer, *J. Am. Chem. Soc.* **2012**, 134, 8324–8327.
- [143] J. Roque, D. Havrylyuk, P. C. Barrett, T. Sainuddin, J. McCain, K. Colón, W. T. Sparks, E. Bradner, S. Monro, D. Heidary, C. Cameron, E. C. Glazer, S. McFarland, *Photochem. Photobiol.* **2020**, 96, 327–339.
- [144] J.-A. Cuello-Garibo, M. S. Meijer, S. Bonnet, *Chem. Commun.* **2017**, 53, 6768–6771.
- [145] L. Gourdon, K. Cariou, G. Gasser, *Chem. Soc. Rev.* **2022**, 51, 1167–1195.
- [146] Y. Chen, L. Bai, P. Zhang, H. Zhao, Q. Zhou, *Molecules* **2021**, 26, 5679.
- [147] H. Shi, O. W. L. Carter, F. Ponte, C. Imberti, M. A. Gomez-Gonzalez, F. Cacho-Nerin, P. D. Quinn, J. E. Parker, E. Sicilia, H. Huang, P. J. Sadler, *Angew. Chem. Int. Ed.* **2024**, 63, e202400476.
- [148] R. Edmondson, J. J. Broglie, A. F. Adcock, L. Yang, *Assay Drug Dev. Technol.* **2014**, 12, 207–218.
- [149] K. Poornima, A. P. Francis, M. Hoda, M. A. Eladl, S. Subramanian, V. P. Veeraraghavan, M. El-Sherbiny, S. M. Asseri, A. B. A. Hussamuldin, K. M. Surapaneni, U. Mony, R. Rajagopalan, *Front. Oncol.* **2022**, 12, 891673.
- [150] A. Cacciamali, R. Villa, S. Dotti, *Front. Physiol.* **2022**, 13, 836480.

- [151] B. Pinto, A. C. Henriques, P. M. A. Silva, H. Bousbaa, *Pharmaceutics* **2020**, *12*, 1186.
- [152] R. C. Curley, C. S. Burke, K. S. Gkika, S. Noorani, N. Walsh, T. E. Keyes, *Inorg. Chem.* **2023**, *62*, 13089–13102.
- [153] L. Schneider, M. Kalt, S. Koch, S. Sithamparanathan, V. Villiger, J. Mattiat, F. Kradolfer, E. Slyshkina, S. Lubner, M. Bonmarin, C. Maake, B. Spingler, *J. Am. Chem. Soc.* **2023**, *145*, 4534–4544.
- [154] K. S. Gkika, S. Noorani, N. Walsh, T. E. Keyes, *Inorg. Chem.* **2021**, *60*, 8123–8134.
- [155] O. Sirenko, T. Mitlo, J. Hesley, S. Luke, W. Owens, E. F. Cromwell, *Assay Drug Dev. Technol.* **2015**, *13*, 402–414.
- [156] J. Kulesza, M. Pawłowska, E. Augustin, *Molecules* **2021**, *26*, 6262.
- [157] J. Karges, S. Kuang, F. Maschietto, O. Blacque, I. Ciofini, H. Chao, G. Gasser, *Nat. Commun.* **2020**, *11*, 3262.
- [158] K. Mizukami, A. Katano, S. Shiozaki, T. Yoshihara, N. Goda, S. Tobita, *Sci. Rep.* **2020**, *10*, 21053.
- [159] L. Zeng, P. Gupta, Y. Chen, E. Wang, L. Ji, H. Chao, Z.-S. Chen, *Chem. Soc. Rev.* **2017**, *46*, 5771–5804.
- [160] E. Alessio, L. Messori, *Molecules* **2019**, *24*, 1995.
- [161] D. A. Smithen, H. Yin, M. H. R. Beh, M. Hetu, T. S. Cameron, S. A. McFarland, A. Thompson, *Inorg. Chem.* **2017**, *56*, 4121–4132.
- [162] C. Sonkar, S. Sarkar, S. Mukhopadhyay, *RSC Med. Chem.* **2022**, *13*, 22–38.
- [163] E. Alessio, *Eur. J. Inorg. Chem.* **2017**, *2017*, 1549–1560.
- [164] J. M. Rademaker-Lakhai, D. van den Bongard, D. Pluim, J. H. Beijnen, J. H. M. Schellens, *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* **2004**, *10*, 3717–3727.
- [165] B. Happl, T. Balber, P. Heffeter, C. Denk, J. M. Welch, U. Köster, C. Alliot, A.-C. Bonraisin, M. Brandt, F. Haddad, J. H. Sterba, W. Kandioller, M. Mitterhauser, M. Hacker, B. K. Keppler, T. L. Mindt, *Dalton Trans.* **2024**, *53*, 6031–6040.
- [166] D. Baier, T. Mendrina, B. Schoenhacker-Alte, C. Pirker, T. Mohr, M. Rusz, B. Regner, M. Schaier, N. Sgarioto, N. J. -M. Raynal, K. Nowikovsky, W. M. Schmidt, P. Heffeter, S. M. Meier-Menches, G. Koellensperger, B. K. Keppler, W. Berger, *Adv. Sci.* **2023**, *10*, 2301939.
- [167] D. Baier, B. Schoenhacker-Alte, M. Rusz, C. Pirker, T. Mohr, T. Mendrina, D. Kirchhofer, S. M. Meier-Menches, K. Hohenwallner, M. Schaier, E. Rampler, G. Koellensperger, P. Heffeter, B. Keppler, W. Berger, *Pharmaceutics* **2022**, *14*, 238.
- [168] M. V. Liberti, J. W. Locasale, *Trends Biochem. Sci.* **2016**, *41*, 211–218.

- [169] S. Bakewell, I. Conde, Y. Fallah, M. McCoy, L. Jin, A. N. Shajahan-Haq, *Cancers* **2020**, *12*, 2647.
- [170] B. J. Park, P. Raha, J. Pankovich, M. Bazett, *Cancers* **2022**, *15*, 28.
- [171] S. Monro, K. L. Colón, H. Yin, J. I. Roque, P. Konda, S. Gujar, R. P. Thummel, L. Lilge, C. G. Cameron, S. A. McFarland, *Chem. Rev.* **2019**, *119*, 797–828.
- [172] S. Chamberlain, H. D. Cole, J. Roque, D. Bellnier, S. A. McFarland, G. Shafirstein, *Pharmaceuticals* **2020**, *13*, 137.
- [173] S. Y. Lee, C. Y. Kim, T.-G. Nam, *Drug Des. Devel. Ther.* **2020**, *14*, 5375–5392.
- [174] B. Bertrand, K. Passador, C. Goze, F. Denat, E. Bodio, M. Salmain, *Coord. Chem. Rev.* **2018**, *358*, 108–124.
- [175] A. Jabłońska-Wawrzycka, P. Rogala, S. Michałkiewicz, M. Hodorowicz, B. Barszcz, *Dalton Trans.* **2013**, *42*, 6092–6101.
- [176] M. Pal, U. Nandi, D. Mukherjee, *Eur. J. Med. Chem.* **2018**, *150*, 419–445.
- [177] S. Wang, L. Gai, Y. Chen, X. Ji, H. Lu, Z. Guo, *Chem. Soc. Rev.* **2024**, *53*, 3976–4019.
- [178] F. Ponte, D. M. Scopelliti, N. Sanna, E. Sicilia, G. Mazzone, *Molecules* **2022**, *27*, 5635.
- [179] K. S. Gkika, A. Kargaard, C. S. Burke, C. Dolan, A. Heise, T. E. Keyes, *RSC Chem. Biol.* **2021**, *2*, 1520–1533.
- [180] A. Upadhyay, P. Kundu, V. Ramu, P. Kondaiah, A. R. Chakravarty, *Inorg. Chem.* **2022**, *61*, 1335–1348.
- [181] N. E. Aksakal, E. T. Eçik, H. H. Kazan, G. Y. Çiftçi, F. Yuksel, *Photochem. Photobiol. Sci.* **2019**, *18*, 2012–2022.
- [182] Y. Liu, Z. Li, L. Chen, Z. Xie, *Dyes Pigments* **2017**, *141*, 5–12.
- [183] A. Martin, A. Byrne, C. Dolan, R. J. Forster, T. E. Keyes, *Chem. Commun.* **2015**, *51*, 15839–15841.
- [184] Q. Tao, Y. Wen, W. Liang, L. Wang, R. Guo, M. Ding, M. Luo, J. Yan, F. Gong, C. Cao, L. Li, G. Liu, *Adv. Sens. Energy Mater.* **2024**, *3*, 100106.
- [185] W. Miao, C. Yu, E. Hao, L. Jiao, *Front. Chem.* **2019**, *7*, 825.
- [186] S. Paul, P. Kundu, P. Kondaiah, A. R. Chakravarty, *Inorg. Chem.* **2021**, *60*, 16178–16193.
- [187] L. Qiao, J. Liu, S. Kuang, X. Liao, J. Kou, L. Ji, H. Chao, *Dalton Trans.* **2021**, *50*, 14332–14341.

Chapter 2 : Phototoxicity of Tridentate Ru(II) Polypyridyl Complex with Expanded Bite Angle toward Mammalian Cells and Multicellular Tumour Spheroids

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Contributions:

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Dr. Christopher S. Burke: Synthesis and structural characterisation of the compounds. Discussion and revision of the manuscript.

Dr. Karmel S. Gkika: Supervision of biological experiments, discussion, and revision of the manuscript.

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Supporting information associated with this chapter in **Appendix A**.

Phototoxicity of Tridentate Ru(II) Polypyridyl Complex with Expanded Bite Angle toward Mammalian Cells and Multicellular Tumour Spheroids

2.1 Abstract

Tridentate ligand-coordinated ruthenium(II) polypyridyl complexes with large N–Ru–N bite angles have been shown to promote ligand field splitting and reduce singlet–triplet state mixing leading to dramatically extended emission quantum yields and lifetimes under ambient conditions. These effects are anticipated to enhance their photoinduced singlet oxygen production, promoting prospects for such complexes as type II phototherapeutics. In this contribution, we examined this putative effect for $[\text{Ru}(\text{bqp})(\text{bqpCOOEt})]^{2+}$, Ru-bqp-ester, a heteroleptic complex containing bqp = [2,6-bi(quinolin-8-yl)pyridine], a well-established large bite angle tridentate ligand, as well as its peptide conjugates $[\text{Ru}(\text{bqp})(\text{bqpCONH-ahx-FrFKFrFK(Ac)-CONH}_2)]^{5+}$ (Ru-bqp-MPP) and $[\text{Ru}(\text{bqp})(\text{bqp})(\text{CONH-ahx-RRRRRRRRR-CONH}_2)]^{10+}$ (Ru-bqp-R8) that were prepared in an effort to promote live cell/tissue permeability and targeting of the parent. Membrane permeability of both parent and peptide conjugates were compared across 2D cell monolayers; A549, Chinese hamster ovary, human pancreatic cancer (HPAC), and 3D HPAC multicellular tumour spheroids (MCTS) using confocal microscopy. Both the parent complex and peptide conjugates showed exceptional permeability with rapid uptake in both 2D and 3D cell models but with little distinction in permeability or distribution in cells between the parent or peptide conjugates. Unexpectedly, the uptake was temperature independent and so attributed to passive permeation. Both dark and photo-toxicity of the Ru(II) complexes were assessed across cell types, and the parent showed notably low dark toxicity. In contrast, the parent and conjugates were found to be highly phototoxic, with impressive phototoxic indices (PIs) toward HPAC cell monolayers in particular, with PI values ranging from ~580 to 760. Overall, our data indicate that the Ru(II) parent complex and its peptide conjugates show promise at both cell monolayers and 3D MCTS as photosensitisers for photodynamic therapy.

2.2 Introduction

Photodynamic therapy (PDT) or photosensitised light therapy, is a long-standing medical treatment that has been applied to treatment of cancers as well as infection and skin conditions.^[1,2] The most common mode of application is that the photosensitiser (PS), under illumination by light of non-tissue destructive wavelengths, in visible or NIR range

(typically 630-800 nm), initiates the formation of singlet oxygen, from ambient oxygen at the site of treatment to destroy the targeted cells.^[3,4] In biological environments, singlet oxygen species typically have limited lifetimes ($< 3 \mu\text{s}$), with an estimated half-life of approximately 40 ns and diffusive distances of $< 268 \text{ nm}$.^[5-7] Meaning the sensitiser must permeate the site of therapy. This format of PDT is known as Type II PDT and constitutes the mechanism behind the majority of clinically approved PS for PDT, which have been based on tetrapyrrolic structures, for example porphyrin, chlorin and phthalocyanine.^[8-11] Photofrin, for example is a clinically approved PS hematoporphyrin derivative used for PDT treatment of but not limited to lung and oesophageal cancer.^[12] Many organic PS species traditionally applied to PDT have several drawbacks including poor aqueous solubility, propensity for aggregation, skin photosensitivity and dark toxicity.^[13,14] There is, therefore, demand for the development of new classes of PS that address these issues. An ideal PS should be amphiphilic, display good solubility in biological media or water and exhibit minimum toxicity in the dark, but high toxicity when activated by a light source. Additionally, PS should be photostable given the short diffusive distances of singlet O_2 , they should be cell and tumour permeable.^[15] Luminescent co-ordination compounds such as those of ruthenium are potentially very attractive agents for PDT as they are constitutionally triplet state emitters with high extinction coefficients (minimising the PS dosage required), and have versatile chemistry that can be used to tune the properties of the compound to meet the demanding criteria of a PS. As they are usually charged, they tend not to self-aggregate, into structures that diminish their photophysical properties or solubility and they have favourable photophysical properties including good photostability.^[16]

Metal polypyridyl luminophores are consequently under intensive investigation across a range of biological applications, including as luminescent probes for intracellular imaging, sensing^[17,18] as well as for their phototherapeutic potential in PDT.^[19] Ru(II) complexes in particular are under study as sensitisers for routine Type II PDT applications, and Type II Ru(II) sensitisers have been rationally designed for 2-photon PDT.^[20-23] TLD-1433, a polypyridyl Ru(II) complex designed for the treatment of non-muscle-invasive bladder cancer (NMIBC), was the first Ru(II)-based PDT agent to enter a human clinical trial.^[24] TLD-1433 is administered through bladder instillation 1 hour prior to light treatment and demonstrates high retention in bladder cancer cells.^[25] Furthermore, Chamberlain *et al.* reported successful destruction of A549 lung cancer cells *in vitro* upon irradiation of TLD-1433 at 532 and 630 nm.^[26]

As their excited state is formally a triplet, Ru(II) polypyridyl complexes, in oxygenated environments can undergo Dexter, triplet-triplet energy transfer with molecular oxygen

to form tissue destructive singlet oxygen. However, the $^3\text{MLCT}$ state, is short-lived relative to organic phosphors because the heavy ruthenium atom, tends to mix singlet and triplet character in the MLCT state. Consequently, they tend to be less efficient singlet oxygen sensitizers than, metal porphyrins for example, whose PS efficiency is well established.^[27] However, owing to their synthetic versatility, Ru(II) complexes can be tailored to offer the aforementioned advantages of lower dark cytotoxicity and cell targeting.^[28,29] Means to promote singlet oxygen sensitisation in such complexes include; increasing the triplet character of the excited state e.g. through mixing ^3LC states with the $^3\text{MLCT}$ state or by reducing singlet-triplet mixing of the $^3\text{MLCT}$ state; or by enhancing the $^3\text{MLCT}$ lifetime. The latter is accomplished by manipulation of the relative energy of the triplet metal centred state whose thermal population, likely to be promoted in the warm cellular environment, increases non-radiative decay. This has been achieved in practice, by increasing the energy gap between the $^3\text{MLCT}$ and ^3MC through tuning the σ donor and π accepting ability of the ligands.^[30] Additionally, in the particular case of tridentate ligand coordinated ruthenium(II) polypyridyl complexes it has been very effectively accomplished by expanding the N–Ru–N bite angles of the coordination cage.^[31,32] The resulting release of steric strain, facilitates a less distorted octahedral geometry and has been shown to dramatically extend emission quantum yield and lifetime under ambient conditions.^[33–35] In an example by Schubert *et al.*, it was demonstrated that both approaches can be combined to profoundly lengthen the $^3\text{MLCT}$ excited state.^[36]

A key challenge in biological application of luminescent coordination compounds such as ruthenium, osmium and platinum are membrane permeability and subcellular localisation. Non-specific localisation can result in the activation of the phototherapeutic off-target and thus, in PDT applications, damage to the surrounding cells.^[37,38] The mechanism of cellular uptake of ruthenium complexes can vary depending on complex properties such as its size, charge, and lipophilicity.^[39] Increasing the lipophilicity of a ruthenium complex should increase uptake via passive diffusion, as was demonstrated by Park and co-workers.^[40] However, the mechanism of uptake can also depend on the concentration of the complex administered and the gene expression of the cell line, which can upregulate proteins, such as efflux pumps, and proteins for active transport.^[41,42] Cell penetrating peptides (CPP) such as octa-arginine sequences (RRRRRRRR) and signal peptides such as mitochondrial penetrating peptides (MPP) have proven effective in promoting permeation and targeting of ruthenium polypyridyl complexes in cells, but less is known about their effectiveness in promoting uptake and permeation through tissue.^[39] Peptide vectorisation has been successfully exploited by

our group and others to target metal complex luminophores to specific organelles for imaging and sensing.^[43,44] For example, we previously reported a peptide-bridged dinuclear Ru(II) conjugate, $[(\text{Ru}(\text{bpy})_2\text{phen-Ar})_2\text{-MPP}]^{7+}$ which allowed for precise targeting to the mitochondria. More recently, we demonstrated an octa-arginine conjugated osmium(II) complex, $[\text{Os}(\text{R4})_2]^{10+}$ that successfully penetrated 3D pancreatic cancer tumour spheroids.^[45,46]

Although 2D cell monolayers offer a convenient and well-accepted approach for evaluating permeation in drug development, 3D cellular models such as spheroids and cellular aggregates provide a more physiologically relevant cellular environment.^[47] The layered structure of spheroids mimics the tumour microenvironment more closely than 2D cell monolayers. Nutrient and oxygen gradients in spheroids mimic that of tumours *in vivo* with the vast majority of spheroids comprising three zones, the outer proliferative zone, the senescent zone and the necrotic (hypoxic) core.^[48] Increased cell-to-cell and cell-to-extracellular matrix interactions lead to enhanced physical barriers in spheroids that make drug permeation more difficult, which better reflects drug uptake *in vivo*.^[49] In the last decade, 3D multicellular spheroids and cell aggregates are becoming increasingly popular models for drug discovery and function as an excellent intermediate model between *in vitro* cell monolayers and *in vivo* tumours. Imaging, sensing and therapeutic evaluation in 3D cell models requires luminescent probes that can deeply penetrate a spheroid or cellular aggregate. Significant differences between the permeation and destination of luminescent probes between 2D and 3D cellular models have been reported.^[50] Therefore, in assessing potential PS, permeation through 3D models should ideally also be considered, but rarely have been to date in metal complex species, with only a small number of studies reported to date assessing metal complex photosensitisers in spheroids.^[51,52] Recent work includes lysosomal localising Ru(II) complexes, studied by the Chao group in HeLa spheroids for two photon PDT, irradiating the spheroids at 10 J/cm² at 800 nm and also the study of photoactive iridium(III) complexes in A549 spheroids by the Sadler group.^[53,54]

Herein, we report on the application of tridentate coordinated $[\text{Ru}(\text{bqp})(\text{bqpCOOEt})]^{2+}$ (Ru-bqp-ester), where bqp = 2,6-bi(quinolin-8-yl)pyridine, and its peptide conjugates $[\text{Ru}(\text{bqp})(\text{bqpCONH-ahx-FrFKFrFK(Ac)-CONH}_2)]^{5+}$ (Ru-bqp-MPP) and $[\text{Ru}(\text{bqp})(\text{bqpCONH-ahx-RRRRRRRRR-CONH}_2)]^{10+}$ (Ru-bqp-R8) as potential phototoxic agents. The parent complex, (Figure 2.1) was selected as, when first reported by Hammarström *et al.*, it showed exceptionally long lived ³MLCT excited state facilitated by the expanded coordination bite angle of the 2,6-bi(quinolin-8-yl)pyridine ligand so that the coordination geometry around the metal centre is approximately octahedral.^[33,55] This

has the effect of increasing the ligand field of the complex limiting thermal access to the ^3MC state, but studies have also shown there is dual effect where the ligand also reduces singlet mixing of the triplet excited state which also contributes to the long lifetime.^[56] We rationalised that both effects should yield a complex that is a good singlet oxygen sensitiser, which is why we selected it for study here. To promote cell membrane permeation, Dr. Christopher S. Burke conjugated the parent complex to peptides and the parent and peptide conjugates were investigated here in live cell monolayers and 3D cancer models. We examined the impact of the peptide conjugation on the photophysical behaviour, cell uptake and dark and phototoxicity of the complex in live A549 lung cancer, HPAC pancreatic adenocarcinoma and CHO (Chinese hamster ovary) non-cancer cell monolayers and in 3D HPAC tumour spheroids where we observe high phototoxicity, consistent with the long-lived excited triplet state of the complex.

2.3 Results and Discussion

2.3.1 Synthesis and Characterisation of the Ru(II) polypyridyl complexes and conjugates

Chemical synthesis and characterisation were carried out by Dr. Christopher S. Burke. Ru-bqp-ester was synthesised by modification of a protocol reported by Abrahamsson *et al.* and the asymmetric complex; $[\text{Ru}(\text{bqp})(\text{bqp-COOH})](\text{PF}_6)_2$, (Ru-bqp-Acid) was prepared to allow peptide conjugation through the carboxy handle. The materials were characterised by ^1H NMR (Supplementary Figures S2.1-S2.7 in Appendix A) and HR-MS (Supplementary Figures S2.8-S2.10 in Appendix A) and conformed to previously reported data.^[34,57,58] Peptide conjugation to form Ru-bqp-MPP and Ru-bqp-R8 (Figure 2.1) were prepared according to methods previously reported by us and also confirmed by ^1H NMR.

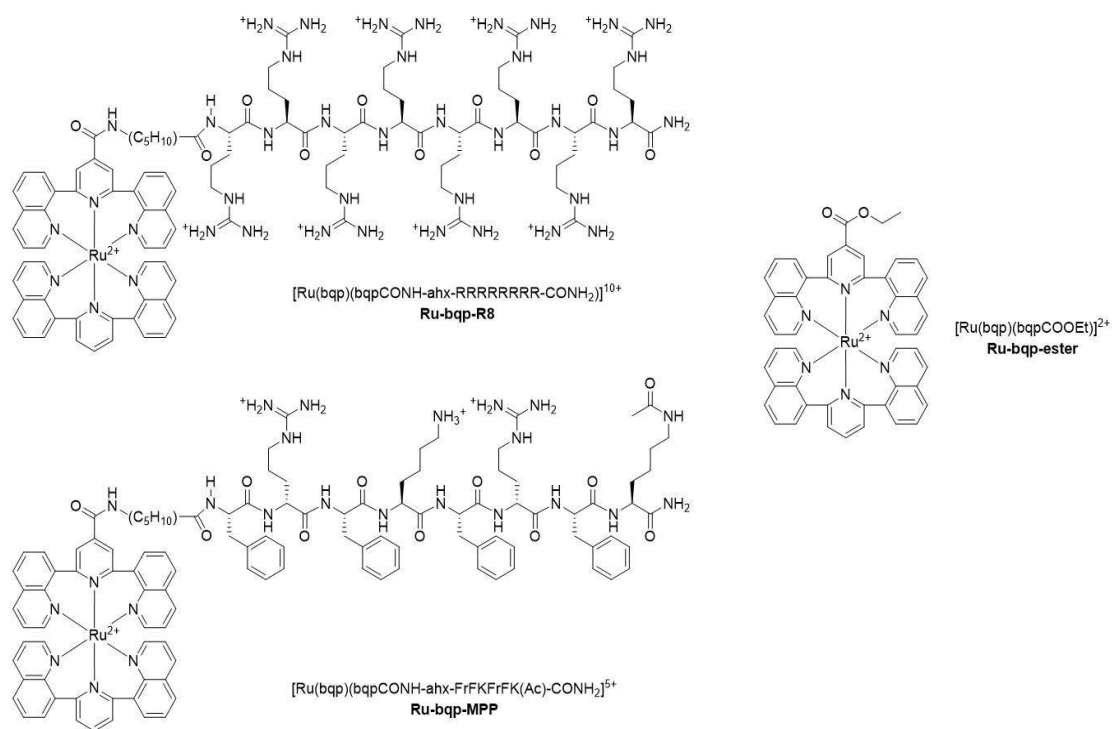


Figure 2.1 Chemical structures of the complexes and conjugates reported in this work; Ru-bqp-ester, Ru-bqp-R8 and Ru-bqp-MPP.

Table 2.1. Photophysical data of Ru-bqp-ester, Ru-bqp-MPP and Ru-bqp-R8 in PBS (pH 7.4) and acetonitrile.

	Solvent	$\lambda_{\text{abs}} (\epsilon)$ nm ($\times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$)	λ_{em} nm	τ_{lum} ns	
				Aerated	Deaerated
Ru-bqp-ester	PBS	272 (20.1), 345 (17.8), 491 (9.6)	699	782 $\pm$ 18	1320 $\pm$ 20
	MeCN	278 (25.6), 344 (23.3), 492 (11.8)	691 (Φ 4%) ^a	384 $\pm$ 1	4300 ^a
Ru-bqp-R8	PBS	280 (32.0), 342 (30.3), 494 (14.1)	691 (Φ 2.7%)	930 $\pm$ 40	1921 $\pm$ 49
Ru-bqp-MPP	PBS	278 (37.6), 342 (31.3), 493 (16.0)	690 (Φ 2.2%)	1077 $\pm$ 33	2011 $\pm$ 21

^a Previously published data, measured at 298 K, in deaerated MeOH/EtOH solution^[57,58]

2.3.2 Photophysical Characterisation of Ru-bqp-ester, Ru-bqp-MPP and Ru-bqp-R8

The absorbance and emission of the ester in acetonitrile conform to previously reported data.^[57,58] The absorbance and emission spectra of the parent, Ru-bqp-ester and peptide conjugates Ru-bqp-MPP and Ru-bqp-R8 in PBS buffer are shown in Figure 2.2. Emission decay across both ester and the conjugates conformed to single exponential kinetics in PBS and in acetonitrile. In phosphate buffered saline, the ester complex Ru-bqp-ester, shows long-lived emission of 782 ± 18 ns, which is nearly twice that of the complex in MeCN and the lifetime doubles again to over 1.3 μ s on deaeration. This is consistent with data reported previously for related complexes in DI water.^[61] Peptide conjugation had no measurable impact on the optical properties of the complex with absorbance maximum attributed to MLCT centred around 490 nm in PBS and λ_{max} emission around 690nm ($^3\text{MLCT}$). However, emission quantum yield and emission decay increased considerably on peptide conjugation and the magnitude of increase varied with peptide to 930 ± 40 ns for R8 and to 1077 ± 33 ns for the MPP conjugate. Similar though more modest effects have been noted previously for ruthenium(II) polypyridyl complexes on peptide conjugation and likely reflects the strong oxygen sensitivity of these species, and some limited oxygen protection exerted by the peptide.^[62]

To be effective as a Type II sensitiser, one would expect the emission lifetime to be strongly influenced by molecular oxygen and indeed on deaeration in PBS buffer (pH 7.4) emission lifetimes approximately doubled extending to over 2 μ s for Ru-bqp-MPP.

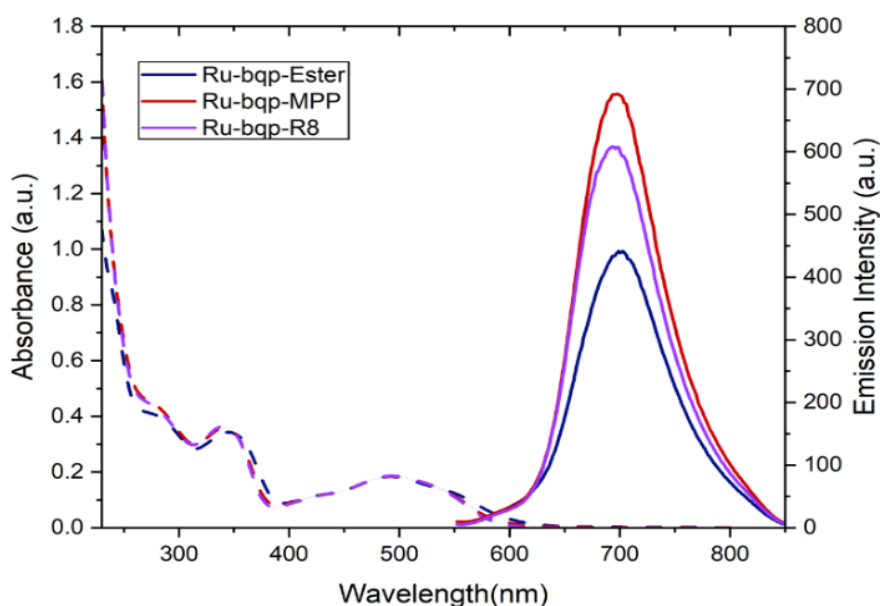


Figure 2.2 Absorbance and emission spectra of Ru-bqp-ester, Ru-bqp-MPP and Ru-bqp-R8 (20 μ M) in aerated PBS (pH 7.4), emissions are excited under absorbance matched conditions at 490 nm so emission intensity directly reflect relative quantum yield changes on peptide conjugation.

2.3.3 Uptake and Distribution in Cell Monolayers

The uptake of Ru-bqp-ester, Ru-bqp-MPP and Ru-bqp-R8 at 37 °C was assessed across three mammalian cell lines, two cancer; A549, HPAC and one non-cancer; CHO, by incubating the complex or conjugates at 30 μ M in phenol red free media. A549 cells were cultured in DMEM, HPAC in RPMI and CHO in DMEM/F-12 Ham supplemented with 1% penicillin/streptomycin and 5% (HPAC) or 10% FBS (A549 and CHO). Media formulations were selected based on the literature and recommendations from reputable vendors such as ATCC.

All three Ru(II) complexes including rather unexpectedly, the parent complex, Ru-bqp-ester, exhibited good membrane permeability and cellular uptake at live cells. Indeed, all showed notably rapid permeation, particularly at CHO and A549 cells where uptake was complete by 30 minutes. Consequently, for cellular imaging, CHO and A549 cells incubation time of just 1 h was applied, while HPAC cells required a minimum incubation of 2 h to achieve complete uptake, with images acquired at this cell line after a 4 h incubation (Figure 2.3).

After uptake, the probes distribution in live cells is remarkably similar across all three complexes, irrespective of peptide conjugation and cell type. In all cases emission was evident throughout the cytoplasm with intense punctate features overlying weaker continuous emission. All three complexes appear to be nuclear excluding, but the emission is particularly bright around the exterior of the nucleus.

Given the notable speed of uptake, temperature dependent uptake studies were carried out in CHO and A549 cells at 4 °C to determine the mechanism of uptake of the Ru(II) complexes. Most reported metal complex peptide conjugates, certainly all reported to date by our group, undergo cell uptake by endocytosis or active transport, where uptake was observed to switch off at 4 °C, indicating an energy dependent pathway.^[23,29,63,64]

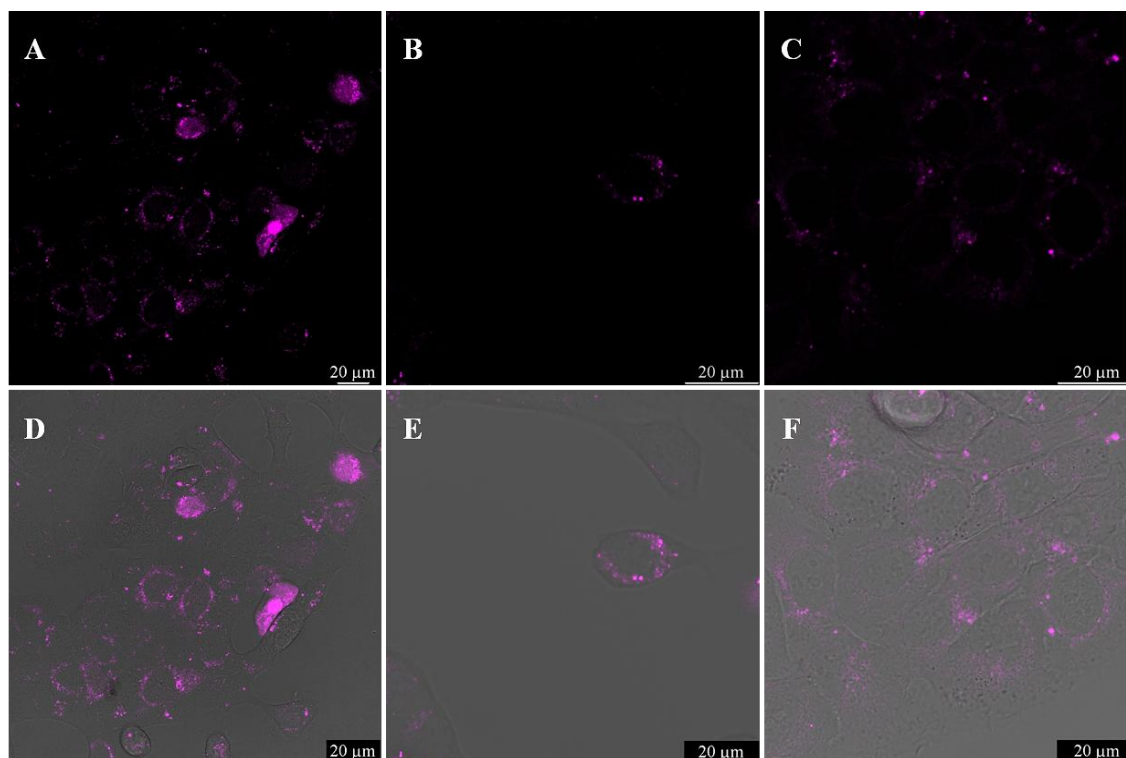


Figure 2.3 Cellular uptake of 30 μ M (A) Ru-bqp-MPP, (B) Ru-bqp-R8 and (C) Ru-bqp-ester in HPAC cell monolayers at 37 °C and 4 hour incubation, with their (D-E) corresponding brightfield overlay images. Live HPAC cells were imaged in phenol red free RPMI media supplemented with 5% FBS and 1% penicillin/streptomycin.

In contrast here, all three compounds remained strongly cell permeable at 4 °C and imaging following incubation for 1 h incubation confirmed, uniquely in our experience, that the mechanism of uptake is energy independent. This accounts for the rapid uptake by live cells. The absence of energy requirement for passive uptake also explains the membrane permeability of the parent Ru-bqp-ester complex and it is reasonable though surprising that the peptide conjugates derived from this complex exhibited the same mode of uptake. Generally, ruthenium complexes without peptide conjugation have poor membrane permeability in the absence of permeabilisation agents such as detergent or DMSO. The parent and peptide conjugates were relatively water soluble, requiring negligible DMSO to prepare the 2 mM starting stock solutions, where overall a minimal final DMSO concentration of <0.003% v/v would have been present in the cell incubation medium.

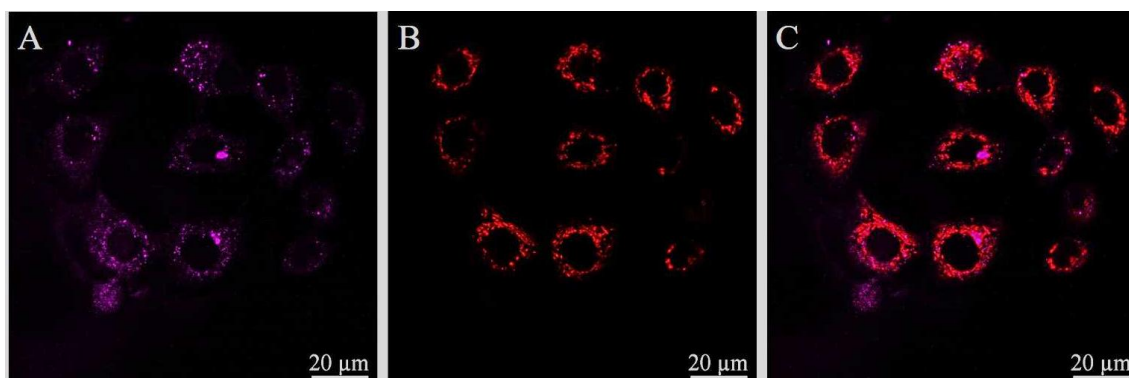


Figure 2.4 Confocal luminescence imaging from cellular uptake and localisation studies of Ru-bqp-MPP in live A549 cells. Cells were incubated for 1 h in the dark with **(A)** Ru-bqp-MPP and co-stained with **(B)** MitoTracker Deep Red (100 nM) for 30 minutes. Co-localisation was low, confirmed in **(C)** the overlay image of Ru-bqp-MPP and MitoTracker Deep Red (Pearson's coefficient = 0.302 ± 0.05). A 470 nm white light laser was used to excite the Ru(II) peptide conjugate and emission was collected between 580 and 700 nm. The MitoTracker Deep Red dye was excited at 644 nm and emission collected between 730-820 nm. Live cells were washed twice with PBS and imaged in fresh phenol red-free DMEM supplemented with 10% FBS and 1% penicillin/streptomycin.

Interestingly, and not previously observed in related complex conjugates, confocal imaging studies of A549 cells indicates efflux of the complexes may be occurring. Cells were incubated as usual at 37 °C in the dark and washed twice with PBS prior to imaging. The wash step serves to both remove any of the complex sitting on top of the cells, but also to remove any dead cells. In live A549 cells, after peaking around 1 hour incubation, emission intensity diminished over time and after a 3 h incubation, no emission could be observed under our routine imaging conditions. However as shown in Figure S2.13, using Ru-bqp-MPP as an example, uptake was observed in dead A549 cells, confirmed by DRAQ7 uptake. This behaviour was not observed in CHO and HPAC cell monolayers, where molecular brightness remained unchanged beyond 3 and 4 h incubation respectively. Further investigation is necessary, but our preliminary data suggest that efflux is occurring from this cell line.

To investigate the distribution of the complexes *in cellulo* further, co-localisation studies with MitoTracker Deep Red were carried out in A549 cells and revealed a surprisingly low correlation between the emission of the Ru-bqp-MPP complex and MitoTracker Deep Red (Figure 2.4) with a Pearson's coefficient of 0.302 ± 0.05 , while for Ru-bqp-R8 a Pearson's coefficient of just 0.163 ± 0.02 was determined. Indicating that although Ru-bqp-MPP does not show specific targeting to the mitochondria, as previously observed for complex conjugates carrying this mitochondrial targeting sequence,^[45,62] it does penetrate the mitochondria compared the general cell penetrating peptide (CPP), R8. Although poor targeting of the Ru-bqp-MPP conjugate was surprising, we have seen less predictable targeting with MPP or nuclear localising signal (NLS) peptides previously when combined with highly lipophilic Ru complexes such as biquinoline or

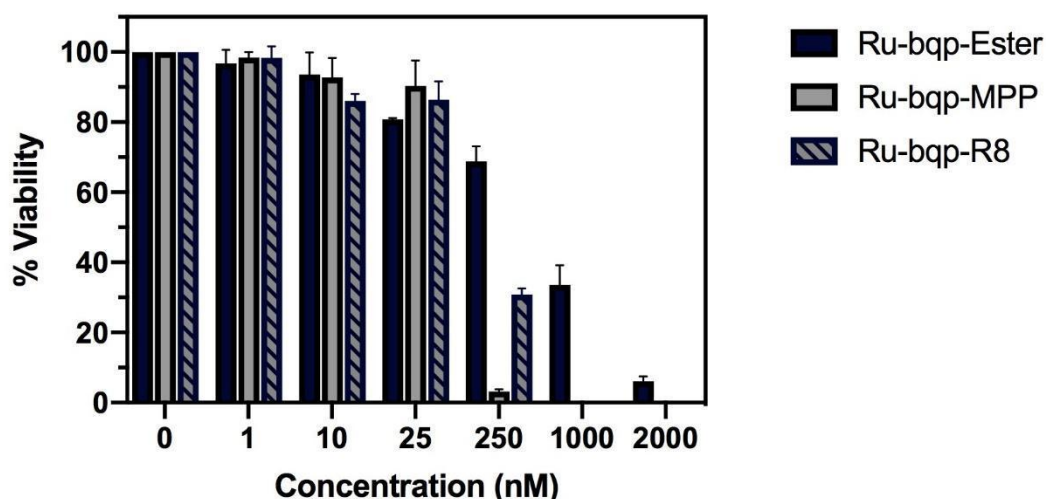


Figure 2.5 Cell viability of HPAC cells after 2 h exposure to Ru-bqp-ester, Ru-bqp-MPP and Ru-bqp-R8 at 0, 1, 10, 25, 250, 1000 and 2000 nM and irradiation with a 470 nm blue light LED to a final irradiation dose of $17 \text{ J/cm}^2 \pm 1.64 \text{ J/cm}^2$ (2 h at $2.37 \pm 0.23 \text{ mW/cm}^2$). Maximum DMSO concentration $<0.003\%$ in RPMI media. RPMI without phenol red was essential for phototoxicity studies as control experiments revealed that cells in phenol red-containing media exhibited some phototoxicity under 470 nm irradiation, potentially confounding the results.

diphenyl phenanthroline $[\text{Ru}(\text{biq})_2(\text{trz-CONH-MPP})]^{4+}$ and $[\text{Ru}(\text{biq})_2(\text{trz-CONH-NLS})]^{5+}$ studied in CHO and HeLa cell lines.^[65,66] Similarly, co-localisation of Ru-bqp-MPP with LysoTracker Green DND-26 in A549 and Rab7a-GFP in A549 and CHO cells, indicated Pearson's values of 0.197 with LysoTracker Green DND-26 and 0.117 and 0.105 for Rab7a-GFP in A549 and CHO cells respectively (A16). This observation and the evidently wide distribution of the three Ru(II) complexes in the cytoplasm along with their rapid uptake by passive diffusion indicates mainly cytoplasmic staining. Similar distribution of the Ru(II) complex and conjugate was observed across all CHO, A549 and HPAC cell monolayers, with no uptake evident in the nucleus of the cells.

2.3.4 Dark Toxicity in Cell Monolayers

The cytotoxicity of Ru-bqp-ester and its peptide conjugates, Ru-bqp-R8 and Ru-bqp-MPP is an important consideration in assessing their suitability as imaging or potential PDT agents, where for both applications dark toxicity should be low. The alamar blue (Resazurin) assay was used to assess cell viability of CHO, A549 and HPAC cell monolayers after 24 h incubation in the dark with the complex and conjugates. In the presence of metabolically active cells the alamar blue dye is reduced to resorufin and changes from navy blue to a pink colour depending on the number of viable cells. Thus, this assay can be used as a simple, direct indicator of cell viability. CHO and A549 cells were treated with the peptide conjugates at concentrations of 0, 1, 5, 10, 15, 25, 50, 75 and 100 μM and HPAC with concentrations; 0, 15, 25, 50, 75, 100 and 150 μM . In the case of Ru-bqp-ester additional concentrations 200 μM , 500 μM and 1 mM) were

Table 2.2 Cytotoxicity and Phototoxicity Half-Maximal Inhibitory Concentration (IC₅₀) Values (μM) in HPAC cell monolayers and multicellular tumour spheroids.

	HPAC Cell Monolayers			HPAC Multicellular Spheroids (3D)		
	Dark (μM)	Light (μM)	PI ^b	Dark (μM)	Light (μM)	PI ^b
<i>Ru-bqp-ester</i>	585 ± 6.28	1.77 ± 0.11	760	>100	>100	n.d.
<i>Ru-bqp-MPP</i>	27.39 ± 1.06	0.047 ± 0.01	582	>100	30.29 ± 3.17	> 3.3
<i>Ru-bqp-R8</i>	64.78 ± 0.55	0.104 ± 0.03	623	>100	41.50 ± 4.12	> 2.5

^a IC₅₀ values in μM, toxicity evaluated as the average of three plates (cells treated in triplicate per plate) ^b PI = phototoxic index, the ratio of dark to light IC₅₀ values. Cell monolayers irradiated at 17 ± 1.64 J/cm² (2.37 ± 0.23 mW/cm² for 2 h) and spheroids at 4.27 ± 0.41 J/cm² (2.37 ± 0.23 mW/cm² for 30 minutes; irradiation dose selected as appropriate for cells or spheroids without inducing toxicity for non-treated control samples). n.d. = not determinable. Studies in both HPAC cell monolayers and spheroids were performed using RPMI media supplemented with 5% FBS and 1% penicillin/streptomycin (phenol red free for phototoxicity studies).

included in the assay to obtain an exact IC₅₀ value, as Ru-bqp-ester was had significantly lower dark toxicity than the peptide conjugates (Figure S2.17).

The parent complex, Ru-bqp-ester was minimally toxic toward all cell lines assessed, with an IC₅₀ value above 100 μM in all cases when incubated for 24 h in the dark. Similarly, in A549 cells, the peptide conjugates showed low dark toxicity with an IC₅₀ value above 100 μM. By comparison, after 24-hour incubation the R8 and MPP peptide conjugates showed modest dark toxicity toward CHO and HPAC cell lines with an IC₅₀ of 50 and 20.56 ± 1.89 μM for MPP and 78 and 42.66 ± 2.52 μM for R8 in CHO and HPAC cells respectively. It is important to note that the IC₅₀ values obtained for our dark control plates and 24 h dark toxicity studies will vary as the recovery time for the dark control plate is significantly longer than for the cytotoxicity studies. Cells in our dark control plates are incubated with Ru(II) for 30 minutes and allowed 23.5 h to recover prior to the alamar blue assay, whereas cytotoxicity studies involve treating the cells for 24 h, and adding the 10% (v/v) alamar blue in media solution immediately after the incubation giving the cells no time to recover. Our findings confirm that cell death is triggered by both the MPP and R8 conjugates in a dose dependent manner that enabled us to identify a safe working concentration for imaging and phototoxicity.

2.3.5 Phototoxicity

The phototoxicity of the Ru-bqp-ester, Ru-bqp-R8 and Ru-bqp-MPP were next compared across the three cell line monolayers. Two identical plates; a dark control and phototoxicity plate, were prepared and incubated with varying concentrations of Ru-bqp-

ester, Ru-bqp-R8 and Ru-bqp-MPP for 30 minutes or 2 h depending on the cell line. The test solutions were then decanted, and the wells replaced with phenol red free media. The phototoxicity plate was irradiated using a 470 nm LED, corresponding which is close to the absorbance maximum associated with the ¹MLCT of the complex, total irradiation dose $17 \pm 1.64 \text{ J/cm}^2$ (constituting 2 h at $2.37 \pm 0.23 \text{ mW/cm}^2$) at 37 °C and the control plate was maintained under the same conditions in the dark. The cells were then incubated for a further 21.5 hours at 37 °C with 5% CO₂ to allow for cell recovery. An alamar blue assay was performed to assess cell viability. For phototoxicity studies, we selected an irradiation dose close to clinically used ranges which are typically between 20 and 200 J/cm² as possible, which also maintained cell viability.^[67] Figure 2.5 shows a bar chart of the % cell viability for HPAC cells and the same plots for CHO and A549 are shown in Appendix A.

The PI values are provided in Table 2.2. To overcome potential efflux of the complexes from the A549 cells, a shorter complex incubation time of 30 minutes was selected prior to irradiation. This was confirmed from imaging to be sufficient incubation period to allow for cell uptake, without loss of emission intensity. In contrast to the dark toxicity controls, cell viability was extremely low in A549 cells following photoirradiation. More than 70% of A549 cells were dead when treated with the parent complex Ru-bqp-ester or the Ru-bqp-MPP and Ru-bqp-R8 peptide conjugates at a dose of < 5 μM i.e. IC₅₀ under irradiation was below 5 μM.

Similar to the phototoxicity studies in A549 cells, an incubation time of 30 minutes was selected for CHO cells to allow uptake. As the parent complex, Ru-bqp-ester was not toxic towards CHO in the dark we were interested to assess the phototoxicity of the complex toward this non-cancerous cell line. Similar to the A549 and HPAC cells, the Ru-bqp-ester complex was found to be phototoxic in CHO with an IC₅₀ of below 5 μM. Cell viability was determined relative to untreated control wells using an alamar blue assay. All plates were performed in triplicate (Figure S2.18).

The HPAC cells required a longer incubation period of 2 hours prior to irradiation to account for slower uptake. Comparing the Ru(II) parent ester complex and its peptide conjugates in HPAC cells (Figure 2.5) an IC₅₀ under irradiation of just $770 \pm 110 \text{ nM}$, $47 \pm 12 \text{ nM}$, and $104 \pm 30 \text{ nM}$ for Ru-bqp-ester, R8, and MPP respectively was determined. Therefore, all compounds showed impressive PI values (phototoxic index) from Ru-bqp-MPP at 582, Ru-bqp-R8 at 623 and Ru-bqp-ester at 760 (Table 2.2). It is important to note that impressive PI values for other ruthenium polypyridyl complexes have been reported recently, most notably a PI value of 5439 in HL-60 cells, however in reported

cases higher irradiation doses, e.g. 28 J/cm² to 100 J cm/cm², was administered in these studies.^[68–71] In comparison to the ruthenium complexes reported in Table 1.1 that were tested under lower irradiation doses (5 to 18 J/cm²), the PI values of the family of ruthenium complexes reported in this chapter show significant enhancement, particularly Ru-bqp-ester with a PI value of 760 in comparison to the PI values in Table 1.1 ranging between 20-210. These results highlight the benefit of ruthenium complexes with expanded bite angle for enhanced ¹O₂ generation and thus higher phototoxic potential.

In summary, Ru-bqp-ester is minimally toxic in the dark, but highly phototoxic in all test cells when activated using a 470 nm LED. Furthermore, the peptide conjugates Ru-bqp-R8 and Ru-bqp-MPP show phototoxic potential in cancerous cell lines A549 and HPAC selected for analysis. The increased uptake of the MPP peptide conjugate into the mitochondria, as determined by our co-localisation studies, could be linked to the higher phototoxicity of the complex in A549 and HPAC cells and also the increased dark toxicity in HPAC and CHO cell monolayers in comparison to the parent and R8 peptide conjugate.

It is important to highlight that direct comparison between other reported phototoxic indices is difficult to make because of the diversity of conditions used to determine PI values. Since PI is influenced by a range of factors, including the cell type extent of permeability of a given agent and the irradiation dose.^[72] Nonetheless, our PI values seem to be amongst the highest reported for Ru(II) polypyridyl complex and exceed many reported PI values of clinically approved PS under comparable conditions.^[73–75] Based on control studies with untreated CHO, A549 and HPAC cell monolayers, we selected an irradiation dose of 17 ± 1.64 J/cm² because this intensity was observed in control not to impact the cells directly and because similar irradiation conditions have been used by others, for example Cloonan et. al applied 18 J/cm² to study the phototoxicity of their photoactive Ru(II) complexes.^[76] For approved PDT agents conditions reported for PI vary for example, the phototoxicity of Photofrin has been extensively studied, with reported PI values of at least 10, though the irradiation dose is often lower than used here. For example, PI of Photofrin was reported to be >10 in HeLa cells with an irradiation dose of 5 J/cm² by Delaey *et al.*^[77] Additionally, PI values of >18 and 4.2 were reported for clinically approved PDT drug aminolevulinic acid (ALA) by Glazer and colleagues in HL-60 leukaemia cells and A549 cell monolayers respectively.^[78]

The thiophene pendants in TLD-1433 are reported to serve to delocalise the excited state of the complex reducing its energy and promoting its triplet ligand character and

lifetime to promoting triplet energy transfer to oxygen.^[79] As described the expansion of the coordination cage in complexes like Ru-bqp, can similarly extend triplet lifetime and promote triplet energy transfer. Thus, it is anticipated that that the origin of phototoxicity is $^1\text{O}_2$ or ROS generation. To identify singlet oxygen production a $^1\text{O}_2$ scavenger study was completed using 1,3-diphenylisobenzofuran (DPBF). In the presence of $^1\text{O}_2$, DPBF forms endoperoxide 1, which decomposes to the colourless 1,2-dibenzoylbenzene (DBB) that can be detected spectrophotometrically through decreases to the DPBF.

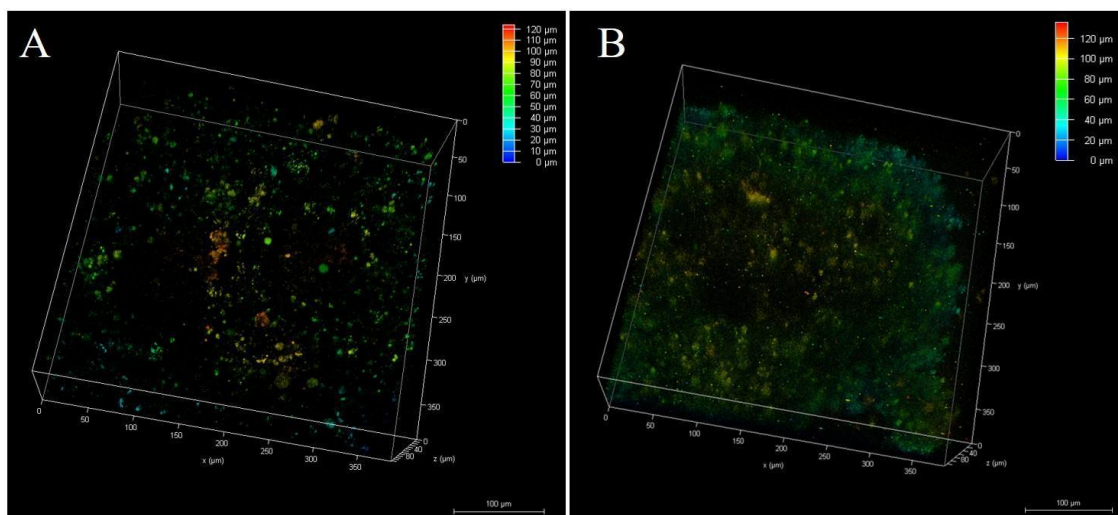


Figure 2.6 3D reconstruction depth coding images of live HPAC spheroids with Ru-bqp-ester at **(A)** 30 μM and **(B)** 100 μM at 24 h incubation in the dark obtained by collecting images in a z-scan from below to above the spheroid. A 490 nm white light laser was used to excite the spheroids with Ru(II), and emission was collected between 580 and 730 nm. Spheroids were imaged in RPMI without phenol red, supplemented with 5% FBS and 1% penicillin/streptomycin.

absorbance at 410 nm. Although highly sensitivity to $^1\text{O}_2$ it is important to note that DPBF can also react with other ROS, for example superoxide anions or hydroxyl radicals. A solution of 10 mM DPBF containing 20 μM of the parent complex Ru-bqp-ester in Ethanol (with 1% DMSO) was assessed for $^1\text{O}_2$ generation by photoirradiating the sample with a 470 nm LED at $2.37 \pm 0.23 \text{ mW/cm}^2$. Within 5 minutes of irradiation of the DPBF-Ru solution was degraded with a decrease in absorbance of $77 \pm 4 \%$ (Table S2.1), indicating the phototoxicity of the Ru(II) complexes is the result $^1\text{O}_2$ generation. A control study was performed by irradiating the DPBF without the Ru-bqp-ester to confirm direct photodecomposition of the DPBF was $<10\%$ decrease in absorbance was observed over $t=0$ and $t = 6$ minutes of irradiation.

2.3.6 3D Cell Culture – Multicellular Tumour Spheroids

While imaging and cytotoxicity of metal complex luminophores have been widely investigated in 2D culture, there has been far less study in multicellular structures, and particularly in the context of PDT, very little evaluation has been extended to study of

metal complexes peptide conjugates in 3D cell models. However, they can provide a more physiologically realistic insight into the potential effectiveness of PDT reagents *in vivo*. We thus evaluated the uptake, toxicity and photocytotoxicity of the parent and conjugates in human pancreatic cell (HPAC) multicellular tumour spheroids (MCTS).

Confocal fluorescence imaging was performed to determine if the cellular uptake observed in 2D culture extends to 3D models. The HPAC MCTS were grown for 96 hours, treated with Ru-bqp-ester, Ru-bqp-MPP or Ru-bqp-R8 for 24 h at 30 and 100 μM

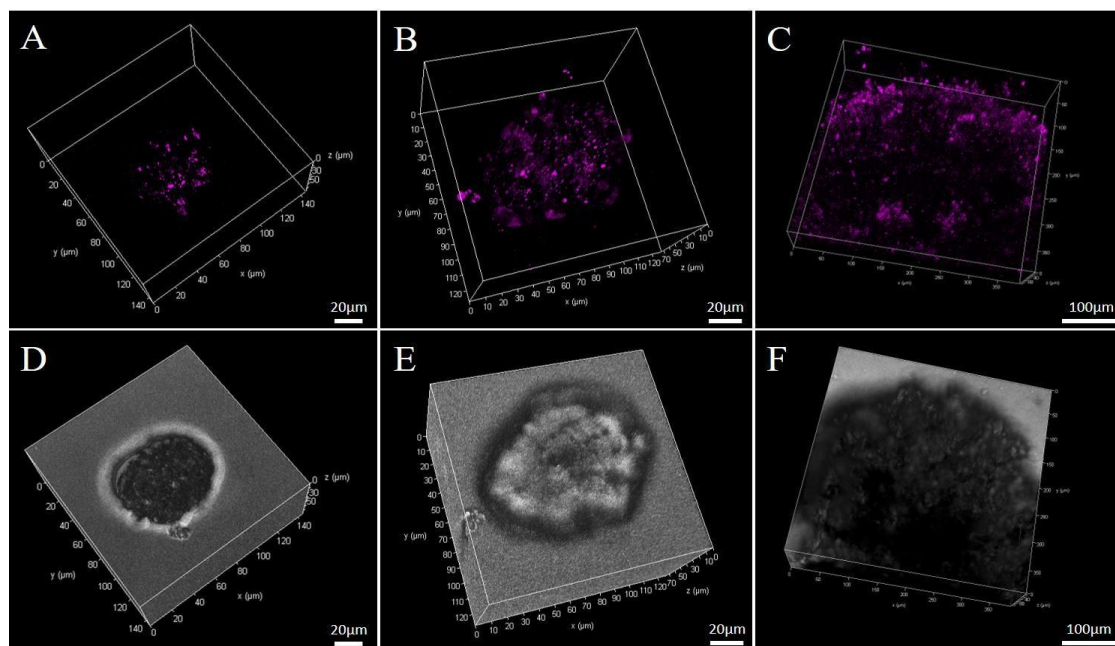


Figure 2.7 3D reconstruction images of live HPAC spheroids treated with (A) Ru-bqp-ester (B) Ru-bqp-MPP and (C) Ru-bqp-R8 at 100 μM for 24 h and (D-E) the brightfield images of the respective spheroids. A 490 nm white light laser was used to excite the spheroids, and emission was collected between 580 and 730 nm.

before imaging. A 24 h incubation window was selected to mimic the majority of current PDT protocols, which involve administration of a PS approximately 24–48 h prior to surgery.^[80] For example, Raza and colleagues studied the uptake of a ruthenium(II) complex $[\{\text{Ru}(\text{TAP}_2)\}_2(\text{tpphz})]^{4+}$ in 3D melanoma spheroids prepared using the C8161 cell line, and confirmed in that case that a 24 hour incubation period of the complex at 100 μM was sufficient to ensure uptake.^[81] Confocal imaging of HPAC spheroids with Ru-bqp-ester, Ru-bqp-MPP and Ru-bqp-R8 confirmed some uptake at 30 μM , the concentration used for imaging of 2D culture. However, a higher concentration of 100 μM ensured complete internalisation of the complex into the 3D structure. This was confirmed from depth coding images of Ru-bqp-ester incubated with 30 and 100 μM for 24 hours prior shown in Figure 2.6, where a clear contrast in the accumulation of ruthenium at 30 μM (Figure 2.6A) and 100 μM (Figure 2.6B) can be seen. As for the 2D

studies, the spheroid studies indicate similar cellular uptake between the parent and peptide conjugates in the spheroids. As shown in Figure 2.7, there is wide distribution of the complexes throughout the spheroids, confirming they are capable of penetrating deeply throughout the spheroid, with no evidence of subcellular localisation. Overall, the data indicates both the parent and peptide conjugates can be delivered effectively to cells in a multicellular environment and thus shows potential for use as therapeutic agents.

2.3.7 Toxicity in a 3D Cell Model (Spheroids)

The dark and phototoxicity of the parent and peptide conjugates were also assessed in HPAC spheroids. Control studies were performed with untreated spheroids to determine the appropriate irradiation dose for phototoxicity studies. The spheroids were treated with 0, 5, 25, 50 and 100 μM of the parent and peptide conjugates for 24 h, fresh phenol red free media added, and the plate irradiated with a 470 nm LED at a total dose of $4.27 \pm 0.41 \text{ J/cm}^2$ (30 min at $2.37 \pm 0.23 \text{ mW/cm}^2$). The spheroids were incubated for a further 23.5 h in the dark to allow for recovery. Cell viability was determined using the CellTiter-Glo 3D luminescence-based assay, and viability assays were performed in triplicate. The CellTiter-Glo 3D reagent can penetrate deeply into 3D cell cultures and detects the presence of ATP, an indicator of cell viability. As in the 2D alamar blue assay protocol, % viability is calculated relative to untreated control spheroids.

Phototoxicity assays performed with HPAC spheroids confirmed that the phototoxicity of the ruthenium complexes observed in 2D cell monolayer studies persists in 3D spheroids. As previously mentioned, 3D cell culture models closely mimic the *in vivo* environment of tumours compared to experiments using cell monolayers. Therefore, there is often a difference in uptake, distribution, and phototoxic effects in 3D versus 2D cell cultures. In comparison to the phototoxicity studies performed in HPAC cell monolayers where the cells required only a 2-hour incubation with the ruthenium complexes, the spheroids were treated for 24 hours prior to irradiation. As outlined in Table 2.2, the phototoxic indices of Ru-bqp-ester, Ru-bqp-MPP and Ru-bqp-R8 were lower when tested in 2D compared to 3D HPAC cell models. While the dark IC_{50} values of the complexes were above 100 μM , indicating low dark toxicity, we confirmed that both the peptide conjugates Ru-bqp-MPP and Ru-bqp-R8 had phototoxic indices of above 2. As per the OECD TG 432,^[82] a PI value between 2 and 5 indicates probable phototoxicity and a PI value above 5 suggests positive phototoxicity, thus as the PI values achieved for Ru-bqp-MPP and Ru-bqp-R8 were in the >2 and <5 category for our

3D toxicity studies in HPAC spheroids, the phototherapeutic potential of the conjugates carries across 2D to 3D studies.

The notably lower phototoxicity of the Ru-bqp-ester complex toward 3D cell models compared to the peptide conjugates was surprising given their relatively similar effects in 2D-cell culture. This we tentatively attribute to potentially improved penetration of Ru-bqp-MPP and Ru-bqp-R8, leading to their potentially higher accumulation into vulnerable organelles, particularly mitochondria in the spheroid compared to the ester. Although uptake on the basis of intensity seemed to be only modestly better in the case of the peptides from confocal imaging, the localisation of Ru-bqp-ester, Ru-bqp-MPP and Ru-bqp-R8 was not distinguished in imaging of the HPAC spheroids, but given improved mitochondrial penetration was observed, particularly in the case of Ru-bqp-MPP in 2D culture, this is a rationale conclusion.

It is important to note that although there was a significant decrease in phototoxicity in spheroids with an IC_{50} of $> 100 \mu M$ for Ru-bqp-ester, $30.29 \pm 3.17 \mu M$ for Ru-bqp-MPP and $41.50 \pm 4.12 \mu M$ for Ru-bqp-R8, this can in large part be ascribed to the reduced irradiation dose the spheroids received in comparison to the cell monolayers. We found that a lower irradiation dose was necessary from control studies performed on the spheroids in media, without sensitiser, that indicated the irradiation dose of $17 \pm 1.64 J/cm^2$ used for cell monolayer studies had direct toxic impact on the spheroids. Therefore, the irradiation time and consequently the total irradiation dose for 3D toxicity studies was reduced to a level that showed no toxicity in the controls which is about 4x lower than that applied to 2D culture. This does not fully account for the difference observed though. While cell monolayers can quickly repair the damage caused by the LED/sensitiser, spheroids have a reduced capacity for repair since their compact nature reduces oxygen availability and nutrients in the inner layers that along with their slower proliferation rate, reduces their recovery from radiation damage compared to cell monolayers. In addition, the inner layers of spheroids, may be shielded somewhat from the irradiation, as scatter will reduce the penetrative power of the light and therefore the ruthenium that is accumulating in this region may not be excited as efficiently by the LED. Oxygen concentration is also expected to be depleted at the spheroid core, depending on the spheroid size, and assuming based on the singlet oxygen studies above that it is a Type II PDT agent, the reduced molecular oxygen at the core will reduce the effectiveness of the Ru(II) complexes if operating through a Type II mechanism. Of note, however, typically spheroids of larger than $200 \mu m$ begin to show a hypoxic centre and require a minimum of 4 days to form the necrotic zone depending on the cell line used, with $500 \mu m$ being the optimum spheroid size for the formation of necrotic

zones.^[83,84] Our spheroids were grown for 4 days, to a diameter of 100 to 250 μm , thus while our spheroids may not have a hypoxic core like larger spheroids, they will still have less oxygen at their core due to their size. Although the complex and conjugates show excellent PI in cell monolayers and spheroid penetrability, the reduced PI at the spheroid reflect better the challenges and performance of the complexes and their phototoxic potential in a tumour environment.

Similar increases in the IC_{50} values and therefore decrease in the phototoxic indices of phototoxic agents between 2D and 3D cell culture models have been noted before. For example, Kucinska *et al.* have reported IC_{50} values of zinc phthalocyanine M2TG3 in spheroids prepared and grown for 3 days using the prostate cancer cell line LNCaP are almost four times higher than the IC_{50} values obtained for 2D cell monolayers of the same cell type under normoxic conditions.^[85] However, in our case, as the irradiation dose selected for the HPAC spheroids is lower than was tested on the cell monolayers, may account for the lower PI values obtained. Overall, the contrast in toxicity values obtained in 3D versus 2D cell models highlights the importance of evaluating the efficacy of PSs in both models to gain a better understanding of their potential for use in PDT.

2.4 Conclusions

A luminescent ruthenium(II) polypyridyl complex Ru-bqp-ester and its peptide conjugates, Ru-bqp-MPP and Ru-bqp-R8, exhibited excellent cell permeability with a rapid energy-independent uptake in HPAC, A549, and CHO cells in growth media. The parent complex was minimally cytotoxic toward A549, CHO, and HPAC cells with IC_{50} values above 100 μM , while the MPP and R8 peptide conjugates showed moderate cytotoxicity toward HPAC and CHO cells. Under conditions used for cellular imaging, cells remained viable, confirmed by the lack of DRAQ7 uptake during the confocal imaging window. However, under continuous photoirradiation using a 470 nm LED, the parent complex and peptide conjugates showed high phototoxicity in both A549 and HPAC cancer cell lines with PI values ranging from 580 to 750 in HPAC cells. The assessment by $^1\text{O}_2$ scavenger, DPBF, indicated that the mechanism is type II, as expected. Interestingly, conjugating the parent complex to a cell penetrating octaarginine sequence or a mitochondrial targeting sequence results in increased dark cytotoxicity in the CHO and HPAC cell lines. Co-localisation studies with MitoTracker Deep Red confirmed mitochondrial penetration, particularly for the MPP conjugate, but showed a low Pearson's coefficient value, which indicated mainly non-specific localisation of Ru-bqp-MPP and R8 within the mitochondria of A549 and CHO cell monolayers. Nonetheless Ru-bqp-MPP was found to be the most toxic of the three Ru(II) complexes toward the test cells used in this study. Although targeting is not specific, the greater

toxicity of Ru-bqp-MPP is likely due to superior penetration of this complex to the mitochondria than the other complexes, reflected in the higher Pearson's coefficient with MitoTracker. The cellular uptake and phototoxicity of these complexes observed in the 2D cell culture was shown to extend to 3D culture in human pancreatic MCTS. The parent and peptides exhibited similar localisation and deep penetration throughout 3D spheroids. Photocytotoxicity was observed in the HPAC spheroids but was reduced, notwithstanding lower irradiation powers, compared to the 2D cell monolayers. Overall, this work demonstrates that ruthenium complexes with enhanced ligand metal-centred triplet states make potentially valuable nominees as phototherapeutics, for example, as photosensitisers for PDT.

2.5 References

- [1] G. Gunaydin, M. E. Gedik, S. Ayan, *Front. Chem.* **2021**, 9.
- [2] L. Zhang, A. Yang, C. Ruan, B.-P. Jiang, X. Guo, H. Liang, W.-S. Kuo, X.-C. Shen, *ACS Appl. Mater. Interfaces* **2023**, 15, 3253–3265.
- [3] N. Meng, P. Xu, C. Wen, H. Liu, C. Gao, X.-C. Shen, H. Liang, *J. Colloid Interface Sci.* **2023**, 644, 437–453.
- [4] C. Gao, W. Guo, X. Guo, Z. Ding, Y. Ding, X.-C. Shen, *Acta Biomater.* **2021**, 129, 220–234.
- [5] C. Mari, V. Pierroz, S. Ferrari, G. Gasser, *Chem. Sci.* **2015**, 6, 2660–2686.
- [6] E. Skovsen, J. W. Snyder, J. D. C. Lambert, P. R. Ogilby, *J. Phys. Chem. B* **2005**, 109, 8570–8573.
- [7] A. P. Castano, T. N. Demidova, M. R. Hamblin, *Photodiagnosis Photodyn. Ther.* **2005**, 2, 1–23.
- [8] J. Karges, F. Heinemann, M. Jakubaszek, F. Maschietto, C. Subecz, M. Dotou, R. Vinck, O. Blacque, M. Tharaud, B. Goud, E. Viñuelas Zahínos, B. Spingler, I. Ciofini, G. Gasser, *J. Am. Chem. Soc.* **2020**, 142, 6578–6587.
- [9] J. H. Correia, J. A. Rodrigues, S. Pimenta, T. Dong, Z. Yang, *Pharmaceutics* **2021**, 13, 1332.
- [10] C. Donohoe, M. O. Senge, L. G. Arnaut, L. C. Gomes-da-Silva, *Biochim. Biophys. Acta BBA - Rev. Cancer* **2019**, 1872, 188308.
- [11] H. Abrahamse, M. R. Hamblin, *Biochem. J.* **2016**, 473, 347–364.
- [12] R. R. Allison, G. H. Downie, R. Cuenca, X.-H. Hu, C. J. Childs, C. H. Sibata, *Photodiagnosis Photodyn. Ther.* **2004**, 1, 27–42.
- [13] J. Shum, P. K.-K. Leung, K. K.-W. Lo, *Inorg. Chem.* **2019**, 58, 2231–2247.

- [14] P. Agostinis, K. Berg, K. A. Cengel, T. H. Foster, A. W. Girotti, S. O. Gollnick, S. M. Hahn, M. R. Hamblin, A. Juzeniene, D. Kessel, M. Korbelik, J. Moan, P. Mroz, D. Nowis, J. Piette, B. C. Wilson, J. Golab, *CA. Cancer J. Clin.* **2011**, *61*, 250–281.
- [15] A. Fikai, A. M. Grumezescu, *Nanostructures for Antimicrobial Therapy*, Elsevier, **2017**.
- [16] Y. Wu, S. Li, Y. Chen, W. He, Z. Guo, *Chem. Sci.* **2022**, *13*, 5085–5106.
- [17] L. C.-C. Lee, K. K.-W. Lo, *Chem. – Asian J.* **2022**, *17*, e202200840.
- [18] K. K.-W. Lo, *Acc. Chem. Res.* **2015**, *48*, 2985–2995.
- [19] J. Karges, S. Kuang, F. Maschietto, O. Blacque, I. Ciofini, H. Chao, G. Gasser, *Nat. Commun.* **2020**, *11*, 3262.
- [20] J. Li, Z. Zhuang, Z. Zhao, B. Z. Tang, *VIEW* **2022**, *3*, 20200121.
- [21] M. Ochsner, *J. Photochem. Photobiol. B* **1997**, *39*, 1–18.
- [22] L. M. Lifshits, J. A. R. Iii, E. Ramasamy, R. P. Thummel, C. G. Cameron, S. A. McFarland, *J. Photochem. Photobiol.* **2021**, *8*, 100067.
- [23] S. Paul, P. Kundu, P. Kondaiah, A. R. Chakravarty, *Inorg. Chem.* **2021**, *60*, 16178–16193.
- [24] S. Monro, K. L. Colón, H. Yin, J. I. Roque, P. Konda, S. Gujar, R. P. Thummel, L. Lilge, C. G. Cameron, S. A. McFarland, *Chem. Rev.* **2019**, *119*, 797–828.
- [25] S. A. McFarland, A. Mandel, R. Dumoulin-White, G. Gasser, *Curr. Opin. Chem. Biol.* **2020**, *56*, 23–27.
- [26] S. Chamberlain, H. D. Cole, J. Roque, D. Bellnier, S. A. McFarland, G. Shafirstein, *Pharmaceuticals* **2020**, *13*, 137.
- [27] K. Rybicka-Jasińska, W. Shan, K. Zawada, K. M. Kadish, D. Gryko, *J. Am. Chem. Soc.* **2016**, *138*, 15451–15458.
- [28] L. Cosgrave, M. Devocelle, R. J. Forster, T. E. Keyes, *Chem. Commun.* **2010**, *46*, 103–105.
- [29] L. Holden, K. S. Gkika, C. S. Burke, C. Long, T. E. Keyes, *Inorg. Chem.* **2023**, *62*, 2213–2227.
- [30] M. T. Rupp, N. Shevchenko, G. S. Hanan, D. G. Kurth, *Coord. Chem. Rev.* **2021**, *446*, 214127.
- [31] A. Breivogel, C. Förster, K. Heinze, *Inorg. Chem.* **2010**, *49*, 7052–7056.
- [32] E. A. Medlycott, G. S. Hanan, *Chem. Soc. Rev.* **2005**, *34*, 133–142.
- [33] T. Schlotthauer, B. Suchland, H. Görls, G. A. Parada, L. Hammarström, U. S. Schubert, M. Jäger, *Inorg. Chem.* **2016**, *55*, 5405–5416.
- [34] M. Abrahamsson, M. Jäger, T. Österman, L. Eriksson, P. Persson, H.-C. Becker, O. Johansson, L. Hammarström, *J. Am. Chem. Soc.* **2006**, *128*, 12616–12617.

- [35] F. Schramm, V. Meded, H. Fliegl, K. Fink, O. Fuhr, Z. Qu, W. Kloppe, S. Finn, T. E. Keyes, M. Ruben, *Inorg. Chem.* **2009**, *48*, 5677–5684.
- [36] D. G. Brown, N. Sanguantrakun, B. Schulze, U. S. Schubert, C. P. Berlinguette, *J. Am. Chem. Soc.* **2012**, *134*, 12354–12357.
- [37] P. Kaspler, A. Mandel, R. Dumoulin-White, M. Roufaiel, P. Kaspler, A. Mandel, R. Dumoulin-White, M. Roufaiel, in *Tumor Progress. Metastasis*, IntechOpen, **2020**.
- [38] D. Van Straten, V. Mashayekhi, H. S. De Bruijn, S. Oliveira, D. J. Robinson, *Cancers* **2017**, *9*, 19.
- [39] L. Holden, C. S. Burke, D. Cullinane, T. E. Keyes, *RSC Chem. Biol.* **2021**, *2*, 1021–1049.
- [40] S. Park, J. L. Gray, S. D. Altman, A. R. Hairston, B. T. Beswick, Y. Kim, E. T. Papish, *J. Inorg. Biochem.* **2020**, *203*, 110922.
- [41] N. Avramović, B. Mandić, A. Savić-Radojević, T. Simić, *Pharmaceutics* **2020**, *12*, 298.
- [42] P. O. Ughachukwu, P. C. Unekwe, *Ann. Med. Health Sci. Res.* **2012**, *2*, 191–198.
- [43] E. M. Hahn, N. Estrada-Ortiz, J. Han, V. F. C. Ferreira, T. G. Kapp, J. D. G. Correia, A. Casini, F. E. Kühn, *Eur. J. Inorg. Chem.* **2017**, *2017*, 1667–1672.
- [44] A. Luengo, I. Marzo, M. Reback, I. M. Daubitz, V. Fernández-Moreira, N. Metzler-Nolte, M. C. Gimeno, *Chem. – Eur. J.* **2020**, *26*, 12158–12167.
- [45] A. Martin, A. Byrne, C. S. Burke, R. J. Forster, T. E. Keyes, *J. Am. Chem. Soc.* **2014**, *136*, 15300–15309.
- [46] K. S. Gkika, S. Noorani, N. Walsh, T. E. Keyes, *Inorg. Chem.* **2021**, *60*, 8123–8134.
- [47] S. A. Langhans, *Front. Pharmacol.* **2018**, *9*.
- [48] Y. Zhu, E. Kang, M. Wilson, T. Basso, E. Chen, Y. Yu, Y.-R. Li, *Organoids* **2022**, *1*, 149–167.
- [49] J. Ro, J. Kim, Y.-K. Cho, *Analyst* **2022**, *147*, 2023–2034.
- [50] H. Huang, P. Zhang, H. Chen, L. Ji, H. Chao, *Chem. – Eur. J.* **2015**, *21*, 715–725.
- [51] U. K. Komarnicka, S. Kozieł, B. Pucelik, A. Barzowska, M. Siczek, M. Malik, D. Wojtala, A. Nioiretini, A. Kyzioł, V. Sebastian, P. Kopel, S. Caramori, A. Bieńko, *Inorg. Chem.* **2022**, *61*, 19261–19273.
- [52] R. Bevernaegie, B. Doix, E. Bastien, A. Diman, A. Decottignies, O. Feron, B. Elias, *J. Am. Chem. Soc.* **2019**, *141*, 18486–18491.
- [53] H. Huang, B. Yu, P. Zhang, J. Huang, Y. Chen, G. Gasser, L. Ji, H. Chao, *Angew. Chem. Int. Ed.* **2015**, *54*, 14049–14052.
- [54] P. Zhang, C. K. C. Chiu, H. Huang, Y. P. Y. Lam, A. Habtemariam, T. Malcomson, M. J. Paterson, G. J. Clarkson, P. B. O'Connor, H. Chao, P. J. Sadler, *Angew. Chem. Int. Ed.* **2017**, *56*, 14898–14902.

- [55] M. Abrahamsson, H.-C. Becker, L. Hammarström, *Dalton Trans.* **2017**, 46, 13314–13321.
- [56] L. A. Fredin, J. Wallenstein, E. Sundin, M. Jarenmark, D. F. Barbosa de Mattos, P. Persson, M. Abrahamsson, *Inorg. Chem.* **2019**, 58, 16354–16363.
- [57] M. Jäger, R. J. Kumar, H. Görls, J. Bergquist, O. Johansson, *Inorg. Chem.* **2009**, 48, 3228–3238.
- [58] M. Abrahamsson, M. Jäger, R. J. Kumar, T. Österman, P. Persson, H.-C. Becker, O. Johansson, L. Hammarström, *J. Am. Chem. Soc.* **2008**, 130, 15533–15542.
- [59] M. Majuran, G. Armendariz-Vidales, S. Carrara, M. A. Haghighatbin, L. Spiccia, P. J. Barnard, G. B. Deacon, C. F. Hogan, K. L. Tuck, *ChemPlusChem* **2020**, 85, 346–352.
- [60] D. Wlodkowic, J. Akagi, J. Dobrucki, R. Errington, P. J. Smith, K. Takeda, Z. Darzynkiewicz, *Curr. Protoc. Cytom.* **2013**, 65, 9.41.1–9.41.8.
- [61] P. Spence, J. Fielden, Zoë. A. E. Waller, *J. Am. Chem. Soc.* **2020**, 142, 13856–13866.
- [62] C. S. Burke, A. Byrne, T. E. Keyes, *Angew. Chem. Int. Ed.* **2018**, 57, 12420–12424.
- [63] C. A. Puckett, J. K. Barton, *Biochemistry* **2008**, 47, 11711–11716.
- [64] S. Finn, A. Byrne, K. S. Gkika, T. E. Keyes, *Front. Chem.* **2020**, 8.
- [65] L. Blackmore, R. Moriarty, C. Dolan, K. Adamson, R. J. Forster, M. Devocelle, T. E. Keyes, *Chem. Commun.* **2013**, 49, 2658–2660.
- [66] D. Cullinane, K. S. Gkika, A. Byrne, T. E. Keyes, *J. Inorg. Biochem.* **2020**, 207, 111032.
- [67] M. M. Kim, A. Darafsheh, *Photochem. Photobiol.* **2020**, 96, 280–294.
- [68] B. A. Albani, B. Peña, N. A. Leed, N. A. B. G. de Paula, C. Pavani, M. S. Baptista, K. R. Dunbar, C. Turro, *J. Am. Chem. Soc.* **2014**, 136, 17095–17101.
- [69] X. Hu, N.-Y. Liu, Y.-Q. Deng, S. Wang, T. Liu, X.-W. Liu, *Molecules* **2021**, 26, 3471.
- [70] G. Ghosh, H. Yin, S. M. A. Monro, T. Sainuddin, L. Lapoot, A. Greer, S. A. McFarland, *Photochem. Photobiol.* **2020**, 96, 349–357.
- [71] D. A. Smithen, S. Monro, M. Pinto, J. Roque, R. M. Diaz-Rodriguez, H. Yin, C. G. Cameron, A. Thompson, S. A. McFarland, *Chem. Sci.* **2020**, 11, 12047–12069.
- [72] M. Ankathatti Munegowda, A. Manalac, M. Weersink, S. A. McFarland, L. Lilge, *Coord. Chem. Rev.* **2022**, 470, 214712.
- [73] E. T. Papish, O. E. Oladipupo, *Curr. Opin. Chem. Biol.* **2022**, 68, 102143.
- [74] F. Heinemann, J. Karges, G. Gasser, *Acc. Chem. Res.* **2017**, 50, 2727–2736.
- [75] Q. Chen, V. Ramu, Y. Aydar, A. Groenewoud, X.-Q. Zhou, M. J. Jager, H. Cole, C. G. Cameron, S. A. McFarland, S. Bonnet, B. E. Snaar-Jagalska, *Cancers* **2020**, 12, 587.

- [76] S. M. Cloonan, R. B. P. Elmes, M. Erby, S. A. Bright, F. E. Poynton, D. E. Nolan, S. J. Quinn, T. Gunnlaugsson, D. C. Williams, *J. Med. Chem.* **2015**, *58*, 4494–4505.
- [77] E. Delaey, F. van Laar, D. De Vos, A. Kamuhabwa, P. Jacobs, P. de Witte, J. *Photochem. Photobiol. B* **2000**, *55*, 27–36.
- [78] B. S. Howerton, D. K. Heidary, E. C. Glazer, *J. Am. Chem. Soc.* **2012**, *134*, 8324–8327.
- [79] C. Reichardt, S. Monro, F. H. Sobotta, K. L. Colón, T. Sainuddin, M. Stephenson, E. Sampson, J. I. Roque, H. Yin, J. C. Brendel, C. G. Cameron, S. McFarland, B. Dietzek, *Inorg. Chem.* **2019**, *58*, 3156–3166.
- [80] G. Shafirstein, A. Battoo, K. Harris, H. Baumann, S. O. Gollnick, J. Lindenmann, C. E. Nwogu, *Ann. Am. Thorac. Soc.* **2016**, *13*, 265–275.
- [81] A. Raza, S. A. Archer, S. D. Fairbanks, K. L. Smitten, S. W. Botchway, J. A. Thomas, S. MacNeil, J. W. Haycock, *J. Am. Chem. Soc.* **2020**, *142*, 4639–4647.
- [82] Organization for Economic Co-Operation and Development-OECD. Test No. 432: In Vitro 3T3 NRU Phototoxicity Test, **2018**.
- [83] D. Khaitan, S. Chandna, M. B. Arya, B. S. Dwarakanath, *J. Transl. Med.* **2006**, *4*, 12.
- [84] S. Däster, N. Amatruda, D. Calabrese, R. Ivanek, E. Turrini, R. A. Drieser, P. Zajac, C. Fimognari, G. C. Spagnoli, G. Iezzi, V. Mele, M. G. Muraro, *Oncotarget* **2016**, *8*, 1725–1736.
- [85] M. Kucinska, A. Plewinski, W. Szczolko, M. Kaczmarek, T. Goslinski, M. Murias, *Free Radic. Biol. Med.* **2021**, *162*, 309–326.

Chapter 3 : Visualising Stress Granule Dynamics with an RNA Guanine Quadruplex Targeted Ruthenium(II) Peptide Conjugate

Submitted

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Contributions:

Rhianne C. Curley: Performed all cellular studies including assays and imaging. Co-first author and contributor to experimental design, execution, analysis of cellular work. Manuscript preparation and revision.

Dr. Lorcan Holden: Synthesis and photophysical characterisation of Ru-RHAU. Co-first author and contributor to experimental design, execution, analysis of all DNA/RNA studies. Manuscript preparation and revision.

Professor Tia E. Keyes: Project conceptualisation and management, discussion, and revision of the manuscript.

Supporting information associated with this chapter in **Appendix B**.

Visualising Stress Granule Dynamics with an RNA Guanine Quadruplex Targeted Ruthenium(II) Peptide Conjugate

3.1 Abstract

Stress granules (SGs) are membraneless ribonucleoprotein assemblies that form in response to cellular stress. They have been linked to cell survival and cancer progression, though many questions remain regarding their structure, function and therapeutic potential. Live cell fluorescence imaging is key to advancing understanding of SGs, but there are very few small molecule probes reported that selectively image these organelles. RNA G-quadruplex (rG4) folding is believed to play a role in initiation of SG formation. Thus, to create a probe for SGs, we exploited a G4 binding domain peptide from RNA helicase associated with AU-rich element (RHAU) conjugated to a luminescent $[\text{Ru}(\text{bpy})_2(\text{PIC-COOH})]^{2+}$, Ru-RHAU, designed to target rG4s and thus SGs in live cells. Studies *in cellulo* demonstrate that Ru-RHAU can induce SG formation in a concentration and time dependent manner and immunolabelling confirmed the complex remains associated with rG4s in the SG, so we attribute SG stimulation to stabilisation of rG4 by Ru-RHAU. Ru-RHAU shows low cytotoxicity under imaging conditions, permitting prolonged observation in live cells. Under more intense photoirradiation, Ru-RHAU induces phototoxicity through an apoptotic pathway. Ru-RHAU is a versatile tool for probing SG dynamics and function in cellular stress responses and for heretofore unconsidered potential phototherapeutic applications targeting SGs.

3.2 Introduction

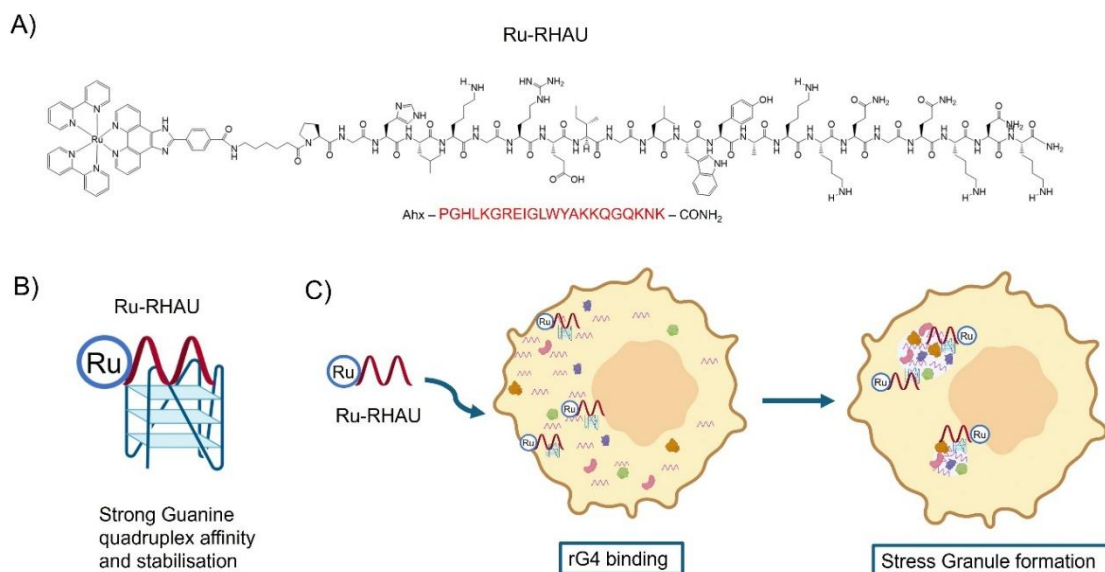
Guanine quadruplexes (G4s) are non-canonical secondary structures that assemble in guanine rich sequences through a combination of Hoogsteen hydrogen bonding, pi stacking and Van der Waals forces. They occur in both DNA and RNA sequences. Compared to DNA, guanine in RNA contains a 2'-hydroxyl group at ribose, that facilitates greater intramolecular interaction, increasing RNA G4s (rG4s) *in vitro* stability. Another key difference is the rG4 topological preference for parallel structure, attributed to steric constraints from the presence of the 2' hydroxyl-group.^[1] In recent years, numerous studies have provided evidence for the formation of rG4s in a live cell environment including imaging, computational, and molecular biology studies, linking their formation to essential biological processes.^[2] Cytoplasmic rG4s have been implicated in RNA translation, stability, transport, and RNA-binding protein (RBP) sequestering although their roles and prevalence has yet to be fully elucidated.^[3] In particular, due to RNA role in translation, rG4s have emerged as an exciting target for small molecule drugs, with a

view to stabilise rG4s and inhibit translation by preventing ribosomal activity or translation machinery.

Recently, a link has emerged between the formation of cytoplasmic rG4s and stress granules (SGs).^[4] Theoretical and experimental studies indicate that rG4 sequences are ubiquitous in the 5' and 3' untranslated regions (UTRs) of mRNAs and that rG4 folding is stimulated in mRNA 3'-UTRs under stress conditions.^[5-7] Furthermore, evidence suggests rG4 folding in UTRs stimulates interactions with RNA-binding proteins seeding the formation of ribonucleoprotein biomolecular condensates including stress granules (SGs). Recent studies have provided direct evidence for rG4 role in the assembly of SGs.^[8,9] For example Turner et al. have reported that G3BP1, a key SG nucleating protein, interacts with rG4s in a stress-dependent manner.^[10]

SGs are non-membranous phase separated organelles that assemble in response to various cell stress response pathways. Some stressors include heat shock or arsenic salt concentrations, and exposure to UV-light. By sequestering UTRs into condensates, the SGs are believed to provide protective effect sequestered mRNAs and SG formation in response to cellular stress has notable advantages for cell survival by reducing energy expenditure and minimising damage. However, their role and function is not yet fully elucidated.

Aberrant, persistent SGs result from SG dysregulation and have been linked to cancer progression as well as several human neurodegenerative disorders, including amyotrophic lateral sclerosis (ALS).^[11-13] While it is clear that SGs are of great importance to cellular homeostasis and survival under stress conditions. There are many open questions regarding their structure, mechanism and dynamics of formation and disassembly. There are currently limited probes available for their study in live cells. Immunofluorescent labelling or fluorescence in situ hybridisation (FISH) are the most widely used approaches but are not amenable to live cell studies.^[14-16] Ideally to investigate and visualise the composition of SGs, to monitor their assembly and disassembly in a live cell environment and to unravel their connection to rG4s, small molecule, cell permeable probes that recognize rG4s are required.^[17]



Scheme 3.1 **(A)** Chemical structure of Ru-RHAU. **(B)** Illustration of Ru-RHAU binding to parallel quadruplex. **(C)** Proposed cellular mechanism for the formation of stress granules.

To date, the first and only small molecule sensor for imaging SGs in live cells was recently reported by Shao et al. who demonstrated a cell permeable benzothiazole cyanine fluorophore that appears to bind both to RNA and SG protein that led to fluorescence turn-on attributed to viscosity sensing in live HeLa cells.^[18]

Ruthenium(II) polypyridyl probes have emerged as promising imaging agents, due to their excellent photostability, large Stoke shifts and long lived ³MLCT excited state, rendering them sensitive to their local environment.^[19] They have been implemented with advanced live imaging techniques such as stimulated depletion microscopy (STED)^[20] and structured illumination microscopy (SIM).^[21] However, a drawback commonly encountered when Ru(II) polypyridyl complexes are explored for biological applications is either poor membrane permeability or endosomal entrapment, where their localisation is limited to endosomes, preventing interaction with desired biomolecules or organelles and limiting their efficacy and potential. To overcome this, our group has previously demonstrated that judicious ligand design and bio-conjugations, particularly implementation of cell penetrating peptides (CPPs) or signal peptides, can promote permeability and facilitate organelle targeting of these complexes.^[22–25] The use of CPPs promotes cell uptake and can offer selective organelle targeting, for example we have reported that Ru(II) conjugations with NLS and MPP can traverse the cell membrane and localise within the nucleus and mitochondria respectively.^[26,27] Similarly, other researchers have explored the functionalisation of Ru(II) complexes with diverse peptides to study DNA interaction, facilitate cellular uptake or expand their therapeutic applications.^[28,29]

Ruthenium(II) complexes are potent G4 probes, offering advantages in stability, specificity, and photophysical properties.^[30] A number of G4-targeted ruthenium complexes have been reported recently including two G4 targeted probes Ru-PDC3 and Ru-TAP-PDC3 studied by our group.^[31,32] Furthermore Yang et al. studied the ultrafast excited state dynamics and light-switching of the commonly studied Ru(II) complex $[\text{Ru}(\text{phen})_2(\text{dppz})]^{2+}$ in G4 DNA.^[33]

Recently, peptides capable of selective G4 binding have shown promise in biological applications but they are, to date, relatively underexplored as targeting vectors.^[34] For example, peptides derived from the RNA helicase associated with AU-rich element (RHAU) contain a helical G4 binding domain with selectivity for parallel G4s, offering exciting prospectives for rG4 monitoring and targeted therapies.^[35,36] RHAU helicase derived peptides and their chemically modified counterparts are effective G4 binding ligands. A key advantage of RHAU peptides as vectors is that they can show very high selectivity for G4 structures and even sub-topology specificity that is very difficult to accomplish in small molecule probes. The 53 amino acid long peptide, Rhau53, binds to various parallel G4s but does not bind to other G4s or duplex DNA.^[37] There are some drawbacks of G4 targeted peptides including limited cellular uptake in their linear form and stability issues in a cell environment. Efforts to directly modify the peptide through stapling have resulted in a locked helical form with increased stability but critically, diminished selectivity for G4s.^[38] Additionally, there is a significant lack of cell-based studies to investigate the function of peptide-based leads for therapeutic applications.^[39]

Herein, we present the first example of a metal-based SG probe comprising a Ru(II)-peptide bioconjugate, Ru-RHAU, that incorporates a peptide derived from the binding domain of the RHAU helicase protein in order to achieve rG4 binding. The bioconjugate was designed to increase peptide stability, rG4 stabilisation and live cell visualisation using fluorescence microscopy. We demonstrate that the probe can be used to explore rG4 and SG dynamics in a concentration and time dependent manner, inspired by reports of the RHAU helicase sequestering to SGs as a cellular stress response. Furthermore, we report to the best of our knowledge, the first example of an imaging dye capable of generating and imaging SGs in live cells, an important advancement for understanding SG dynamics.

3.3 Results & Discussion

3.3.1 Synthesis and Photophysical Characterisation

The parent complex, $[\text{Ru}(\text{bpy})_2(\text{Pic-COOH})]^{2+}$ (Ru-PIC), also known as Ru-PIP, was prepared by Dr. Lorcan Holden and characterised as previously described.^[25,40] The

peptide conjugate Ru- RHAU was synthesised through NHS coupling in an analogous route to one previously reported by our group ^[41], with structural confirmation from ¹H NMR, mass spectrometry and HPLC analysis. The peptide, PGHLKGREIGLWYAKKGQKNK, derived from the N-terminal domain of the RHAU helicase protein was synthesized by Celtek Peptides, incorporating a terminal hexyl amino linker as the site of peptide coupling. Conjugation of the parent complex to the peptide was achieved through a NHS coupling, stirring in a DMF and PBS (pH 7.4) solution. The complex was initially precipitated out as a PF₆ salt before conversion to chloride salt by stirring overnight on amberlite chloride exchange resin. Successful conjugation and purity of Ru-RHAU was confirmed by HPLC analysis at 280 nm and 450 nm (Figure S3.2). The structure was confirmed through ¹H NMR and mass spec analysis. Photophysical characterisation of Ru-RHAU and its conjugate was completed in deionized (D.I.) water, potassium phosphate buffer and MeCN, representative absorbance and luminescence data are shown in Figure 3.1. Ru-RHAU shows MLCT maxima at 460 nm in D.I. water, 455 in KPi buffer (10 mM potassium phosphate and 100 mM KCl) and 458 nm in PBS. When excited at 490 nm, the emission maximum of Ru-RHAU is centred in D.I. at 608 nm, in PBS at 601 nm and in KPi at 600 nm. The emission conforms to mono-exponential decay kinetics of 487 ns in D.I. water, 580 ns in KPi and 584 ns in PBS.

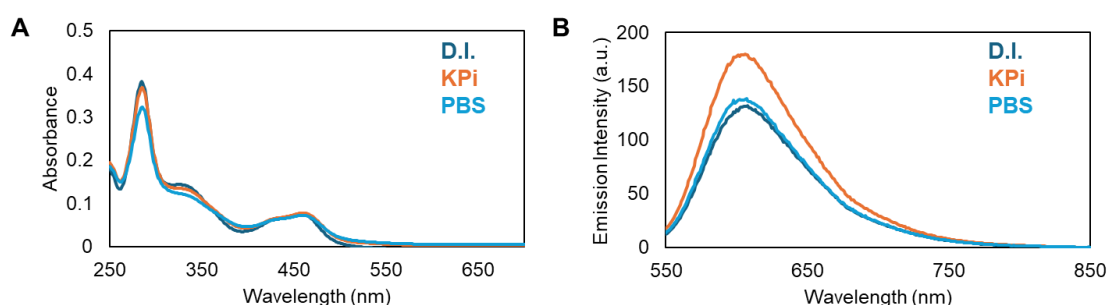


Figure 3.1 (A) Absorbance and **(B)** emission spectra of Ru-RHAU (5 μM) in D.I. water (dark blue), KPi buffer (orange) and PBS (light blue). Courtesy of Dr. Lorcan Holden.

3.3.2 DNA and RNA Binding

To compare the DNA and RNA binding capabilities of the Ru-RHAU conjugate, absorbance and luminescence titrations were performed. The oligonucleotides included G4 forming sequences; KRAS, Pu24T, CMYC, 22AG, NRAS and dsDNA (ctDNA and ds26). The DNA G4 oligonucleotides were annealed in KPi buffer at a DNA strand concentration of 1 mM, prior to use. RNase free buffer was used to prepare stocks of the RNA oligonucleotides with a strand concentration of 0.1 mM, similarly, with the

Table 3.1 Ru-RHAU Binding and Stabilisation. Courtesy of Dr. Lorcan Holden.

Sequence ^[a]	Binding affinities		F/F ₀	ΔT_m (°C) ^[c]
	(M ⁻¹)	SSR(%) ^[b]		
Pu24T	3.2 x10 ⁵ (±0.4)	4.0	1.43 (±0.50)	17.6 (±0.6)
CMYC	1.5 x10 ⁷ (±0.2)	6.5	1.34 (±0.08)	9.0 (±0.5)
KRAS	1.0 x10 ⁶ (±0.1)	5.3	1.62 (±0.15)	10.9 (±1.0)
22AG	5.2 x10 ⁶ (±0.8)	3.7	1.13 (±0.03)	2.9 (±0.6)
NRAS	1.9 x10 ⁶ (±0.2)	5.2	1.25 (±0.06)	26.4 (±0.3)
ctDNA	6.2 x10 ⁵ (±1.2)	4.3	0.80 (±0.15)	n.d.
ds26	2.5 x10 ⁵ (±0.5)	3.9	0.68 (±.14)	n.d.

^[a] Full sequences can be found in Table S3.1, ^[b] SSR = sum of the square residuals, based on deviation from the Scatchard plots in Figure S3.4 ^[c] n.d. = not determined

addition of potassium phosphate and potassium chloride. Representative absorption and emission titration plots for Ru-RHAU against dsDNA and NRAS are illustrated in Figure 3.2. Upon addition of DNA oligonucleotides, a decrease in the MLCT absorption of Ru-RHAU was observed. The absorbance intensity data were fit to a modified Scatchard equation to fit the binding curves and obtain binding constants, Table 3.1. Based on control studies, 7 minutes were allowed after each aliquot of nucleic acid before collecting the spectra to ensure binding equilibrium had been reached. Representative binding curves used to extract binding affinities are shown in Figure S3.4. Ru-RHAU showed greatest binding affinity for the parallel quadruplex CMYC with a K_b of 1.5 (±0.2) x10⁷. The unmodified 23 amino acid RHAU peptide has been reported to show preferential binding to CMYC (with similar K_d of 112 nM), this suggests the conjugate is associating through the peptide in the same binding mode as the unconjugated peptide.^[38] KRAS and 22AG binding affinities are estimated as 1.0 (±0.1) x10⁶ and 5.2(±0.8) x10⁶ respectively. Conjugate binding affinities toward duplex DNA; ctDNA and oligonucleotide ds26 were determined as 6.2 (±1.2) x10⁵ and 2.5 (±0.5) x10⁵ respectively, i.e., roughly an order of magnitude weaker than for G4 structures. Attempts to apply the modified Scatchard equation model to absorption data for RNA parallel quadruplex NRAS were unsuccessful. Therefore, binding data was instead extracted

from luminescence response of Ru-RHAU on NRAS titration, where K_b was estimated as $1.9 (\pm 0.2) \times 10^6$ which is comparable to DNA G4 affinity.

Luminescence titrations were performed by Dr. Lorcan Holden to understand and compare the impact of DNA and G4 association on photophysical properties of the Ru-RHAU conjugate. An excitation wavelength of 490 nm was selected as it lies in a pseudo isosbestic point in the absorption data. Interestingly, opposing effects were observed in spectral data on incubation with G4 forming oligos and dsDNA. Hyperchromic responses were observed when Ru-RHAU was bound to the parallel G4s KRAS, Pu24T, and CMYC were 62%, 43% and 34% respectively and 25% for the rG4 NRAS. Whereas a hypochromic effect was observed on dsDNA association of 20% and 32% on saturation binding for ctDNA and ds26 respectively (Figure 3.2C).

Control experiments on the association of the parent complex, $[\text{Ru}(\text{bpy})_2(\text{Pic}-\text{COOH})]^{2+}$ (Ru-PIC), with ctDNA, CMYC and NRAS (Figure S3.6-3.7) were completed and showed that Ru-PIC did not elicit a strong absorbance spectral response on incubation with ctDNA but notably, did result in an increase in luminescent intensity. This contrast with Ru-RHAU suggesting a different binding mode is at play. It has been reported that Ru(II) complexes bearing 'pic-like' ligands can intercalate between base pairs leading to increase in emission intensities.^[42] It is likely that conjugation to the RHAU peptide inhibits the Ru centre from binding to dsDNA in a similar manner to the free complex. A decrease in the Ru-PIC MLCT absorption upon association with CMYC and an increase in luminescent intensity occurred with increasing G4 concentrations. Interestingly the binding of Ru-PIC to NRAS had negligible impact on either the absorbance or emission spectra, indicating weak interaction with this sequence. It is possible that the increased stability of NRAS caused by the 2' hydroxyl group in ribose may prevent Ru-PIC from binding as effectively when compared to the DNA G4 CMYC. In contrast, the increase in luminescence intensity on Ru-RHAU association with NRAS suggests that this association protects the ruthenium centre decreasing non-radiative decay rate.

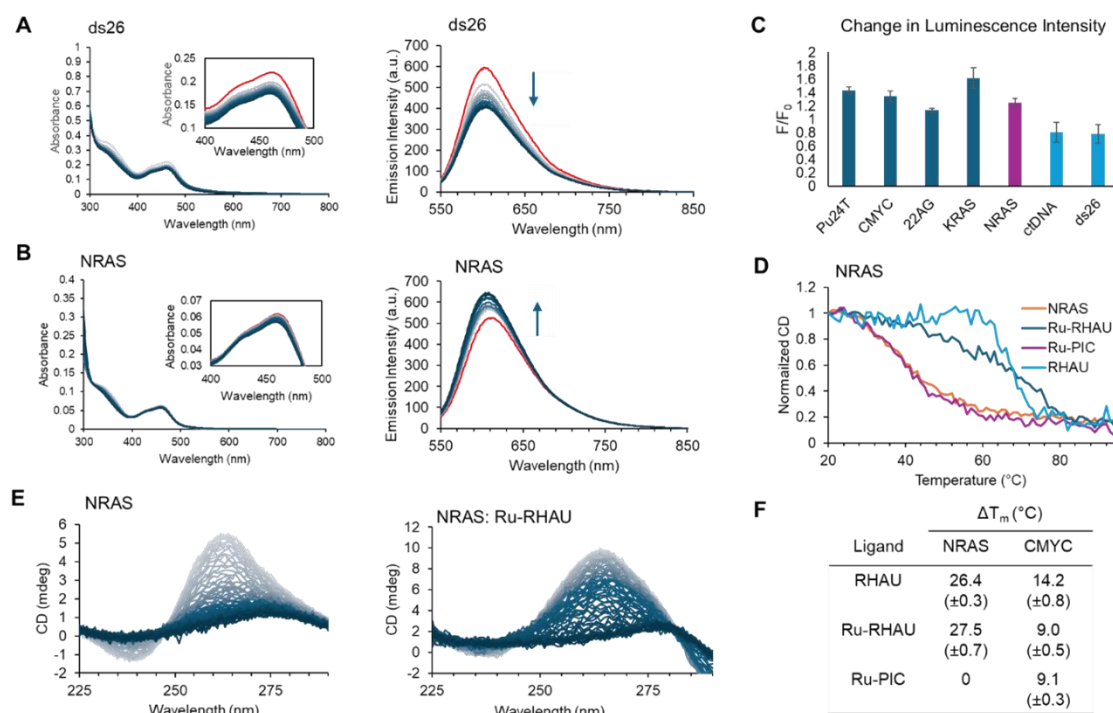


Figure 3.2 (A) Absorbance and emission titration of Ru-RHAU (10 μ M) with increasing concentrations of ds26 (0-30 μ M). (B) Absorbance and emission titration of Ru-RHAU (2 μ M) with increasing concentrations of ds26 (0-6 μ M). (C) Normalised changes in fluorescence upon association with 3 equivalents of oligonucleotides. (D) Normalised changes in the CD signal of NRAS at 265 nm when bound to 2 equivalents of RHAU, Ru-PIC or Ru-RHAU with increasing temperature. (E) Circular dichroism melting curves of NRAS (5 μ M) unbound and bound to Ru-RHAU (10 μ M) from 20–94 $^{\circ}$ C. (F) Changes in melting temperatures of NRAS and CMYC when bound to 2 equivalents of RHAU, Ru-PIC or Ru-RHAU.

3.3.3 Circular Dichroism Melting Studies

To determine the stabilisation capabilities of Ru-RHAU towards nucleic acids, CD melting studies were employed by Dr. Lorcan Holden. Melting studies with KRAS, Pu24T and 22AG oligonucleotides were conducted using a buffer solution of 10 mM potassium phosphate and 100 mM KCl. Due to the high stability of CMYC and NRAS in buffers with high potassium concentrations, a weaker stabilising buffer was required to obtain melting temperature curves, comprised of LiCl (10 mM) and lithium cacodylate (100 mM) at pH 7. In the case of the parallel G4s, Pu24T and KRAS, stabilisation was evident. Binding to Pu24T caused an increase in ΔT_m of 17.6 (± 0.6) $^{\circ}$ C and an increase of 9.0 (± 0.5) $^{\circ}$ C towards CMYC and 10.9 (± 1.0) $^{\circ}$ C towards KRAS. Although strong binding to 22AG was calculated based on, binding to Ru-RHAU did not result in notable G4 stabilisation where ΔT_m was 2.9 (± 0.6) $^{\circ}$ C. Also of note, the CD spectra of 22AG indicates that on binding to Ru-RHAU, conformational changes from a hybrid structure to an anti-parallel conformation occur. The minimal changes in ΔT_m observed could be the result of differences the thermal stability between different confirmations. In contrast, when Ru-

RHAU associates with NRAS a ΔT_m of 26.4 (± 0.3) °C was observed, indicating considerable stabilisation of RNA G4s.

An important consideration in Ru-RHAU binding and stabilisation with G4s, is the impact that the separate supramolecular components; the parent complex and RHAU peptide have on stability of the G4s since both Ru(II)-PIC complexes and RHAU derived peptides are reported G4 binders. Thus, individual stabilising capabilities were studied using melting studies with NRAS and CMYC for both Ru-PIC and the free, unconjugated RHAU peptide (Figure 3.2). Upon incubation with 2 equivalents of RHAU very strong stabilisation of 27.5 (± 0.7) °C was observed for CMYC. The extent of stabilisation matches very closely that of the Ru-RHAU conjugate, indicating similar binding in each case. Conversely, when Ru-PIC was incubated with NRAS no stabilisation was observed. When Ru-PIC was bound to CMYC, a moderate increase in T_m of 9.1 (± 0.3) °C was observed. Association of RHAU to CMYC resulted in a T_m of 14.2 (± 0.8) °C (Figure S3.8).

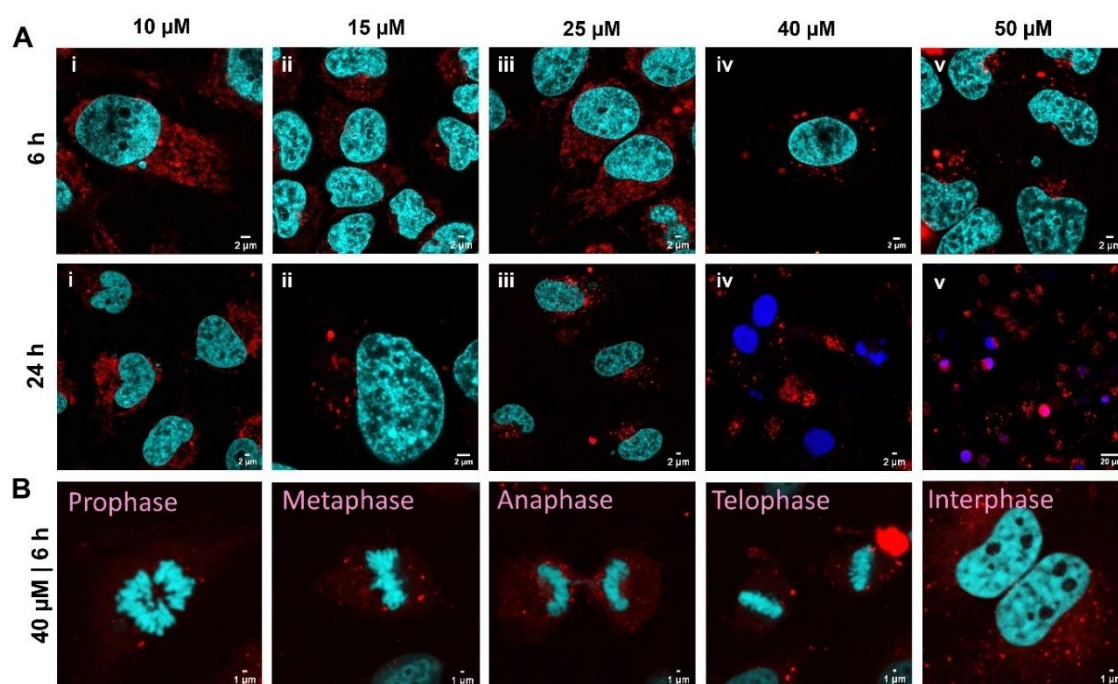


Figure 3.3 Confocal imaging showing **(A)** uptake of Ru-RHAU in live HeLa cells at varying concentrations after 6 and 24 h incubation where DRAQ7 uptake (3 μM) is evident in cells treated with 40 μM and 50 μM Ru-RHAU for 24 h, confirming cytotoxicity. Cells were incubated in the dark with Ru-RHAU at 37 °C and 5% CO₂ in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin and were washed twice with warm PBS prior to imaging **(B)** confocal images of fixed HeLa cells with Ru-RHAU (40 μM, 6 h) at various cell cycle phases.

3.3.4 Cellular Uptake and Assembly of Stress Induced Foci by Ru-RHAU

Cellular uptake was assessed by incubating HeLa cells with Ru-RHAU over a range of concentrations and incubation times. Figure 3.3 shows the assembly of large foci in the cytoplasm on uptake of Ru-RHAU that is both concentration and time dependent. Notably, large foci (approx. 2 μm) form between 30-40 μM Ru-RHAU after a relatively short incubation of 6 h incubation (Figure 3.3 and S3.11) and at ca. 15 μM after a 24 h incubation. In both cases the foci are thought to be assembly of stress granules (SGs). Uptake of DRAQ7 (3 μM) at 40 and 50 μM Ru-RHAU (24 h) indicates cells are dead or damaged under these conditions.

Two time/concentration points were then selected for intracellular imaging of the SGs: 6 h incubation at concentration of 40 μM and a 24 h incubation with a concentration of 25 μM . These concentrations were selected for study since punctate foci could be readily observed without DRAQ7 uptake, indicating the cells remain in good health. SGs have been reported to form independent of the cell cycle phase, and preliminary imaging corroborates this finding (Figure 3.3B). To explore if uptake and SG stimulation is cell line dependent, MCF-7 breast cancer cells were treated with Ru-RHAU at 25 μM (24 h) and 40 μM (6 h) and similar large cytoplasmic foci were observed (Figure S3.15), confirming induction of SGs by Ru-RHAU and that it is not unique to HeLa cells. It is tempting to speculate that SG induction by Ru-RHAU is attributed to the propensity for Ru-RHAU to stabilise rG4 structure as demonstrated in solution *vide supra*. Since rG4s are purported to provide the seed for SG assembly if these structures are stabilised, they will likely provide a thermodynamic impetus for SG assembly.^[4]

Ru-RHAU was found to be readily permeable to live cells, without addition of solvent or another permeant. To understand this, uptake was evaluated after the cells were treated with a combination of metabolic inhibitor oligomycin and 2-deoxy-D-glucose (2DG) to determine if cellular uptake is energy-dependent. When cells are treated with oligomycin, they become more reliant on glycolysis for energy production. 2DG further disrupts this process by inhibiting glycolysis, thus combining both inhibitors creates an energy-starved environment.^[43] Confocal imaging of cells pre-treated with oligomycin and 2DG for 40 minutes before a 24-h incubation with 25 μM Ru-RHAU confirmed strong emission of the complex from the cell interior (Figure S3.15), indicating uptake occurs independent of oligomycin/2DG treatment suggesting it is an energy-independent, i.e. passive process.

3.3.5 Ru-RHAU Localisation: From Lysosomes to Stress Granules

Since the RHAU protein can be found in both the nucleus and cytoplasm of cells,^[44] co-localisation studies were performed to confirm the exact location of the punctate Ru-RHAU staining we observe in live HeLa cells. Confocal imaging of co-staining with commercial lysosome dye LysoTracker Deep Red confirmed that when Ru-RHAU is not sequestered within SGs, it localises in the lysosomes of HeLa cells at incubation times of 6 h (40 μ M Ru-RHAU, Pearson's coefficient $r = 0.47 \pm 0.06$) or 24 h (25 μ M Ru-RHAU, Pearson's coefficient $r = 0.52 \pm 0.08$) as shown in Figures S16-17. This is not surprising as lysosomes are closely linked to SGs, with lysosomal damage reportedly causing cell stress and SG assembly.^[45] Moreover, SG marker G3BP1 has been shown to co-localise with the LAMP-1 lysosome protein.^[46–48]

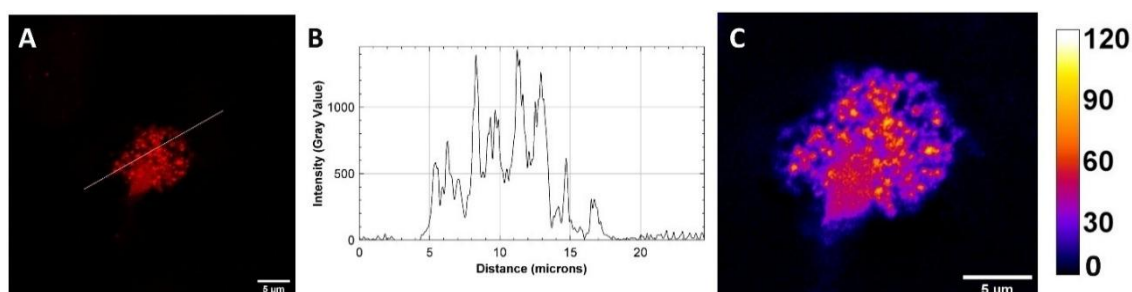


Figure 3.4 Super-resolution imaging (Zeiss Airyscan) of fixed HeLa cells with Ru-RHAU (25 μ M, 24 h) shown in figure (A) with region of interest used to generate (B) the corresponding plot profile. A zoomed image of the SG with intensity map representing relative gray scale intensity is shown in (C). Scale bars measure 5 μ m.

3.3.6 Super-Resolution Imaging

To obtain higher resolution images of the SGs and gain a better insight into the dynamics of the Ru-RHAU induced SGs, airyscanning technology was employed using the ZEISS LSM 980 microscope. This method uses a 32-channel GaAsP area detector instead of the traditional single photomultiplier detector used in conventional confocal microscopy to achieve a lateral resolution of approximately 120 nm, a significant enhancement in comparison to conventional confocal microscopy (250 nm).^[49] As shown in Figure 3.4.B, imaging with super-resolution mode allows us to resolve the structure of the stress granules, revealing they are made up of numerous smaller foci. Further analysis of foci in HeLa cells treated with Ru-RHAU at 40 μ M (6 h) or 25 μ M (24 h) measuring 150-160 foci (per condition) ranging from 0-10 μ m² indicate SGs are of similar size across both conditions with average foci of 5.33 ± 0.45 and 5.66 ± 0.39 for 40 μ M (6 h) and 25 μ M (24 h) respectively. As shown in Figure 3.4.C the concentration of Ru-RHAU varies within the SGs, with distinct areas of higher intensity. This result is similar to data

reported for TASG by Shao et al. for live cell imaging of SGs, where findings indicated that the dye was mainly restricted to SG cores.^[18] In the present case as the peptide associates with rG4, it likely remains associated with these structures in the SG giving rise to the punctuated distribution around the SG.

3.3.7 Immunostaining with Stress Granule Markers

To validate that Ru-RHAU is localised specifically to stress granules immunostaining was carried out. There are many proteins found in SGs, however G3BP1 and TIA-1 have been the most widely studied.^[50,51] Reports suggest the interaction of G3BP1, an rG4-binding protein, and rG4s play a role in SG formation.^[10] Anti-bodies tagged with AlexaFluor-647 were employed to stain the SG localising proteins, G3BP1 and TIA-1. We used the AlexaFluor-647 tagged G3BP1 and TIA-1 to confirm the assembly of SGs in HeLa cells incubated with Ru-RHAU at both 6 h (40 μ M) and 24 h (25 μ M) incubation times. Strong co-localisation of the larger foci of Ru-RHAU with G3BP1 and TIA-1 was observed (Figure 3.5) supporting the idea that Ru-RHAU is capable of initiating SG assembly. Control studies were performed in HeLa cells using the SG markers under both normal conditions where there were no SGs in the cells and on heat shock induced SGs (42 °C, 1 h) to confirm the reliability of the G3BP1 and TIA-1 antibodies in detecting SGs under the imaging conditions used in this work. These controls validate the accuracy of SG detection observed in the presence of the dye (Figure S3.20).

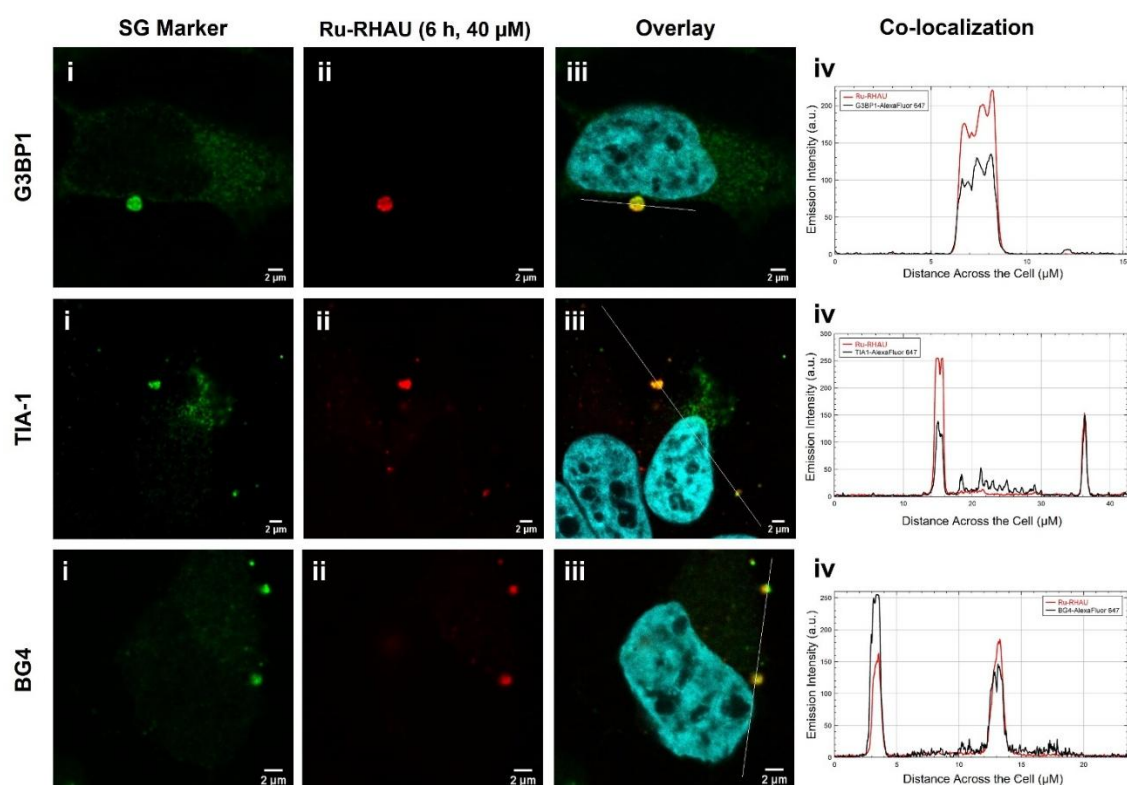


Figure 3.5 Confocal imaging of (i) G3BP1, TIA-1 or BG4 antibodies visualised using AlexaFluor 647 in fixed HeLa cells stained with (ii) Ru-RHAU at 40 μ M for 6 h. The nucleus is indicated by Hoechst 33342 in (iii) the overlay images of (i) and (ii). The white lines indicate a selected region of interest to generate the corresponding co-localisation graphs. Live HeLa cells were treated with Ru-RHAU for 24 h 25 μ M) prior to fixation with 4% paraformaldehyde and permeabilisation with 0.01% Triton-X. Immunostaining with G3BP1, TIA-1 or BG4 were then performed as described in Appendix B. Co-localisation imaging of Ru-RHAU at 25 μ M, 24 h with G3BP1, TIA-1 or BG4 can be found in Appendix B. Scale bars measure 2 μ m.

3.3.8 Ru-RHAU is Binding to rG4s in Stress Granules

As SG condensates contain rG4s bound by G3BP1 in response to cellular stress and endogenous rG4s are enriched within SGs we wanted to confirm that the Ru-RHAU SG labelling is associated with its binding to rG4s in SGs.^[10] To confirm this, co-localisation of Ru-RHAU with immunofluorescence staining of BG4 was examined. BG4 is a single-chain variable fragment (scFv) antibody that has high affinity for both DNA and RNA G4s. It is not permeable and does not usually fold effectively in live mammalian cells, thus we carried out the study in fixed HeLa cells.^[52] HeLa cells treated with Ru-RHAU (40 μ M, 6 h or 25 μ M, 24 h) were fixed and then stained with anti-G4 antibody, BG4 (Merck). In the BG4 channel, staining can be seen throughout the entire cell with notable foci (approx. 2 μ m) in the cytoplasm. The increased intensity of BG4 in comparison to control cells not treated with Ru-RHAU is striking, indicating Ru-RHAU role in SGs generation.

Based on confocal imaging, strong co-localisation is evident between BG4 and Ru-RHAU in the cytoplasm which is consistent with rG4 binding. This is further confirmed in

the co-localisation graph shown in Figure 3.5. The co-localisation with BG4 in combination with the stabilisation of rG4 by Ru-RHAU and its evident ability to induce SGs, supports previously reported association between rG4s and SGs.^[4]

3.3.9 Stress Granule Disassembly

Recovery of SGs following exposure to stressors such as sodium arsenite, hydrogen peroxide and heat shock have been reported to occur between 60 and 120 minutes after stressor removal. To test recovery of live HeLa cells post Ru-RHAU treatment, cells were treated with Ru-RHAU for 6 h (40 μ M) or 24 h (25 μ M) and imaged every 15 minutes for a total of 150 minutes (Figure S3.28-3.29) after the removal of Ru-RHAU. Imaging over time suggests the shorter incubation of 6 h enables the formation of early stage SGs, most of which were observed to disassemble over time following removal of Ru-RHAU. Longer, 24 h incubation with Ru-RHAU resulted in the formation of some large pathological, persistent SGs that were observed not to disassemble within a period of 150 minutes after the removal of the Ru(II) complex. Pathological SGs have been implicated in the development of various cancers including breast and lung.^[53] The phase contrast images (Figure S3.28-3.29) show changes in cell morphology over time and indicate that as the SGs disassemble, cell damage and ultimately cell death occurs. 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.^[54]

3.3.10 Ru-RHAU as a Live Cell Imaging Dye for Stress Granules Induced by External Stress Stimuli

To consider Ru-RHAU as a probe for SG formation under application of external stressors, we examined it under stressor conditions at lower concentration and shorter incubation times than reported *vide supra*.

HeLa cells were incubated with Ru-RHAU at 10 μ M for 3 or 6 h. A 10 μ M concentration was selected as it was well below the concentration at which the Ru-RHAU complex induces SGs. After the incubation with Ru-RHAU, the contact solution (Ru in media) was removed from the cells and they were treated with heat shock (42 °C, 1 h) in phenol red free media. Images were acquired immediately and localisation in SGs was observed. To determine the rate of uptake of Ru-RHAU (10 μ M) into the SGs, untreated HeLa cells were subjected to heat shock and the uptake of Ru-RHAU was imaged every 15 minutes from $t = 0$ i.e. from the addition of Ru-RHAU. Confocal imaging was completed using a heated stage to ensure no SG disassembly and rapid accumulation of the dye into SGs was observed, as shown in Figure 3.6. The concentration and time dependency of Ru-

RHAU induced SG assembly means the dye can be used for both live cell imaging of SGs induced using the Ru-RHAU complex itself, or alternatively at lower concentrations and shorter incubation times, can be used as a dye for live cell imaging of SGs induced through external stimuli.

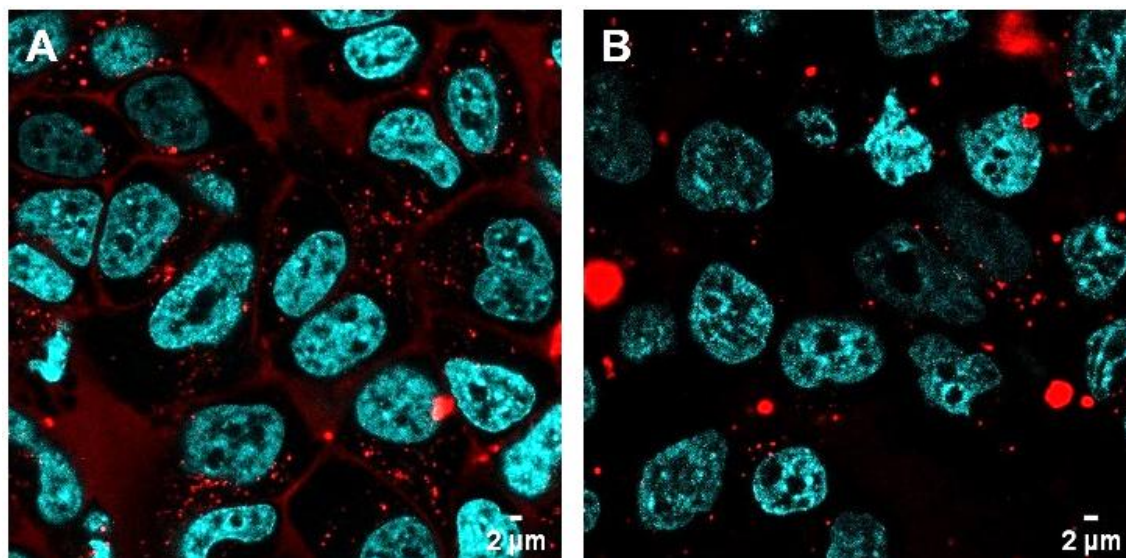


Figure 3.6 HeLa cells treated with Ru-RHAU (10 μ M) **(A)** at $t = 0$ and **(B)** $t = 30$ min after the addition of Ru-RHAU. SGs were induced by heat shock (42 $^{\circ}$ C, 1 h) where cells were incubated in DMEM supplemented with 10% FBS and 1% penicillin streptomycin prior to the addition of Ru-RHAU. A 10 μ M solution of Ru-RHAU in media without phenol red was prepared and added to the cells to monitor live uptake of the complex into SGs and a heated stage was used during confocal imaging to ensure there was no disassembly of the SGs. Hoechst-33342 (cyan) indicates the nucleus. Scale bars measure 2 μ m.

3.3.11 Toxicity of Ru-RHAU in HeLa Cells

Identifying the factors that contribute to SG persistence may reveal potential therapeutic targets for diseases associated with persistent or pathological SGs. For example, inhibition of SG formation or promotion of SG disassembly may sensitize cancer cells to chemotherapy and improve treatment outcomes. The prospect of developing a SG targeting therapeutic is thus appealing, particularly given their prevalence in chemotherapeutic resistance.^[55,56] To this end, MTT toxicity assays were performed in HeLa cells following the removal of Ru-RHAU after 6 h or 24 h incubations in the dark at 37 $^{\circ}$ C. The complex was not very toxic with an $IC_{50} > 100$ μ M (6 h) and 59 ± 0.98 μ M (24 h) which is significantly higher than the concentrations selected for imaging. Although some large SGs assemble after 6 h incubation, the shorter incubation period may support a reduced level of cellular stress during the MTT assay incubation following the removal of Ru-RHAU, resulting in the higher dark IC_{50} of > 100 μ M. In contrast, a 24-hour incubation period likely exacerbates stress responses, leading to a lower IC_{50} of 59 ± 0.98 μ M. To determine the mechanism of cell death, autophagy and apoptosis assays were completed with cells treated with 50 and 60 μ M Ru-RHAU. The assay results

indicates that a 24 h incubation with Ru-RHAU induces cell death through apoptosis, confirmed by the FLICA polycaspase assay where 60-62% of cells were apoptotic relative to control cells treated with Staurosporine. Furthermore, it was confirmed that the cells are not undergoing autophagy, with <3% increase in autophagic vacuoles relative to untreated control cells, in comparison to an 81% increase in autophagy induced control cells treated with Rapamycin and Chloroquine. In contrast, after a 6 h incubation some cells exhibited signs of autophagic cell death, with approximately 20% of cells displaying markers of autophagy. Interestingly, comparable levels of apoptosis were observed both after 6 h and 24 h incubation periods where approximately 57-58% of cells treated with Ru-RHAU showed apoptotic features after a 6 h incubation. The FLICA polycaspase assay detects multiple activated caspases, including caspase-3 and -7 which have been linked to apoptosis.^[57]

As changes to cell morphology indicate decreased cell health during SG disassembly, we were interested to study the dark toxicity after 2 h removal of Ru-RHAU in cells that had been treated for 24 h. Allowing SG disassembly to begin (2 h) prior to the MTT cell viability assay resulted in a significant change in the dark IC₅₀ of the cells after a 24 h incubation from $59 \pm 0.98 \mu\text{M}$ to $28 \pm 4.33 \mu\text{M}$. This observation indicates that the SGs may provide protection to the cells while Ru-RHAU is present, however once the complex is removed, the SGs begin to disassemble and cell viability decreases. It is important to note however that a 24 h incubation with Ru-RHAU is an exceptionally long time for the cells to be under stress, therefore it is not surprising that we see dark toxicity over these time windows.

Ruthenium complexes have been extensively investigated as potential phototherapeutics.^[58–63] To explore the phototoxicity of Ru-RHAU, the impact of photoirradiation on cell health after Ru-RHAU induced SG formation was evaluated. Cells were irradiated at a total dose of $5 \pm 0.29 \text{ J/cm}^2$ (0.5 h at $2.63 \pm 0.16 \text{ mW/cm}^2$) using a 470 nm LED under normoxic conditions after the removal of Ru-RHAU. Light IC₅₀ values of $14 \pm 0.19 \mu\text{M}$ and $15 \pm 0.16 \mu\text{M}$ were recorded after an incubation period of 6 h and 24 h respectively. The similarity in light IC₅₀ values indicate that prolonged incubation does not impact the phototoxic effect of Ru-RHAU upon irradiation. These results suggest the potential of SGs as a target for phototherapy. It is important to note that imaging was performed under significantly lower irradiation power density and the absence of DRAQ7 uptake ($3 \mu\text{M}$) under imaging conditions confirmed cells remain healthy.

3.4 Conclusions

We have developed an rG4 targeted Ru(II) probe, Ru-RHAU, that binds with high affinity and strongly stabilises DNA and RNA G4s. Ru-RHAU, can be used to visualise SGs in live cells, successfully inducing them in a concentration and time dependent manner and is to the best of our knowledge, the first reported dye to do this. Additionally, at lower concentrations and shorter incubation times, this dye can be used to visualise the dynamics of SGs induced using external stress stimuli. G4 binding was established in solution with several G4 sequences, where the highest binding affinity was shown for CMYC and significant stabilisation was observed for NRAS, an rG4 sequence (T_m increased by 26 °C). G4 binding was confirmed in fixed HeLa and MCF-7 cells by immunostaining studies with BG4. SG assembly was confirmed by fixed cell imaging of Ru-RHAU treated cells with SG markers G3BP1 and TIA-1 where exceptional overlap was displayed in the SGs. As Ru-RHAU can be applied for imaging SGs in both live and fixed cells, it offers an opportunity to overcome the current limitations of commercially available dyes, which are restricted to visualising SGs in fixed cells only. Furthermore, Ru-RHAU displayed mild cytotoxicity after a 24 h incubation, however the addition of a 2 h window to allow for SG disassembly, there is a significant increase in cytotoxicity indicating the presence and disassembly of SGs could play a critical role in cytotoxicity. Additionally, Ru-RHAU exhibited high phototoxicity after both 6 h or 24 h incubation, suggesting SGs are worthwhile phototherapeutic targets with potential for overcoming therapy resistant cancer. Overall, these results indicate Ru-RHAU is a promising probe for visualising SG dynamics in a live cell environment, and further studies with this probe in additional cell lines, e.g. neuronal cells, could enhance the understanding of the role of SGs in disease pathology.

3.5 References

- [1] P. Kharel, P. Ivanov, in *RNA Struct. Funct.* (Ed.: J. Barciszewski), Springer International Publishing, Cham, **2023**, pp. 183–203.
- [2] M. M. Fay, S. M. Lyons, P. Ivanov, *J. Mol. Biol.* **2017**, 429, 2127–2147.
- [3] L. Dumas, P. Herviou, E. Dassi, A. Cammas, S. Millevoi, *Trends Biochem. Sci.* **2021**, 46, 270–283.
- [4] P. Kharel, P. Ivanov, *Trends Cell Biol.* **2024**, S0962-8924(24)00005–9.
- [5] P. Kharel, M. Fay, E. V. Manasova, P. J. Anderson, A. V. Kurkin, J. U. Guo, P. Ivanov, *Nat. Commun.* **2023**, 14, 205.

- [6] T. Li, Z. Zeng, C. Fan, W. Xiong, *Biochim. Biophys. Acta Rev. Cancer* **2023**, 1878, 189006.
- [7] S. Y. Yang, P. Lejault, S. Chevrier, R. Boidot, A. G. Robertson, J. M. Y. Wong, D. Monchaud, *Nat. Commun.* **2018**, 9, 4730.
- [8] Y. M. Danino, L. Molitor, T. Rosenbaum-Cohen, S. Kaiser, Y. Cohen, Z. Porat, H. Marmor-Kollet, C. Katina, A. Savidor, R. Rotkopf, E. Ben-Isaac, O. Golani, Y. Levin, D. Monchaud, I. D. Hickson, E. Hornstein, *Nucleic Acids Res.* **2023**, 51, 9369–9384.
- [9] S. Asamitsu, Y. Yabuki, K. Matsuo, M. Kawasaki, Y. Hirose, G. Kashiwazaki, A. Chandran, T. Bando, D. O. Wang, H. Sugiyama, N. Shioda, *Sci. Adv.* **2023**, 9, eade2035.
- [10] M. Turner, Y. M. Danino, M. Barshai, N. S. Yacovzada, Y. Cohen, T. Olender, R. Rotkopf, D. Monchaud, E. Hornstein, Y. Orenstein, *Nucleic Acids Res.* **2022**, 50, 11426–11441.
- [11] P. Anderson, N. Kedersha, P. Ivanov, *Biochim. Biophys. Acta* **2015**, 1849, 861–870.
- [12] J. L. Martin, S. J. Dawson, J. E. Gale, *Hear. Res.* **2022**, 426, 108634.
- [13] V. M. Advani, P. Ivanov, *Cell. Mol. Life Sci. CMLS* **2020**, 77, 4827–4845.
- [14] N. Kedersha, M. R. Cho, W. Li, P. W. Yacono, S. Chen, N. Gilks, D. E. Golan, P. Anderson, *J. Cell Biol.* **2000**, 151, 1257–1268.
- [15] S. Mollet, N. Cougot, A. Wilczynska, F. Dautry, M. Kress, E. Bertrand, D. Weil, *Mol. Biol. Cell* **2008**, 19, 4469.
- [16] N. Kedersha, M. D. Panas, C. A. Achorn, S. Lyons, S. Tisdale, T. Hickman, M. Thomas, J. Lieberman, G. M. McInerney, P. Ivanov, P. Anderson, *J. Cell Biol.* **2016**, 212, 845–860.
- [17] B. V. Treeck, R. Parker, *Cold Spring Harb. Perspect. Biol.* **2019**, 11, a033068.
- [18] W. Shao, S.-T. Zeng, Z.-Y. Yu, G.-X. Tang, S.-B. Chen, Z.-S. Huang, X.-C. Chen, J.-H. Tan, *Anal. Chem.* **2021**, 93, 16297–16301.
- [19] L. C.-C. Lee, K. K.-W. Lo, *Chem. Rev.* **2024**, DOI 10.1021/acs.chemrev.3c00629.
- [20] A. Byrne, C. S. Burke, T. E. Keyes, *Chem. Sci.* **2016**, 7, 6551–6562.
- [21] S. Sreedharan, M. R. Gill, E. Garcia, H. K. Saeed, D. Robinson, A. Byrne, A. Cadby, T. E. Keyes, C. Smythe, P. Pellett, J. Bernardino de la Serna, J. A. Thomas, *J. Am. Chem. Soc.* **2017**, 139, 15907–15913.
- [22] L. Holden, C. S. Burke, D. Cullinane, T. E. Keyes, *RSC Chem. Biol.* **2021**, 2, 1021–1049.
- [23] C. S. Burke, A. Byrne, T. E. Keyes, *Angew. Chem. Int. Ed.* **2018**, 57, 12420–12424.
- [24] C. S. Burke, A. Byrne, T. E. Keyes, *J. Am. Chem. Soc.* **2018**, 140, 6945–6955.
- [25] K. Adamson, C. Dolan, N. Moran, R. J. Forster, T. E. Keyes, *Bioconjug. Chem.* **2014**, 25, 928–944.

- [26] L. Blackmore, R. Moriarty, C. Dolan, K. Adamson, R. J. Forster, M. Devocelle, T. E. Keyes, *Chem. Commun.* **2013**, 49, 2658–2660.
- [27] K. S. Gkika, A. Byrne, T. E. Keyes, *Dalton Trans.* **2019**, 48, 17461–17471.
- [28] E. M. Hahn, N. Estrada-Ortiz, J. Han, V. F. C. Ferreira, T. G. Kapp, J. D. G. Correia, A. Casini, F. E. Kühn, *Eur. J. Inorg. Chem.* **2017**, 2017, 1667–1672.
- [29] D. Obitz, R. G. Miller, N. Metzler-Nolte, *Dalton Trans.* **2021**, 50, 13768–13777.
- [30] J. Jiang, T. Teunens, J. Tisaun, L. Denuit, C. Moucheron, *Molecules* **2022**, 27, 1541.
- [31] L. Holden, K. S. Gkika, C. S. Burke, C. Long, T. E. Keyes, *Inorg. Chem.* **2023**, 62, 2213–2227.
- [32] L. Holden, R. C. Curley, G. Avella, C. Long, Keyes, Tia, *Angew. Chem. Int. Ed.* **2024**, e202408581.
- [33] C. Yang, Q. Zhou, Z. Jiao, H. Zhao, C.-H. Huang, B.-Z. Zhu, H. Su, *Commun. Chem.* **2021**, 4, 1–10.
- [34] F. Merlino, S. Marzano, P. Zizza, F. D’Aria, N. Grasso, A. Carachino, S. Iachettini, A. Biroccio, S. D. Fonzo, P. Grieco, A. Randazzo, J. Amato, B. Pagano, *Nucleic Acids Res.* **2024**, 52, 6748–6762.
- [35] L. T. A. Nguyen, D. T. Dang, *Mol. Biotechnol.* **2023**, 65, 291–299.
- [36] J. Wang, J. Qiao, W. Zheng, H. Lian, *Int. J. Mol. Sci.* **2023**, 24, 1438.
- [37] B. Heddi, V. V. Cheong, H. Martadinata, A. T. Phan, *Proc. Natl. Acad. Sci. U. S. A.* **2015**, 112, 9608–9613.
- [38] A. Minard, D. Morgan, F. Raguseo, A. D. Porzio, D. Liano, A. G. Jamieson, M. D. Antonio, *Chem. Commun.* **2020**, 56, 8940–8943.
- [39] T. Sharma, N. Kundu, S. Kaur, J. Shankaraswamy, S. Saxena, *J. Pept. Sci. Off. Publ. Eur. Pept. Soc.* **2023**, 29, e3491.
- [40] Y. Pellegrin, R. J. Forster, T. E. Keyes, *Inorganica Chim. Acta* **2008**, 361, 2683–2691.
- [41] M. Clement, G. Daniel, M. Trelles, *J. Cosmet. Laser Ther.* **2005**, 7, 177–189.
- [42] J.-Z. Wu, B.-H. Ye, L. Wang, L.-N. Ji, J.-Y. Zhou, R.-H. Li, Z.-Y. Zhou, *J. Chem. Soc. Dalton Trans.* **1997**, 1395–1402.
- [43] B. Pajak, E. Siwiak, M. Sołtyka, A. Priebe, R. Zieliński, I. Fokt, M. Ziemniak, A. Jaśkiewicz, R. Borowski, T. Domoradzki, W. Priebe, *Int. J. Mol. Sci.* **2019**, 21, 234.
- [44] K. Chalupníková, S. Lattmann, N. Selak, F. Iwamoto, Y. Fujiki, Y. Nagamine, *J. Biol. Chem.* **2008**, 283, 35186–35198.
- [45] J. Jia, F. Wang, Z. Bhujabal, R. Peters, M. Mudd, T. Duque, L. Allers, R. Javed, M. Salemi, C. Behrends, B. Phinney, T. Johansen, V. Deretic, *J. Cell Biol.* **2022**, 221, e202207091.

- [46] S. Li, Z. Liao, H. Yin, O. Liu, W. Hua, X. Wu, Y. Zhang, Y. Gao, C. Yang, *Cell Prolif.* **2022**, 56, e13368.
- [47] M. T. Prentzell, U. Rehbein, M. C. Sandoval, A.-S. D. Meulemeester, R. Baumeister, L. Brohée, B. Berdel, M. Bockwoldt, B. Carroll, S. R. Chowdhury, A. von Deimling, C. Demetriades, G. Figlia, M. E. G. de Araujo, A. M. Heberle, I. Heiland, B. Holzwarth, L. A. Huber, J. Jaworski, M. Kedra, K. Kern, A. Kopach, V. I. Korolchuk, I. van 't Land-Kuper, M. Macias, M. Nellist, W. Palm, S. Pusch, J. M. R. Pittol, M. Reil, A. Reintjes, F. Reuter, J. R. Sampson, C. Scheldeman, A. Siekierska, E. Stefan, A. A. Teleman, L. E. Thomas, O. Torres-Quesada, S. Trump, H. D. West, P. de Witte, S. Woltering, T. E. Yordanov, J. Zmorzynska, C. A. Opitz, K. Thedieck, *Cell* **2021**, 184, 655-674.e27.
- [48] Y.-C. Liao, M. S. Fernandopulle, G. Wang, H. Choi, L. Hao, C. M. Drerup, R. Patel, S. Qamar, J. Nixon-Abell, Y. Shen, W. Meadows, M. Vendruscolo, T. P. J. Knowles, M. Nelson, M. A. Czekalska, G. Musteikyte, M. A. Gachechiladze, C. A. Stephens, H. A. Pasolli, L. R. Forrest, P. St George-Hyslop, J. Lippincott-Schwartz, M. E. Ward, *Cell* **2019**, 179, 147-164.e20.
- [49] X. Wu, J. A. Hammer, *Methods Mol. Biol.* **2021**, 2304, 111–130.
- [50] A. Tweedie, T. Nissan, *Front. Mol. Biosci.* **2021**, 8, 647884.
- [51] H. Mahboubi, U. Stochaj, *Biochim. Biophys. Acta BBA - Mol. Basis Dis.* **2017**, 1863, 884–895.
- [52] S. Galli, L. Melidis, S. M. Flynn, D. Varshney, A. Simeone, J. Spiegel, S. K. Madden, D. Tannahill, S. Balasubramanian, *J. Am. Chem. Soc.* **2022**, 144, 23096–23103.
- [53] H. Zhou, J. Luo, K. Mou, L. Peng, X. Li, Y. Lei, J. Wang, S. Lin, Y. Luo, L. Xiang, *Cell Biosci.* **2023**, 13, 86.
- [54] D. Fujikawa, T. Nakamura, D. Yoshioka, Z. Li, H. Moriizumi, M. Taguchi, N. Tokai-Nishizumi, H. Kozuka-Hata, M. Oyama, M. Takekawa, *Curr. Biol.* **2023**, 33, 1967-1981.e8.
- [55] T. Hu, W. Hou, E. Xiao, M. Long, *Genes Dis.* **2022**, 9, 659–667.
- [56] Y. Zhan, H. Wang, Y. Ning, H. Zheng, S. Liu, Y. Yang, M. Zhou, S. Fan, *Am. J. Cancer Res.* **2020**, 10, 2226–2241.
- [57] M. Zhra, R. J. Qasem, F. Aldossari, R. Saleem, A. Aljada, *Int. J. Mol. Sci.* **2024**, 25, 5460.
- [58] S. Monro, K. L. Colón, H. Yin, J. I. Roque, P. Konda, S. Gujar, R. P. Thummel, L. Lilge, C. G. Cameron, S. A. McFarland, *Chem. Rev.* **2019**, 119, 797–828.
- [59] S. Bonnet, *J. Am. Chem. Soc.* **2023**, 145, 23397–23415.
- [60] E. Rafic, C. Ma, B. B. Shih, H. Miller, R. Yuste, T. Palomero, R. Etchenique, *J. Am. Chem. Soc.* **2024**, 146, 13317–13325.

- [61] D. Havrylyuk, D. K. Heidary, Y. Sun, S. Parkin, E. C. Glazer, *ACS Omega* **2020**, *5*, 18894–18906.
- [62] M. Denison, C. Turro, J. J. Kodanko, in *Photochem. Vol. 51* (Eds.: S. Crespi, S. Protti), Royal Society Of Chemistry, **2023**, pp. 535–561.
- [63] F. Heinemann, J. Karges, G. Gasser, *Acc. Chem. Res.* **2017**, *50*, 2727–2736.
- [64] U. Neugebauer, Y. Pellegrin, M. Devocelle, R. J. Forster, W. Signac, N. Moran, T. E. Keyes, *Chem. Commun.* **2008**, 5307.
- [65] B. P. Sullivan, D. J. Salmon, T. J. Meyer, *Inorg. Chem.* **1978**, *17*, 3334–3341.
- [66] A. Marchand, V. Gabelica, *Nucleic Acids Res.* **2016**, *44*, 10999–11012.
- [67] I. Renard, M. Grandmougin, A. Roux, S. Y. Yang, P. Lejault, M. Pirrotta, J. M. Y. Wong, D. Monchaud, *Nucleic Acids Res.* **2019**, *47*, 5502–5510.
- [68] K. McQuaid, J. P. Hall, L. Baumgaertner, D. J. Cardin, C. J. Cardin, *Chem. Commun.* **2019**, *55*, 9116–9119.
- [69] R. V. Reshetnikov, J. Sponer, O. I. Rassokhina, A. M. Kopylov, P. O. Tsvetkov, A. A. Makarov, A. V. Golovin, *Nucleic Acids Res.* **2011**, *39*, 9789–9802.
- [70] F. D'Aria, V. M. D'Amore, F. S. Di Leva, J. Amato, M. Caterino, P. Russomanno, S. Salerno, E. Barresi, M. De Leo, A. M. Marini, S. Taliani, F. Da Settimo, G. F. Salgado, L. Pompili, P. Zizza, S. Shirasawa, E. Novellino, A. Biroccio, L. Marinelli, C. Giancola, *Eur. J. Pharm. Sci.* **2020**, *149*, 105337.

Chapter 4 : Targeting Mitochondrial Guanine Quadruplexes for Photoactivatable Chemotherapy in Normoxic and Hypoxic Environments

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Contributions:

Dr. Lorcan Holden: Synthesis and photophysical characterisation of the compounds. Co-first author and contributor to experimental design, execution, and analysis of all DNA studies. Manuscript preparation and revision.

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Dr. Giuseppe Avella: Gel electrophoresis assays, discussion and revision of the manuscript.

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Professor Tia E. Keyes: Project conceptualisation and management, discussion, and revision of the manuscript.

Supporting information associated with this chapter in **Appendix C**.

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

4.1 Abstract

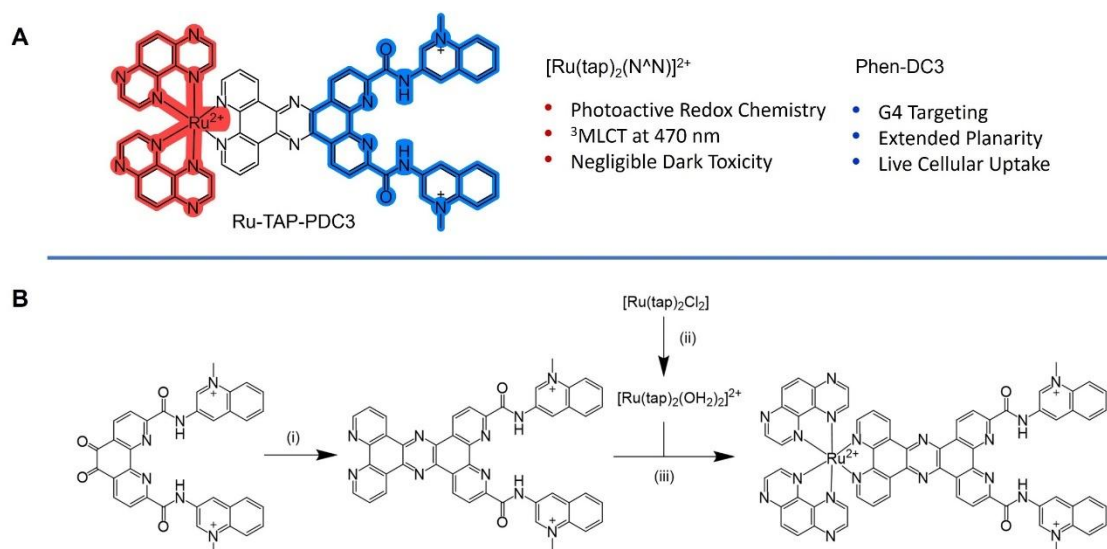
A first example of a mitochondrial G-quadruplex (mitoG4s) targeted Ru(II) photooxidant complex is reported. The complex, Ru-TAP-PDC3 induces photodamage toward guanine quadruplexes (G4s) located in the mitochondrial genome under hypoxic and normoxic conditions. Ru-TAP-PDC3 shows high affinity for mitoG4s and localises within mitochondria of live HeLa cells. Immunolabelling with anti-G4 antibody, BG4, confirms Ru-TAP-PDC3 associates with G4s within the mitochondria of fixed cells. The complex induces depletion of mtDNA in live cells under irradiation at 405 nm, confirmed by loss of PicoGreen signal from mitochondria. Biochemical studies confirm this process induces apoptosis. The complex shows low dark toxicity and an impressive phototoxicity index (PI) of >89 was determined in HeLa under very low intensity irradiation, 5 J/cm². The phototoxicity is thought to operate through both Type II singlet oxygen and Type III pathways depending on normoxic or hypoxic conditions, from live cell assays and plasmid DNA cleavage. Overall, we demonstrate targeting mitoG4s and mtDNA with a photooxidant is a potent route to achieving apoptosis under hypoxic conditions that can be extended to phototherapy.

4.2 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, a challenge for conventional chemotherapy.^[1,2] The majority of photosensitisers (PSs) operate through a Type II mechanism where cytotoxic singlet oxygen (¹O₂) is generated as a result of Dexter energy transfer between a triplet state of the PS and triplet oxygen (³O₂). A limitation of Type II PDT is its dependence on oxygen as a co-reagent within the tissue environment, since hypoxia is a feature of 90% of solid tumours.^[3,4] Type I processes can also accompany Type II where the excited PS undergoes electron transfer either via substrate oxidation or to molecular oxygen to form superoxide (O₂^{•-}) leading to subsequent formation of other ROS.^[5] An alternative Type III process has been suggested where PSs can damage biological substrates independently of oxygen.^[6,7] The majority of Ru(II) complexes reported as PSs are Type II systems.^[8] They are widely investigated in this regard due to their chemical stability, photostability, and their long-lived ³MLCT that can promote triplet photosensitisation for both ¹O₂ and ROS

generation.^[9–11] Ruthenium(II) polypyridyl complexes have been increasingly used in PACT development, where s/p ligand character can tune photoreactivity and photoinduced ligand dissociation, releasing cytotoxic ligands or metal centres with anticancer activity that may be oxygen independent.^[12–19] An additional and potentially important class of PACT agents are those capable of direct photooxidation or photoactivatable crosslinking DNA, for example, Ru(II) complexes bearing electron deficient tetraazaphenanthrene (TAP) ligands.^[20–24] Such ligands shift the photooxidation potential of the complex to provide the thermodynamic driving force to directly photooxidise guanine nucleobases through what is believed to be a proton coupled electron transfer (PCET).^[25] PCET has been shown to effectively cleave DNA, and in some instances also produce covalent photoadducts through an oxygen independent pathway.^[26,27] We recently reported strategies to drive complexes of this type to the nucleus of live cells for the first time where they showed significant phototoxicity but low dark cytotoxicity.^[28]

Many phototherapeutics tested to date are not discretely targeted within the cell, but even with broad dispersion through the cell activity often arises from damage to duplex DNA, most frequently guanine residues, as they are the most easily oxidised. G-rich DNA and RNA sequences can form guanine quadruplexes (G4s) under physiological conditions, where guanines are stabilised through Hoogsteen hydrogen bonding and Van der Waals forces to form tetrads that are further stabilised between monovalent cations.^[29] These structures have been confirmed to form in cells and are associated with essential biological processes.^[30–32] In particular, G4 forming sequences, $G_nN_xG_nN_xG_nN_xG_n$; $n \geq 2$, $x \leq 7$, have been shown to be abundant in untranslated and promoter regions of oncogenes.^[33] Thus ligands that bind G4s have potential as anticancer therapeutics, by stalling cell proliferation, inducing DNA damage and causing apoptosis.^[34–37] G4s offer a unique target for redox active therapeutics, due to the vulnerability of guanine as the most readily oxidised base. Additionally, when multiple guanines are contiguous in a DNA sequence, the oxidation potential of the 5' most guanine becomes further reduced, this is an exciting and underexplored prospect for specific targeting.^[38] The Thomas group developed and investigated the bimetallic $[Ru(tap)_2]_2(tpphz)]^{4+}$ complex for its G4 binding capacity where the complex was shown to photodamage G4s in solution and induced phototoxicity in human C8161 melanoma cells.^[23] Elias *et al.* have reported recent examples of Ru(II)-TAP containing complexes



Scheme 4.1 (A) Structure of Ru-TAP-PDC3 and its associated features. **(B)** Synthetic scheme of Ru-TAP-PDC3, **(i)** reflux in dry MeCN under argon with 5,6-diamino-phenanthroline, 18 h. **(ii)** reflux in water with AgCl for 4 h. **(iii)** reflux overnight in ethylene glycol/water (4/1). Courtesy of Dr. Lorcan Holden.

that associate with non-canonical DNA structures G4s in solution.^[30,39] In the latter case, cell uptake was observed with wide distribution of complex in fixed cells. The complex showed phototoxicity, but the origin of this effect was not identified. However, it was noted the complex reached the nucleus. Mitochondrial G4 structures (mitoG4s) have undergone less investigation 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 their essential role in cellular energy generation in the form of ATP through oxidative phosphorylation, in cell death signalling and in maintaining redox homeostasis.^[40–44] 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, mitochondria present favourable K^+ rich conditions for G4 formation (150–180 mM). G-rich sequences are prevalent in mammalian mtDNA with >200 putative quadruplex forming sequences identified in the mitochondrial genome.^[45] 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. However there is currently a lack of probes that specifically target mitoG4s to enable study and no mitoG4 targeted phototherapeutics to date.^[46,47] Furthermore, while the majority of cellular studies investigating PSs to date have been centred around 2D cell monolayer testbeds, the use of 3D cellular models, such as multicellular tumour spheroids (MCTS), is increasing steadily as they offer a more physiologically relevant intermediate between *in vitro* cell monolayer and *in vivo* studies.

For example, we recently reported an osmium(II) complex, $[\text{Os}-(\text{R4})_2]^{10+}$, that successfully penetrated pancreatic MCTS, and a ruthenium(II) complex, $[\text{Ru}(\text{bqp})(\text{bqpCOOEt})]^{2+}$, and its bioconjugates that showed excellent phototoxic potential in pancreatic MCTS.^[48,49] Other examples include work by Raza *et al.* with human melanoma MCTS to study the phototoxic capacity of their $[\{\text{Ru}(\text{tap})_2\}_2(\text{tpphz})]^{4+}$ complex in a 3D cellular environment and Karges *et al.* who prepared exceptionally large (800 μm) MCTS.^[50–52] The layered structure of MCTS more accurately mimics the tumour microenvironment with proliferative, quiescent, senescent and hypoxic or necrotic regions making them an ideal choice of 3D cell model.

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

4.3 Results and Discussion

The synthetic route to Ru-TAP-PDC3 is illustrated in Scheme 4.1B and was performed by Dr. Lorcan Holden. Phendione-DC3 was synthesised as recently reported.^[53] The TPPHz-PDC3 ligand was generated through Schiff-base condensation between phendione-DC3 and 5,6-diamino-phenanthroline, followed by direct complexation to the metal centre. Since $[\text{Ru}(\text{tap})_2\text{Cl}_2]$ is less reactive than similar polypyridyl complexes, the Ru-aquo intermediate ($[\text{Ru}(\text{tap})_2(\text{H}_2\text{O})_2]$) was prepared by treatment of $[\text{Ru}(\text{tap})_2\text{Cl}_2]$ with silver triflate prior to TPPHz-PDC3 complexation. The complex was purified on C-18 preparation silica plates. Characterisation was completed using ^1H NMR and high-resolution mass spec (Figures S4.2 and S4.6) and purity was confirmed through HPLC (Figure S4.7).

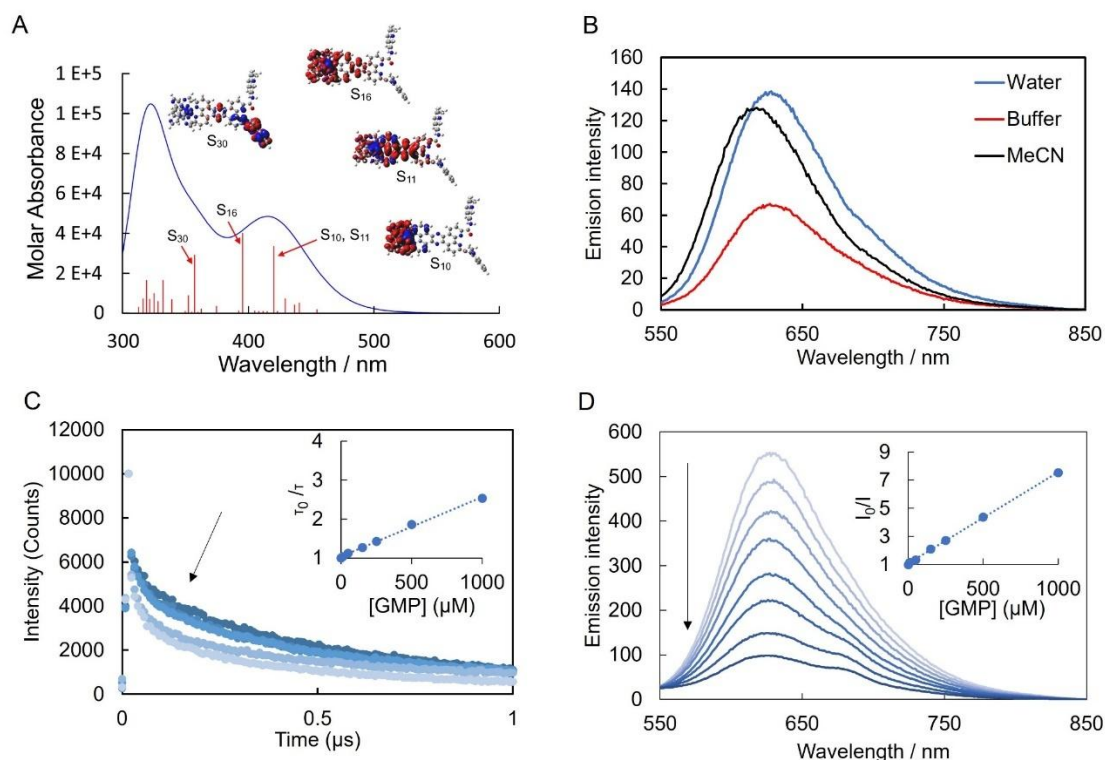


Figure 4.1 (A) Theoretical absorption spectrum modelled and electron density difference maps for highest oscillator strength transitions from DFT calculations in water (see SI for details) (B) Absorption and emission spectra of Ru-TAP-PDC3 (10 μ M) in DI H₂O, MeCN and KPi buffer (10 mM potassium phosphate, 100 mM Potassium chloride 7 pH), with excitation at 470 nm. (C) Lifetime and (D) Luminescent quenching of Ru-TAP-PDC3 (10 μ M) lifetimes with increasing concentrations of GMP (0 mM- 1 mM) in KPi buffer, insets: Stern-Volmer plot of (C) lifetimes and (D) luminescent intensity. Courtesy of Dr. Lorcan Holden.

As shown in Figure 4.1B, 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 ¹MLCT 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 Figure 1B, results in an intense emission with maxima at 628 nm (H₂O), 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 $\tau_2 = 93$ ns (30%). Similar biexponential decay was observed from the previously reported phenanthroline derivative, RuPDC3.^[53]

Given the low-lying states on the PDC3, we first estimated the photoreduction potential of the complex to anticipate if there is sufficient driving force for guanine photooxidation. From voltametric and 77 °K emission studies (Figure S4.8 and S4.9) Ru-TAP-PDC3 has an E^*_{red} of ~ 1.3 eV, which is indeed sufficient.^[54] Correspondingly, in titration of GMP (guanosine monophosphate) against Ru-TAP-PDC3 (Figure 4.2C) both steady state emission intensity and luminescence lifetime decreased. The inset of Figure 4.1C and Figure 4.1D show the resulting Stern-Volmer plots revealing a modest difference in the slopes of each plot (0.0015 and 0.0065 respectively). This indicates the quenching observed is a combination of static and dynamic process, the static component attributed to ion pairing between the cationic complex and anionic GMP. The lifetime data can be used to estimate the quenching constant; Stern-Volmer plot derived from the lifetime quenching studies (inset Figure 4.1C) was used to calculate the quenching constant, $k_q = 2.5 \times 10^9 \text{ s}^{-1}$, consistent with a diffusion-controlled process for the dynamic component of quenching attributed to photoinduced electron transfer.

Association of Ru-TAP-PDC3 with DNA was investigated initially by absorbance and luminescence spectroscopy on titration against representative nuclear G4, mitoG4s and dsDNA sequences. The selected oligonucleotides were; the human telomeric quadruplex; 22AG, putative mitoG4s; KSS, PMPS, and HRCC and the hairpin oligonucleotide; ds26. 22AG forms a mixed hybrid structure when annealed in buffer with a high K^+ concentration (100 mM KCl). Whereas the three mitoG4s all form parallel structures under these same conditions. G4 assembly was confirmed using circular dichroism (CD) (Figure S4.13). Titrations were carried out in KPi buffer (10 mM potassium phosphate buffer with 100 mM KCl at 7.4 pH). Luminescent and lifetime quenching studies of Ru-TAP-PDC3 were completed with the mitoG4s selected, indicating a primary contribution from static quenching when Ru-TAP-PDC3 is fully bound to mitoG4s (Figure S4.10). For all titrations in this study, measurements were made after sufficient time to ensure no further spectral changes occurred ensure equilibrium had been reached.

Absorbance titrations of the dsDNA hairpin oligonucleotide, ds26, are shown in Figure 4.2A. Ru-TAP-PDC3 titrated with increasing concentrations of dsDNA induced a hypochromic response, at 415 nm and 465 nm with a saturable response.

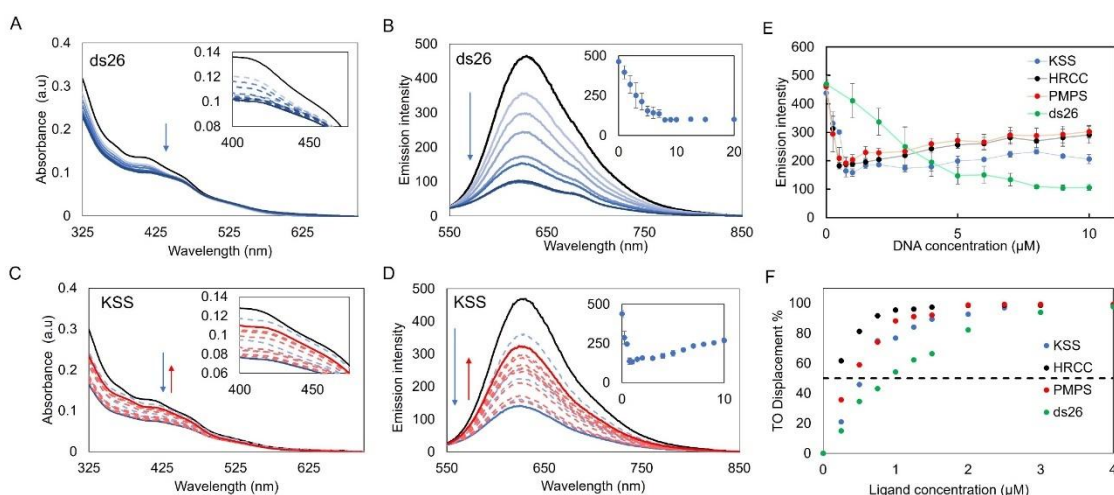


Figure 4.2 Representative UV-Vis absorption for Ru-TAP-PDC3 (10 μ M) when titrated against **(A)** ds26 (dsDNA) and **(C)** KSS (mitoG4), decreases in absorbance are represented in blue and increases are represented in red. insets: Absorption spectra of **(A)** ds26 and **(C)** KSS titration from 400 nm to 475 nm. Representative luminescence titration of Ru-TAP-PDC3 (10 μ M) when titrated with **(B)** ds26 and **(D)** KSS ($\lambda_{\text{exc}} = 465$ nm), inset: luminescence at 630 nm when titrated with **(B)** ds26 and **(D)** KSS. **(E)** Luminescent quenching curves of Ru-TAP-PDC3 (10 μ M) against a series of DNA oligonucleotides (KSS, HRCC, ctDNA and PMPS). **(F)** G4-FID curves of Ru-TAP-PDC3 against a series of DNA oligonucleotides (HRCC, KSS, PMPS and ds26). Error bars show standard deviation of $n=3$ replicate measurements. Figure courtesy of Dr. Lorcan Holden.

In contrast, for parallel mitoG4s, there is hypochromism on initial Ru-TAP-PDC3 binding, however, this is followed by a hyperchromic shift at higher G4 concentration. These hyperchromic and hypochromic changes in the absorption binding studies are reflected also in the luminescence spectral binding studies (Figure S4.11 and S4.12).

When Ru-TAP-PDC3 is titrated with nuclear G4s and mitoG4s the luminescence is partially quenched. Given that G4s comprise contiguous guanine sequences, the decreased emission intensity is taken to indicate that PET occurs on Ru-TAP-PDC3 binding. Following this initial emission decrease, signal saturates at a binding ratio of 4:1, Ru-TAP-PDC3 to G4 followed by a modest increase in luminescence at increasing concentrations of G4s. When the luminescence is corrected to account for changes to absorbance cross section, the modest increase in luminescence remains. This likely also indicates multiple G4 binding modes occur for the complex that vary in electron transfer efficiency, e.g. through distance effects. When titrated against the dsDNA oligo, ds26, quenching is also observed however the response is linear (Figure 4.2B). The McGhee-von Hippel binding model may typically be applied to extract binding affinities, but, 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.^[55]

Instead, 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.^[56] Affinities are reported as concentrations required to displace 50% of TO (DC_{50}). Figure 4.2F illustrates the results of the G4-FID assay for Ru-TAP-PDC3. Ru-TAP-PDC3 showed outstanding displacement of TO across all oligonucleotides tested with near 100% displacement at saturation (Table 4.1). In particular it is evident from Figure 2F that Ru-TAP-PDC3 binds with higher affinity to G4s when compared to ds26. The complex exhibits particular 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 that Ru-TAP-PDC3 is 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 medium affinity ligand, likewise, dsDNA had a DC_{50} of 0.91 μ M. In comparison to literature reported of Phen-DC3 G4-FID experiments, Ru-TAP-PDC3 shows comparable displacement values. Phen-DC3 has a significant DC_{50} 0.31 μ M when binding to 22AG and 0.25 μ M binding to c-kit under the same conditions.^[56]

Circular Dichroism (CD) measurements were carried out to evaluate any impact of Ru-TAP-PDC3 on the G4 structures. Interestingly, when Ru-TAP-PDC3 binds to mitoG4s, no significant change in CD or induced CD is observed. These observations in combination with significant displacement of TO are suggestive of end-capping as the primary 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 S4.13). 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 the tetrad. As Ru-TAP-PDC3 incorporates Phen-DC3, this binding mode should also be considered as a possibility to explain the difference in binding affinities.^[57] Particularly interesting is the markedly different behaviour of Ru-TAP-PDC3 compared to related complex Ru-phen-PDC3, where binding resulted in significant structural disruption of nuclear G4s of parallel topology.^[53]

Table 4.1 Photophysical measurements and G4-FID results

Solution	Absorbance features (nm)	Emission (nm)	DC ₅₀ (μM)
MeCN	373, 419, 455	615	
DI H ₂ O	373, 413, 464	628	
Buffer	376, 410, 461	625	
KSS	378, 429	630	0.40
HRCC	382, 423	627	0.20
PMPS	373, 414	627	0.53
ds26	374, 424	620	0.91

Ru-TAP-PDC3 was examined in live HeLa and HEK-293 cells using confocal fluorescence microscopy. HeLa cells were selected for this study due to their mtDNA rich mitochondria.^[58] To investigate cellular uptake HeLa cells were incubated with Ru-TAP-PDC3, at varying concentrations between 30 μM to 200 μM for 24 h (Figure S4.14). 50 μM Ru-TAP-PDC3 was used thereafter as the optimum concentration for imaging by balancing intensity and phototoxicity. Assessing cellular uptake of Ru-TAP-PDC3 over time (3-24 h), a 24 h incubation was found to be optimum (Figure S4.15). Uptake was then tested in HEK-293 cells at 50 μM (24 h). Localisation of Ru-TAP-PDC3 in HeLa and HEK-293 cells was determined using co-localisation studies with well-established commercial probes (Table S4.2). The complex was found to localise mainly in the mitochondria of HeLa cells following 24 h incubation with 50 μM Ru-TAP-PDC3. However, following 48 h incubation, the mitochondrial accumulation of the complex was reduced and the Pearson's coefficient value with BioTracker 488 mitochondrial dye reduced from 0.60 ± 0.11 at 24 h incubation to 0.33 ± 0.04 at 48 h. This is likely due to the impact of the complex on mitochondrial membrane integrity after prolonged uptake. Moreover, a Pearson's coefficient of 0.46 ± 0.08 and 0.39 ± 0.06 with LysoTracker Green at 24 h or 48 h incubation of Ru-TAP-PDC3 respectively indicates that the majority of the complex is localised in the mitochondria and lysosomes of HeLa cells.

In contrast, in non-cancer HEK-293 cells, the complex was found to suffer endosomal entrapment, localising mainly in the late endosomes and lysosomes, with a Pearson's coefficient of 0.49 ± 0.14 (Late Endosomes-GFP) and 0.39 ± 0.01 (LysoTracker Green) respectively at 24 h. The localisation of Ru-TAP-PDC3 in the mitochondria of HEK-293 cells at 24 h incubation was low with a Pearson's coefficient of 0.30 ± 0.04 with

BioTracker 488, likely attributed to late arrival to the mitochondria from the endosome. If this distinction arises between cancer and non-cancer cells it may offer a therapeutic advantage.

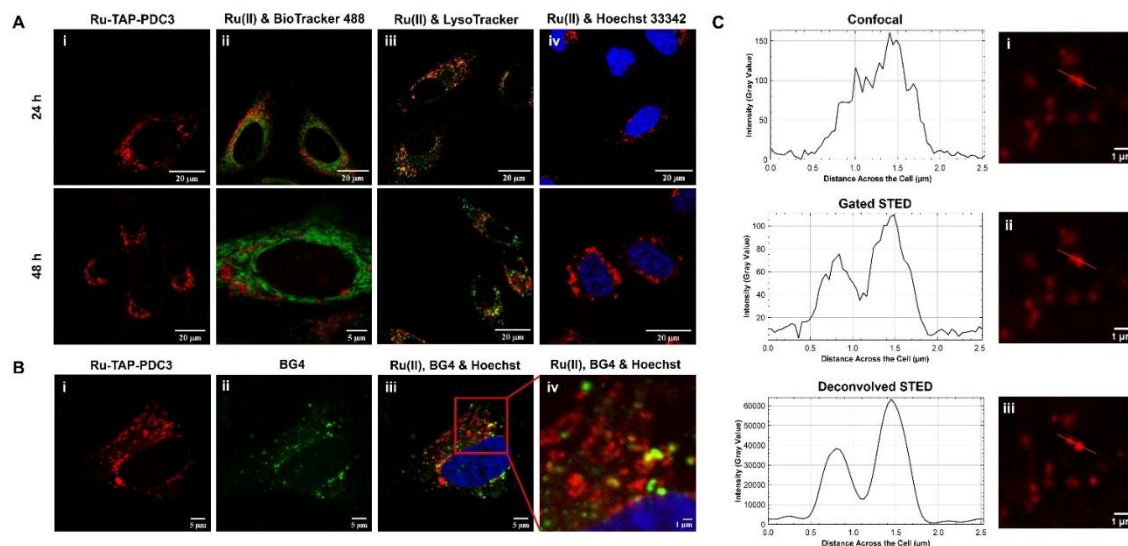


Figure 4.3 Confocal imaging of **(A)(i)** Ru-TAP-PDC3 (50 μM) in live HeLa cells after 24 or 48 h incubation and overlaid with **(ii)** BioTracker 488, **(iii)** LysoTracker green and **(iv)** Hoechst 33342 and **(B)** shows fixed HeLa cells with **(i)** Ru-TAP-PDC3 (50 μM, 24 h), **(ii)** BG4 and **(iii & iv)** the overlay with Hoechst 33342. Scale bars read 5 μm (**i, ii & iii**) and 1 μm (**iv**). Live HeLa cells were washed twice with PBS to remove any remaining dye prior to imaging where they were covered with pre-warmed DMEM without phenol red, supplemented with 10% FBS and 1% penicillin/streptomycin. **(C)** Depicts **(i)** confocal, **(ii)** gated STED and **(iii)** deconvolved STED images of Ru-TAP-PDC3 (50 μM, 24 h) in a live HeLa cell with corresponding profile plots (generated using ImageJ). An image of the full cell, with the region of interest labelled, can be found in Figure S4.20 ($\lambda = 405$ nm). STED images were deconvolved using Huygens Professional deconvolution software. Scale bars read 1 μm.

To understand if Ru-TAP-PDC3 is associating with G4s in the mitochondria we evaluated its co-localisation with the anti-G4 antibody BG4. BG4 is capable of labelling G4s throughout the cell including nuclear, cytoplasmic RNA and recently it has been confirmed to label mitoG4s.^[31,59] Co-stained images of cells with BG4 and Ru-TAP-PDC3 are shown in Figure 3B, where there is clearly excellent co-localisation between BG4 and the Ru-TAP-PDC3 (Pearson's coefficient of 0.63 ± 0.07). It is expected that some BG4 and Ru-TAP-PDC3 foci do not overlap since BG4 also labels RNA G4s and there is partial lysosomal localisation of Ru-TAP-PDC3. Importantly, in the regions ascribed to mitochondria, the overlap is the strongest.

STED microscopy was used to improve resolution of the Ru-TAP-PDC3 foci. 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 live HeLa cell treated with Ru-TAP-PDC3 showed a clear improvement in resolution of the foci (Figure 4.3C). The full cell image, showing the selected ROI, can be found in Figure S20. Dark

toxicity studies of Ru-TAP-PDC3 determined that in the absence of light the IC₅₀ was >200 µM in both HeLa and HEK-293 cells lines, confirming Ru-TAP-PDC3 shows negligible dark toxicity. In contrast, 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 (Figure S4.24). Table 4.2. summarises the measured phototoxicity indices (PI), it is important to note that since PI is the ratio of light and dark toxicity and the exact dark IC₅₀ value could not be determined, the PI values of the complex are likely significantly higher than reported here. The highest phototoxicity was observed in HeLa cells following 24 h incubation with Ru-TAP-PDC3 where a PI of >89 was measured under very low, 5 J/cm² irradiation conditions and >161 under 15 J/cm² fluence (470 nm LED used for both). By comparison after 48 h incubation with the complex the PI values decreased to >69 (24 h) and >170 (48 h) at 15 J/cm². Varying incubation time had minimal impact on the light IC₅₀ values. Given the mitochondrial localisation diminishes between 24 h and 48 h, the data suggest that mitochondrial localisation may not alone be essential for phototoxic effect.

Overall PI values are very impressive, compared to PSs reported under comparable conditions including clinically approved PSs.^[60,61] For example, Delaey *et al.* have reported a PI of >10 (light IC₅₀ 2.57 ± 0.17 µM & dark IC₅₀ >25 µM) for Photofrin (a clinically approved PS) in HeLa cells at a total irradiation dose of 5 J/cm², under very similar conditions to the lower irradiation studies under normoxic conditions reported here.^[62] It's important to note that although we tried to match the irradiance power to this study, direct comparison of PI values across studies is challenging since different spectral bandwidths of irradiance are used.^[8,62]

Table 4.2 Toxicity of Ru-TAP-PDC3

Cell Model	Incub. time	^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	n.d.	>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	n.d.	36.61 ± 0.13	1.6	n.d.

[a] Phototoxicity studies at $5 \pm 0.54 \text{ J/cm}^2$ (0.5 h at $2.81 \pm 0.30 \text{ mW/cm}^2$) and [b] $15 \pm 1.6 \text{ J/cm}^2$ (1.5 h at $2.81 \pm 0.30 \text{ mW/cm}^2$) using a 470 nm LED, where the phototoxicity index (PI) is the ratio of dark IC₅₀ to light IC₅₀. n.d. = not determined.

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 (CoCl₂) was selected for phototoxicity studies as it is a widely used chemical hypoxia model for *in vitro* studies.^[63] With this method, the stabilisation of hypoxia inducible factor 1 alpha subunit (HIF-1α) is maintained for some hours after treatment, allowing a greater window for experiments when compared to low-oxygen induced hypoxia.^[63] The chemical induction of hypoxia with CoCl₂ was confirmed in our test cells using an enzyme linked immunosorbent assay to detect HIF-1α in cell lysates.^[64]

Under low irradiation conditions (5 J/cm^2) a hypoxic light IC₅₀ of $>100 \text{ μM}$ was determined, this value compares with a normoxic light IC₅₀ of 2.24 ± 0.04 , indicating oxygen plays a significant role in phototoxicity observed under lower intensity irradiation. However, increasing irradiation dose to 15 J/cm^2 under hypoxic conditions Ru-TAP-PDC3 notably displayed strong phototoxicity towards HeLa cells with a PI of >13 (light IC₅₀, $16 \pm 1.17 \text{ μM}$). Although a higher light dosage is required under hypoxic conditions, 15 J/cm^2 irradiation intensity remains a modest light dosage and well within intensities reported for PDT studies.^[65] The light/concentration effect observed under hypoxic conditions is expected, as the probability of PET to guanine depends on localisation, binding, and orientation within the G4 structure. This means that at low flux or concentration, the probability of PET is lower than for a Type III mechanism. Nonetheless, it can be promoted with a higher total irradiation dose rather than increased concentration, which is an important distinction for a chemotherapeutic.

Overall, the data indicates that both oxygen dependent and independent pathways operate for phototoxicity of Ru-TAP-PDC3. It is also important to highlight that a single

molecule of Ru-TAP-PDC3 can turnover $^1\text{O}_2$ in an oxygen rich environment, 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 binding of Ru(II) to a G4 structure, and clearly, as the complex remains emissive in the cellular environment, not all Ru(II) reaches G4s. This results in a lower effective concentration and consequently a higher light intensity requirement for effect, suggesting that under hypoxic conditions there may be a light intensity threshold to oxygen independent PET and that ROS generation contributes to toxicity in the presence of oxygen.

Finally, gel electrophoresis studies were carried out by Dr. Giuseppe Avella using supercoiled pUC19 plasmid DNA exposed to Ru-TAP-PDC3, and $^1\text{O}_2$ scavenger, sodium azide (NaN_3). Normoxic control gels were carried out in the absence of NaN_3 . The resulting gels (Figure S4.30) indicated that Ru-TAP-PDC3 cleaves DNA highly efficiently under normoxic conditions, likely through singlet oxygen generation but cleavage persists in the presence of singlet oxygen trap, consistent with data from the cell studies. In addition, there was no emergence of bands that could be associated with photoadducts or covalent bond formation under irradiation conditions selected to mimic phototoxicity assays. This indicates that phototoxicity induced by Ru-TAP-PDC3 is induced through cleavage and not photoadduct formation.

To understand the mechanism of phototoxicity, the impact of photolysis on mitochondrial membrane potential ($\Delta\Psi\text{m}$) was investigated, using the commercial dye JC-1 (Figure 4.4D). By determining the $\Delta\Psi\text{m}$ 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. As shown in Figure 4.4D, there is a significant decrease in $\Delta\Psi\text{m}$ of HeLa cells treated with Ru-TAP-PDC3 (3 μM for 24 h) immediately after photoirradiation, with a decrease of 51% following a total irradiation dose of 5 J/cm^2 , 74% at 15 J/cm^2 under normoxic conditions and 69% at 15 J/cm^2 under hypoxic conditions. The data show that photoactivation of Ru-TAP-PDC3 causes significant mitochondrial depolarisation. Control studies, with Ru-TAP-PDC3 treated cells maintained in the dark, confirmed there was minimal disruption to the mitochondrial membrane potential without irradiation. A positive control using mitochondrial membrane disruptor, CCCP (carbonyl cyanide m-chlorophenyl hydrazone; 20 μM , 2 h) resulted in a 37% decrease in $\Delta\Psi\text{m}$ relative to untreated control cells incubated in the dark.

To investigate direct mtDNA damage caused Ru-TAP-PDC3 photoactivation, the commercial PicoGreen probe was used. PicoGreen stains both nuclear dsDNA and mtDNA (punctate cytoplasmic staining) in live cells.^[66] Fluorescent signal in the cytoplasm arising from PicoGreen emission is directly correlated to the quantity of

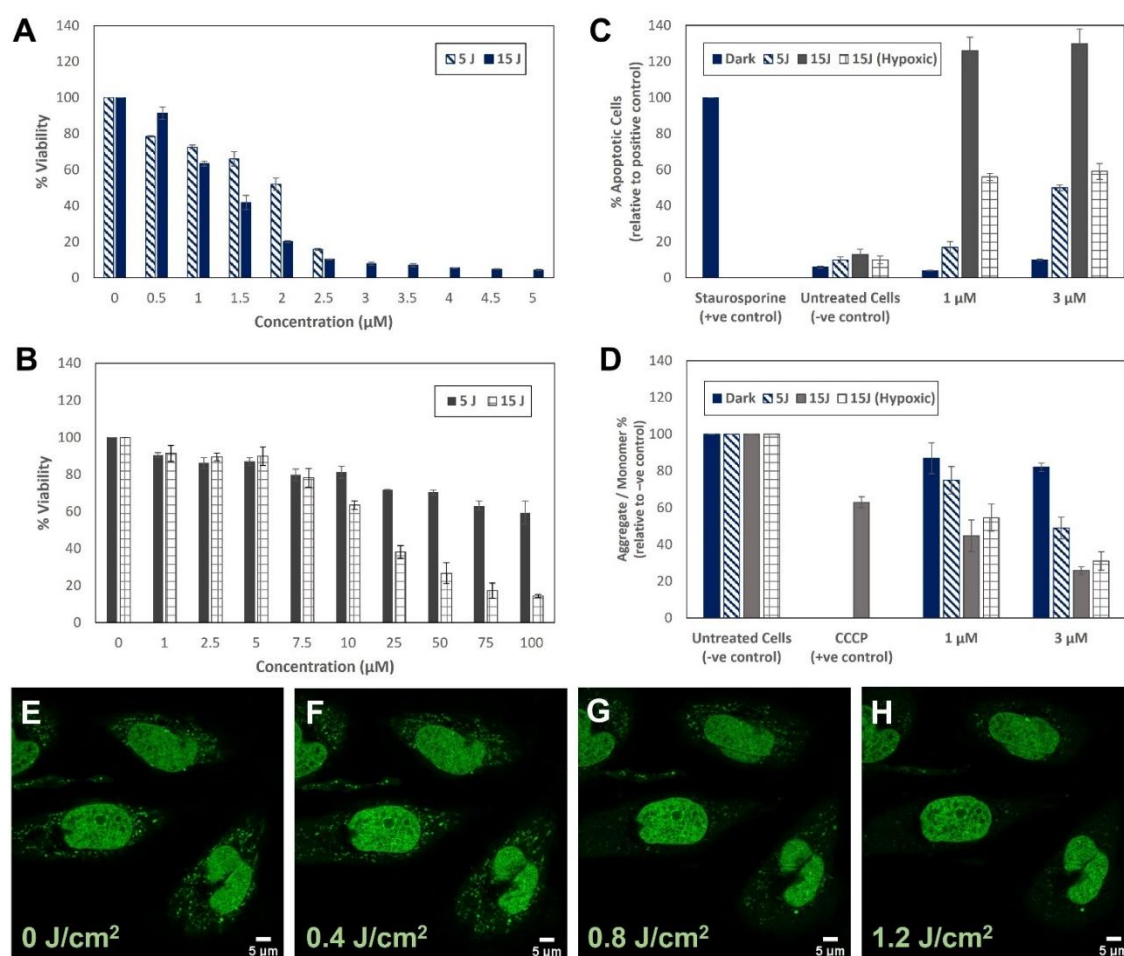


Figure 4.4 Viability of HeLa cells treated with Ru-TAP-PDC3 for 24 h and irradiated in **(A)** normoxic or **(B)** hypoxic conditions (total irradiation dose of 5 J/cm² or 15 J/cm² using a 470 nm LED, and results of the **(C)** FAM-FLICA polycaspase activity assay where % apoptotic cells are reported relative to a positive control with Staurosporine (1 μM , 3 h) and **(D)** JC-1 mitochondrial membrane potential assays in HeLa cells (24 h incubation) where a decrease in the ratio of red (J-aggregates) to green (J-monomers) emission intensity indicates mitochondrial depolarisation. 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. Confocal images in live HeLa cells pre-treated with Ru-TAP-PDC3 (3 μM , 24 h) show PicoGreen **(E)** prior to irradiation at 405 nm and after **(F)** 5, **(G)** 10 or **(H)** 15 minutes of continuous irradiation. PicoGreen was excited at 495 nm with emission collected between 520-530 nm (total irradiation dose after 15 minutes = 1.2 J/cm²).

mtDNA nucleoids within a cell and reduction of this signal is indicative of mtDNA depletion. HeLa cells treated with Ru-TAP-PDC3 for 24 h (2 μM) were counterstained with 2 $\mu\text{L/mL}$ PicoGreen. As shown in Figure 4.4G and 4.4H, after 10 or 15 minutes irradiation of the Ru(II) complex (405 nm, 1.2 J/cm²), clear reduction of PicoGreen emission in the mitochondrial region is observed. This marks a reduction in the number of mitochondrial nucleoids, indicating mtDNA damage is occurring. Importantly, this result supports localisation of Ru-TAP-PDC3 to the mitochondria and that direct binding of mtDNA to the Ru(II) complex occurs. Excitation at 405 nm was used to irradiate cells in Figure 4.4E-D, as we observed in control studies that PicoGreen photobleached under direct irradiation at 470 nm but was photostable under 405 nm excitation. This again

confirms that a combination of the Ru(II) complex and light exposure leads to mtDNA damage. Furthermore, comparing BG4 staining of HeLa cells containing Ru-TAP-PDC3 (3 μ M, 24 h) fixed and treated with BG4 before and after photoirradiation (470 nm, 5 J/cm²) as shown in Figure S4.19, we observed a loss of the punctate staining by BG4, observed outside the nucleus on irradiation. This we tentatively attribute to loss of mitochondrial G4 foci caused by their photocleavage.

To assess the mechanism of cell death when Ru-TAP-PDC3 is activated by light, a FAM FLICA polycaspase assay kit was used. The FLICA assay labels active caspase enzymes present during apoptosis to establish if cell death is by apoptosis. The results from the FLICA polycaspase activity assay (Figure 4.4D) 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 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 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 mtDNA 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.^[40,69] Combining the results of the studies with JC-1, which indicate 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.

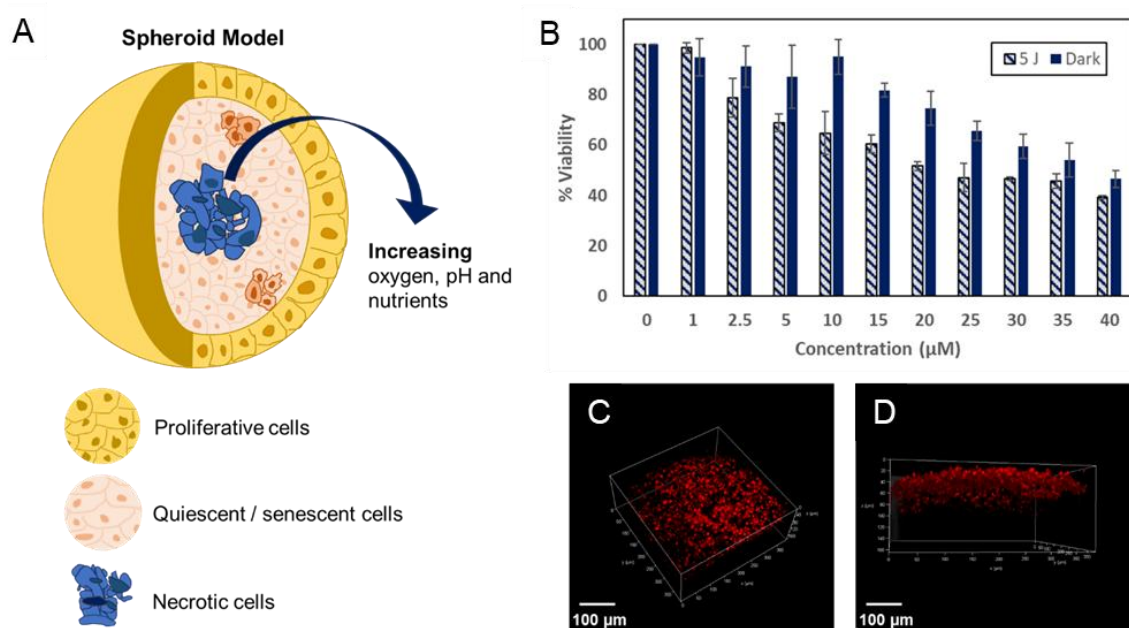


Figure 4.5 (A) Graphic of a 3D spheroid, (B) viability of HeLa MCTS treated with varying concentrations of Ru-TAP-PDC3 for 24 h and irradiated at a total dose of 5 J/cm² compared to control MCTS kept in the dark. (C) and (D) show 3D reconstruction images of Ru-TAP-PDC3 in a live HeLa spheroid (100 µM, 24 h) in fresh DMEM without phenol red, supplemented with 10% FBS and 1% penicillin/streptomycin. Scale bars measure 100 µm.

As 3D cell cultures are a more representative model of the tumour microenvironment compared to cell monolayers, we assessed if the phototoxicity of Ru-TAP-PDC3 carries through from HeLa cell monolayers to MCTS. MCTS typically produce weaker luminescence signal intensity compared to cell monolayers due to their 3D volume 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 imaging conditions (confirmed by lack of DRAQ7 uptake) suggesting the cytotoxic effects occurring within the MCTS are not apparent during imaging. For MCTS toxicity studies, the HeLa spheroids were ≥500 µm in size and appear to have developed a necrotic core (Figure S4.29). 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 (Figure S4.26). It's important to note that the emission of Ru-TAP-PDC3 is reduced when bound to DNA so it's possible some uptake cannot be visualised using confocal microscopy. As a result, it's not possible to be certain if this influences the phototoxicity of the complex. The penetration depth of the LED light through the MCTS may also impact the light IC₅₀ of the complex, resulting in a higher light IC₅₀ value of 23 ± 3.31 µM in HeLa MCTS at 5 J/cm² (470 nm) compared to cell monolayers. Notably,

Ru-TAP-PDC3 is far more toxic toward MCTS than cell monolayers since this value constitutes the denominator in the PI value a much lower PI value of 1.6 was observed for the spheroids. Control studies with untreated HeLa cells at 15 J/cm² (2 x 7.5 J/cm²) indicated this irradiation dose was toxic to the MCTS, and this limited phototoxicity studies to low light fluence. The significant increase in the dark IC₅₀ value in MCTS compared to cell monolayers (>200 µM) may be a result of their layered structure and lower number of proliferating cells, i.e. cells in 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. Differences in the penetration or toxicity patterns of luminescent probes have been reported previously.^[49,70] It is important to note that IC₅₀ 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 x 6 J/cm², 465 nm), in HepG2 liver cancer MCTS using growth curves.^[71] Further, Raza *et al.* studied the live/dead (propidium iodide) cell response to light and changes in cell morphology in C8161 human melanoma MCTS with their ruthenium TAP complex, [Ru(TAP)₂]⁴⁺.^[50] These studies show that although IC₅₀ values are informative, in MCTS, phototherapeutic potential can be further investigated through a variety of additional studies such as measuring MCTS growth or morphological changes over time.

4.4 Conclusions

We present one of the first examples of a phototherapeutic directed toward G-quadruplex mitochondrial DNA. The complex, Ru-TAP-PDC3, combines the high affinity G-quadruplex ligand, Phen-DC3 with Ru(II)TAP centre to drive PCET with DNA through both O₂ dependent and O₂ independent pathways.

The complex showed high affinity for mitoG4s with preference over dsDNA and was found to readily permeate the cell membrane. Ru-TAP-PDC3 preferentially accumulates in the mitochondria of HeLa cells where immunolabelling with anti-G4 antibody, BG4, confirms it associates with mitochondrial G4s. It is non-toxic in the dark with IC₅₀ values >200 µM in both HeLa and HEK-293 cells, but under photoirradiation it exhibits an exceptional phototoxicity index (PI) of >89 under low light conditions (5 J/cm²) and >160 at 15 J/cm², evident in notable loss of ΔΨ_m, mtDNA depletion and ultimately apoptosis upon irradiation at 470 nm. Under hypoxic conditions in HeLa cells Ru-TAP-PDC3 maintained its phototherapeutic potential, although PI was reduced from >160 to >13, under 15 J/cm² fluence. HeLa MCTS studies showed a decreased PI value of 1.6, which

was attributed to poor penetration of the complex through the MCTS core. Overall, the results suggest that Ru-TAP-PDC3 is a great candidate for phototherapy and can be activated under both normoxic and hypoxic cellular conditions and more broadly that mitoG4s are an important potential target for phototherapy. Performance can be improved by applying reliable delivery approaches to maximise tissue penetration and mitochondrial targeting.

4.5 References

- [1] J. Karges, *Angewandte Chemie International Edition* **2022**, *61*, e202112236.
- [2] S. Monro, K. L. Colón, H. Yin, J. I. Roque, P. Konda, S. Gujar, R. P. Thummel, L. Lilge, C. G. Cameron, S. A. McFarland, *Chem. Rev.* **2019**, *119*, 797–828.
- [3] S. Hackbarth, R. Islam, V. Šubr, T. Etrych, J. Fang, *J Pers Med* **2022**, *12*, 891.
- [4] Z. Chen, F. Han, Y. Du, H. Shi, W. Zhou, *Sig Transduct Target Ther* **2023**, *8*, 1–23.
- [5] M. S. Baptista, J. Cadet, P. Di Mascio, A. A. Ghogare, A. Greer, M. R. Hamblin, C. Lorente, S. C. Nunez, M. S. Ribeiro, A. H. Thomas, M. Vignoni, T. M. Yoshimura, *Photochemistry and Photobiology* **2017**, *93*, 912–919.
- [6] R. R. Allison, C. H. Sibata, *Photodiagnosis and Photodynamic Therapy* **2010**, *7*, 61–75.
- [7] Q. Yao, J. Fan, S. Long, X. Zhao, H. Li, J. Du, K. Shao, X. Peng, *Chem* **2022**, *8*, 197–209.
- [8] M. Ankathatti Munegowda, A. Manalac, M. Weersink, S. A. McFarland, L. Lilge, *Coordination Chemistry Reviews* **2022**, *470*, 214712.
- [9] L. Holden, C. S. Burke, D. Cullinane, T. E. Keyes, *RSC Chemical Biology* **2021**, *2*, 1021–1049.
- [10] C. S. Burke, A. Byrne, T. E. Keyes, *Angewandte Chemie International Edition* **2018**, *57*, 12420–12424.
- [11] L. C.-C. Lee, K. K.-W. Lo, *J. Am. Chem. Soc.* **2022**, *144*, 14420–14440.
- [12] N. J. Farrer, L. Salassa, P. J. Sadler, *Dalton Trans* **2009**, 10690–10701.
- [13] Y. Chen, L. Bai, P. Zhang, H. Zhao, Q. Zhou, *Molecules* **2021**, *26*, 5679.
- [14] J. D. Knoll, B. A. Albani, C. Turro, *Acc. Chem. Res.* **2015**, *48*, 2280–2287.
- [15] E. P. van Geest, S. K. Götzfried, D. M. Klein, N. Salitra, S. Popal, Y. Husiev, C. J. Van der Griend, X. Zhou, M. A. Siegler, G. F. Schneider, S. Bonnet, *Photochemistry and Photobiology* **2023**, *99*, 777–786.
- [16] L. N. Lameijer, D. Ernst, S. L. Hopkins, M. S. Meijer, S. H. C. Askes, S. E. Le Dévédec, S. Bonnet, *Angewandte Chemie International Edition* **2017**, *56*, 11549–11553.

- [17] J. Roque III, D. Havrylyuk, P. C. Barrett, T. Sainuddin, J. McCain, K. Colón, W. T. Sparks, E. Bradner, S. Monro, D. Heidary, C. G. Cameron, E. C. Glazer, S. A. McFarland, *Photochemistry and Photobiology* **2020**, 96, 327–339.
- [18] E. Wachter, D. Moyá, S. Parkin, E. C. Glazer, *Chemistry – A European Journal* **2016**, 22, 550–559.
- [19] E. Wachter, D. K. Heidary, B. S. Howerton, S. Parkin, E. C. Glazer, *Chemical Communications* **2012**, 48, 9649–9651.
- [20] J.-P. Lecomte, A. K.-D. Mesmaeker, M. M. Feeney, J. M. Kelly, *Inorganic Chemistry* **1995**, 34, 6481–6491.
- [21] J. P. Lecomte, A. Kirsch-De Mesmaeker, J. M. Kelly, A. B. Tossi, H. Görner, *Photochem Photobiol* **1992**, 55, 681–689.
- [22] B. Elias, A. Kirsch-De Mesmaeker, *Coordination Chemistry Reviews* **2006**, 250, 1627–1641.
- [23] S. A. Archer, A. Raza, F. Dröge, C. Robertson, A. J. Auty, D. Chekulaev, J. A. Weinstein, T. Keane, A. J. H. M. Meijer, J. W. Haycock, S. MacNeil, J. A. Thomas, *Chemical Science* **2019**, 10, 3502–3513.
- [24] S. M. Cloonan, R. B. P. Elmes, M. Erby, S. A. Bright, F. E. Poynton, D. E. Nolan, S. J. Quinn, T. Gunnlaugsson, D. C. Williams, *J. Med. Chem.* **2015**, 58, 4494–4505.
- [25] I. Ortmans, B. Elias, J. M. Kelly, C. Moucheron, A. Kirsch-DeMesmaeker, *Dalton Trans.* **2004**, 668–676.
- [26] C. J. Cardin, J. M. Kelly, S. J. Quinn, *Chem. Sci.* **2017**, 8, 4705–4723.
- [27] S. L. Gac, M. Foucart, P. Gerbaux, E. Defrancq, C. Moucheron, A. K.-D. Mesmaeker, *Dalton Trans.* **2010**, 39, 9672–9683.
- [28] C. S. Burke, A. Byrne, Tia. E. Keyes, *J. Am. Chem. Soc.* **2018**, 140, 6945–6955.
- [29] M. L. Bochman, K. Paeschke, V. A. Zakian, *Nat Rev Genet* **2012**, 13, 770–780.
- [30] M. Gillard, G. Piraux, M. Daenen, M. Abraham, L. Troian-Gautier, L. Bar, H. Bonnet, F. Loiseau, H. Jamet, J. Dejeu, E. Defrancq, B. Elias, *Chemistry – A European Journal* **2022**, 28, e202202251.
- [31] G. Biffi, D. Tannahill, J. McCafferty, S. Balasubramanian, *Nat Chem* **2013**, 5, 182–186.
- [32] J. Robinson, F. Raguseo, S. P. Nuccio, D. Liano, M. Di Antonio, *Nucleic Acids Research* **2021**, 49, 8419–8431.
- [33] S. Balasubramanian, L. H. Hurley, S. Neidle, *Nat Rev Drug Discov* **2011**, 10, 261–275.
- [34] T. Santos, G. F. Salgado, E. J. Cabrita, C. Cruz, *Pharmaceuticals (Basel)* **2021**, 14, 769.

- [35] Q. Cao, Y. Li, E. Freisinger, P. Z. Qin, R. K. O. Sigel, Z.-W. Mao, *Inorganic Chemistry Frontiers* **2017**, *4*, 10–32.
- [36] S. Asamitsu, S. Obata, Z. Yu, T. Bando, H. Sugiyama, *Molecules* **2019**, *24*, 429.
- [37] M. Wumaier, J.-J. Shi, T.-M. Yao, X.-C. Hu, R.-R. Gao, S. Shi, *Journal of Inorganic Biochemistry* **2019**, *196*, 110681.
- [38] A. M. Fleming, C. J. Burrows, *J. Am. Chem. Soc.* **2020**, *142*, 1115–1136.
- [39] J. Weynand, A. Diman, M. Abraham, L. Marcélis, H. Jamet, A. Decottignies, J. Dejeu, E. Defrancq, B. Elias, *Chemistry – A European Journal* **2018**, *24*, 19216–19227.
- [40] C. Wang, R. J. Youle, *Annu Rev Genet* **2009**, *43*, 95–118.
- [41] J. R. Friedman, J. Nunnari, *Nature* **2014**, *505*, 335–343.
- [42] S. Chakraborty, B. K. Agrawalla, A. Stumper, N. M. Vegi, S. Fischer, C. Reichardt, M. Kögler, B. Dietzek, M. Feuring-Buske, C. Buske, S. Rau, T. Weil, *J. Am. Chem. Soc.* **2017**, *139*, 2512–2519.
- [43] R. J. Morris, M. Massi, in *Advances in Inorganic Chemistry* (Eds.: P.C. Ford, R. van Eldik), Academic Press, **2022**, pp. 411–509.
- [44] M. König, D. Siegmund, L. J. Raszeja, A. Prokop, N. Metzler-Nolte, *Med. Chem. Commun.* **2018**, *9*, 173–180.
- [45] P. Kumar, A. Pandith, C.-L. Tseng, T. Burnouf, *Journal of Photochemistry and Photobiology C: Photochemistry Reviews* **2023**, *56*, 100619.
- [46] M. Falabella, R. J. Fernandez, F. B. Johnson, B. A. Kaufman, *Curr Med Chem* **2019**, *26*, 2918–2932.
- [47] V. J. Sahayasheela, Z. Yu, T. Hidaka, G. N. Pandian, H. Sugiyama, *Trends in Genetics* **2023**, *39*, 15–30.
- [48] K. S. Gkika, S. Noorani, N. Walsh, T. E. Keyes, *Inorg. Chem.* **2021**, *60*, 8123–8134.
- [49] R. C. Curley, C. S. Burke, K. S. Gkika, S. Noorani, N. Walsh, T. E. Keyes, *Inorg. Chem.* **2023**, *62*, 13089–13102.
- [50] A. Raza, S. A. Archer, S. D. Fairbanks, K. L. Smitten, S. W. Botchway, J. A. Thomas, S. MacNeil, J. W. Haycock, *J. Am. Chem. Soc.* **2020**, *142*, 4639–4647.
- [51] J. Karges, S. Kuang, Y. C. Ong, H. Chao, G. Gasser, *Chemistry – A European Journal* **2021**, *27*, 362–370.
- [52] R. Bevernaegie, B. Doix, E. Bastien, A. Diman, A. Decottignies, O. Feron, B. Elias, *J. Am. Chem. Soc.* **2019**, *141*, 18486–18491.
- [53] L. Holden, K. S. Gkika, C. S. Burke, C. Long, T. E. Keyes, *Inorg. Chem.* **2023**, *62*, 2213–2227.
- [54] D. H. Johnston, C.-C. Cheng, K. J. Campbell, H. H. Thorp, *Inorg. Chem.* **1994**, *33*, 6388–6390.

- [55] J. D. McGhee, P. H. von Hippel, *Journal of Molecular Biology* **1974**, 86, 469–489.
- [56] E. Largy, F. Hamon, M.-P. Teulade-Fichou, *Anal Bioanal Chem* **2011**, 400, 3419–3427.
- [57] A. Ghosh, M. Trajkovski, M.-P. Teulade-Fichou, V. Gabelica, J. Plavec, *Angewandte Chemie International Edition* **2022**, 61, e202207384.
- [58] V. Pavluch, T. Špaček, H. Engstová, A. Dlasková, P. Ježek, *Sci Rep* **2023**, 13, 5788.
- [59] S. Dahal, H. Siddiqua, V. K. Katapadi, D. Iyer, S. C. Raghavan, *The FEBS Journal* **2022**, 289, 163–182.
- [60] E. T. Papish, O. E. Oladipupo, *Current Opinion in Chemical Biology* **2022**, 68, 102143.
- [61] E. T. Papish, O. E. Oladipupo, *Current Opinion in Chemical Biology* **2022**, 68, 102143.
- [62] E. Delaey, F. van Laar, D. De Vos, A. Kamuhabwa, P. Jacobs, P. de Witte, *Journal of Photochemistry and Photobiology B: Biology* **2000**, 55, 27–36.
- [63] J. Muñoz-Sánchez, M. E. Cháñez-Cárdenas, *Journal of Applied Toxicology* **2019**, 39, 556–570.
- [64] D. Wu, P. Yotnda, *J Vis Exp* **2011**, 2899.
- [65] S. Bonnet, *J. Am. Chem. Soc.* **2023**, 145, 23397–23415.
- [66] N. Ashley, D. Harris, J. Poulton, *Experimental Cell Research* **2005**, 303, 432–446.
- [67] N. L. Wilke, H. Burmeister, C. Frias, I. Ott, A. Prokop, *International Journal of Molecular Sciences* **2023**, 24, 952.
- [68] S. Elmore, *Toxicol Pathol* **2007**, 35, 495–516.
- [69] H. M. Al-Aamri, H. R. Irving, C. Bradley, T. Meehan-Andrews, *BMC Cancer* **2021**, 21, 438.
- [70] H. Huang, P. Zhang, H. Chen, L. Ji, H. Chao, *Chemistry – A European Journal* **2015**, 21, 715–725.
- [71] J. Liu, A. W. Prentice, G. J. Clarkson, J. M. Woolley, V. G. Stavros, M. J. Paterson, P. J. Sadler, *Advanced Materials* **2023**, 35, 2210363.

Chapter 5 : Enhancing Phototoxicity in BODIPY-perylene Charge-transfer Dyads by Combined Iodination and Mesylation

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Contributions:

Rhianne C. Curley: Primary author and contributor to experimental design, execution, analysis and manuscript preparation and revision. Photophysical characterisation in aqueous media and cellular studies using 2D and 3D cellular models.

Dr. Ruben Arturo Arellano-Reyes: Synthesis, structural and photophysical characterisation of the compounds. Discussion and revision of the manuscript.

Dr James N. McPherson: Crystallography, discussion and revision of the manuscript.

Professor Vickie McKee: Interpretation of crystallography data, discussion and revision of the manuscript.

Professor Tia E. Keyes: Project conceptualisation and management, discussion, and revision of the manuscript.

Supporting information associated with this chapter in **Appendix D**.

Enhancing Phototoxicity in BODIPY-perylene Charge-transfer Dyads by Combined Iodination and Mesylation

5.1 Abstract

The uptake and phototoxicity of a family of BODIPY-perylene charge-transfer dyads are compared in live cancer and non-cancer cell lines to evaluate their performance in imaging and photodynamic therapy (PDT). The impact of iodination and mesylation of the meso position of the compounds on their optical properties, cell uptake and toxicity are compared. Notably, across all derivatives the probes were minimally dark toxic up to 50 μM , (the maximum concentration tested), but exhibited outstanding phototoxicity with nanomolar IC_{50} values and impressive phototoxic indices (PI, ratio of dark IC_{50} to light IC_{50}), with best performance for the mesylated iodinated derivative MB2PI, which had a PI of >218 and >8.9 in MCF-7 cells and tumour spheroids respectively. This is significantly higher than non-iodinated analogue M2P in MCF-7 cells with an observed PI of >109 and slightly higher than MB2PI in spheroids with a PI of >8. This compound also showed interesting emission spectral variation with localisation that responded to stimulation of inflammation. Additional studies confirmed efficient singlet oxygen generation by the BODIPYs, suggesting a Type II mechanism of phototoxicity. Overall, the data indicates that combining charge-transfer and iodination is an effective strategy for enhancing phototherapeutic capacity of BODIPY PS.

5.2 Introduction

BODIPY (boron-dipyrromethene) derivatives are versatile luminophores^[1] that have been extensively studied as candidates for bio-imaging^[2] and intracellular sensing.^[3–7] This has been driven by their attractive optical properties, including high absorption coefficients and emission quantum yields at wavelengths that can be tuned from visible to NIR through selective synthetic modification of the BODIPY core.^[8–12] The high emission quantum yields of many BODIPY derivatives is associated with their low quantum yield of inter-system crossing (ISC) which also limits their phototoxicity through singlet oxygen ($^1\text{O}_2$) pathways.^[13] There is nonetheless growing interest in BODIPYs as metal-free photosensitisers (PSs) for photodynamic therapy (PDT)^[14–16] with several strategies available to promote their formation of triplet states. These include through substitution with heavy atoms and implementation of charge-transfer in the compound. In BODIPY derivatives, such strategies have expanded their applications in PDT,^[17] and triplet-triplet annihilation up-conversion (TTA-UC).^[18–20] In ISC promoted through spin-orbit charge-transfer intersystem crossing (SOCT-ISC), introducing donor-acceptor

moieties into the system enables photoinduced electron transfer between the subunits, forming a singlet charge-transfer state that undergoes charge recombination, thereby facilitating the transition to a triplet excited state.^[21] This approach is attractive in BODIPY derivatives as they can behave as either excited state donor or acceptor as demonstrated across numerous donor-acceptor structures.^[22,23] Furthermore, optically, charge-transfer transitions build useful spectroscopic properties into the derivatives including large Stokes shifts and environmental sensitivity. We and others have recently developed BODIPY-perylene charge-transfer compounds capable of SOCT-ISC which is promoted by the orthogonality of the donor perylene and acceptor BODIPY.^[24,25] We further demonstrated that SOCT-ISC can be used to facilitate TTA-UC and ISC promoted by co-substitution with iodine via the heavy atom effect.^[26] Additionally, iodination has been applied to promote ISC and $^1\text{O}_2$ generation by BODIPY derivatives to promote phototoxicity.^[27,28] Nagano *et al.* were amongst the first to report an iodinated BODIPY for PDT in 2005,^[29] since then many groups have studied these systems for PDT including Liu *et al.* who prepared an iodinated BODIPY targeted to lysosomes for PDT with a reported IC_{50} of 2.34 μM (525 nm, 4 mW/cm^2 , 15 min).^[30] Furthermore, we have shown that SOCT can combine with heavy atom effect to promote ISC, which in principle can also increase phototoxicity through $^1\text{O}_2$ generation.^[26]

Wang and co-workers demonstrated that a BODIPY-perylene derivative, BDP-pery-2 (referred to as B2P herein), exhibited high phototoxicity towards HeLa cells under an irradiation dose of 9.6 J/cm^2 (500 nm irradiation) with a phototoxic index (PI) of 1041.^[24] Thus, in extending the family of BODIPY-perylene derivatives, we were interested to investigate the phototoxic capacity of B2P and an associated family of BODIPY-perylene PSs developed by our group (Figure 5.1) with different extents of substitution against another cancer cell line, comparing iodine substituted charge-transfer derivatives to their noniodinated counterparts.

Methylation of BODIPY at the meso phenyl group can enhance the emission quantum yield by reducing non-radiative decay rates, potentially increasing molecular brightness for imaging.^[31] Methyl substitution can increase the lipophilicity and improve membrane permeability, facilitating enhanced cellular uptake of the PS resulting in higher intracellular concentrations and can promote localisation to membrane or lipid droplets.^[32] For example, in methyl-aminolevulinic acid (MAL), a lipophilic derivative of the clinically approved PS 5-aminolevulinic acid (ALA), methylation led to enhanced membrane permeability and overall therapeutic efficacy.^[33]

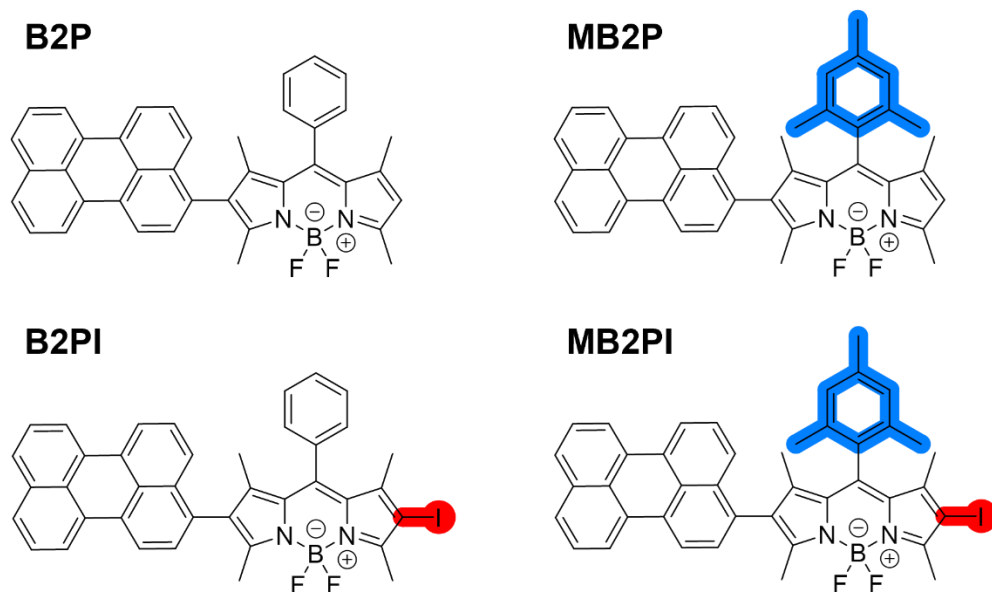
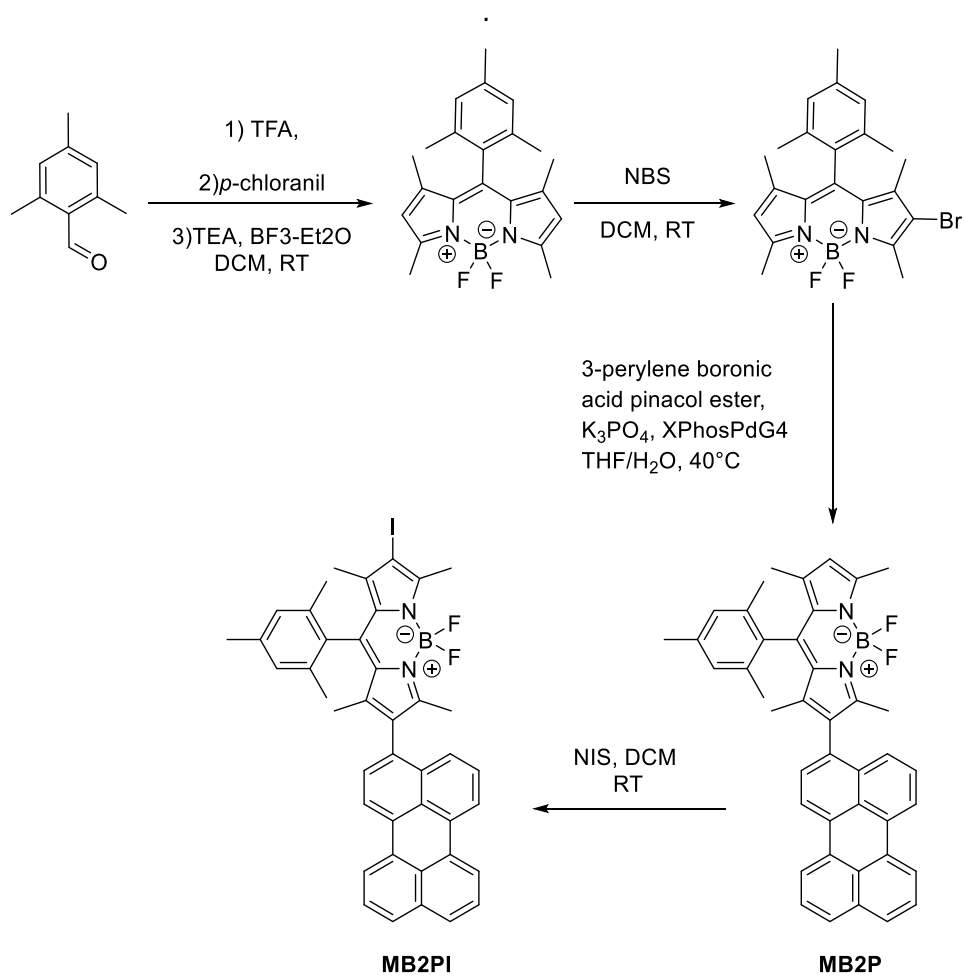


Figure 5.1 Chemical structures of B2P, B2PI, MB2P and MB2PI studied in this work.



Scheme 5.1 Synthetic strategy to prepare MB2P and MB2PI courtesy of Dr. Ruben Arturo Arellano-Reyes where NBS is N-bromosuccinimide and NIS is N-iodosuccinimide.

1,3,5-trimethylbenzene or meso-mesityl substituted BODIPYs, may offer comparable benefits. In the present case, the mesityl group was also introduced to decrease the non-radiative relaxation pathways and improve charge-transfer character of the excited state. We compare the phototoxic effects of the iodinated BODIPY compounds B2PI with MB2PI and their noniodinated analogues in MCF-7 cells, a commonly studied breast cancer cell line, and CHO (Chinese Hamster Ovary) non-cancer cells.

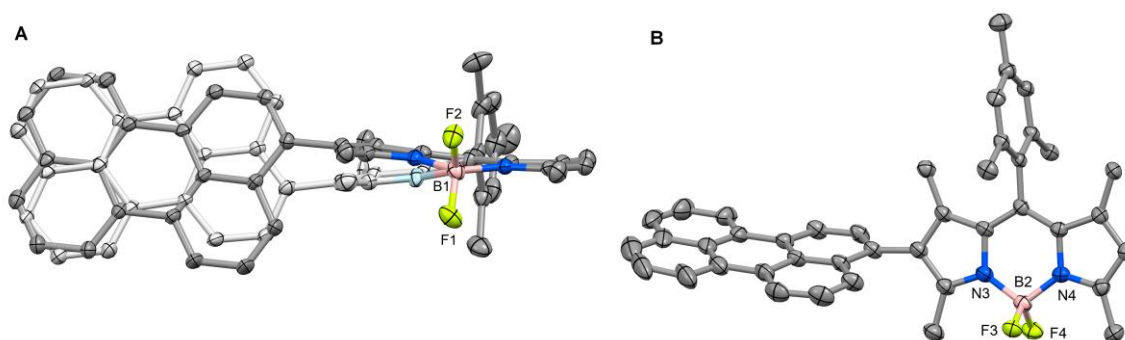


Figure 5.2 (A) MB2P molecule 1, showing disordered perylene ring (minor component shown with paler colours) and **(B)** MB2P molecule 2. Hydrogen atoms omitted for clarity, 30% probability ellipsoids. Courtesy of Dr. James N McPherson and Prof. Vickie McKee.

While BODIPY-derivatives have been widely explored as PSs in PDT, their *in vitro* evaluation has mainly focused on 2D cell culture. 3D spheroid models provide a much closer mimic of the tumour environment for PS including penetration, distribution, and phototoxic effects, but are much less commonly employed.^[34] Campbell *et al.* investigated a tellurophene-appended BODIPY, which showed high phototoxicity in HeLa spheroids (525 nm, 23.60 mW/cm², 5 min)^[35], Ballestri and Marras *et al.* tested the phototoxicity of their free and poly-methyl-methacrylate-bounded BODIPYs in HCT116 colon cancer and MCF-7 spheroids^[36] and Schneider *et al.* grew HeLa spheroids to evaluate their BODIPY-based PSs which showed promise as agents for photothermal and photodynamic therapies.^[37]

In this contribution, we report on the synthesis and spectroscopy of mesylated BODIPY-peryene derivatives extending the family of BODIPY-peryene derivatives with and without iodination. Strategies using charge-transfer and iodination have been exploited to enhance phototoxicity of BODIPY sensitizers, but there has been limited exploration in combining both approaches to amplify phototoxic effects. Thus, we examine and compare uptake, distribution, dark and phototoxicity of the derivatives in MCF-7 (dsmz, ACC-155) and CHO cells with the associated compounds without substitution. The phototoxic potential of the family of probes was assessed under normoxic and hypoxic (MB2PI) conditions, and phototoxic index (PI) values determined. The mechanism of

phototoxicity was determined using $^1\text{O}_2$ scavenger DPBF in solution and ROS dye H_2DCFDA *in cellulose*. Cellular uptake of the BODIPY-erylene derivatives was further confirmed in MCF-7 spheroids by confocal imaging and toxicity was assessed using plate-based assays.

5.3 Results and Discussion

5.3.1 Synthesis and Photophysical Characterisation

The family of BODIPY-erylene derivatives studied, B2P, B2PI, MB2P and MB2PI, are shown in Figure 5.1. MB2P and MB2PI were prepared by Dr. Ruben Arturo Arellano-Reyes according to Scheme 5.1, while the synthesis and photophysical data for B2P and B2PI were reported recently.^[25,26]

5.3.2 Crystallography

Crystallography can offer valuable insight into the relative orientation of donor and acceptor moieties in SOCT-ISC. Crystals of $\text{MB2P}\cdot\text{CHCl}_3$ were obtained for crystallography by vapour diffusion by Dr. Ruben Arturo Arellano-Reyes and crystallography data was obtained and analysed by Dr. James N. McPherson and Prof. Vickie McKee. The asymmetric unit (Figure S5.11) contains two independent $\text{C}_{42}\text{H}_{35}\text{BF}_2\text{N}_2$ molecules and some disordered chloroform solvate. Figure 5.2A shows molecule 1, in which the perylene group is disordered by rotation of 180° about the C–C bond linking it to the BODIPY section, and the site occupancies are refined to 0.74 and 0.26 for the major and minor components, respectively. A slight displacement of the linked 5-membered ring allows a close spatial overlap of the two perylene components. The second molecule (Figure 5.2B) is not significantly disordered. The conformations of the two molecules are similar and the small differences (Figure S5.15) can probably be ascribed to intermolecular interactions in the solid state. In each case the mean plane of the perylene group is rotated with respect to the mean plane of the BODIPY core, these interplanar angles are $69.8(1)^\circ$ and $73.5(3)^\circ$ for the major and minor components of molecule 1 and $73.23(6)^\circ$ for molecule 2. These values are similar to the equivalent interplanar angles for the two independent molecules in the structure of B2P ($73.54(3)^\circ$ and $74.51(4)^\circ$).^[26] The lower value for the major component of molecule 1 may be a consequence of p-p stacking (Figure S5.16).

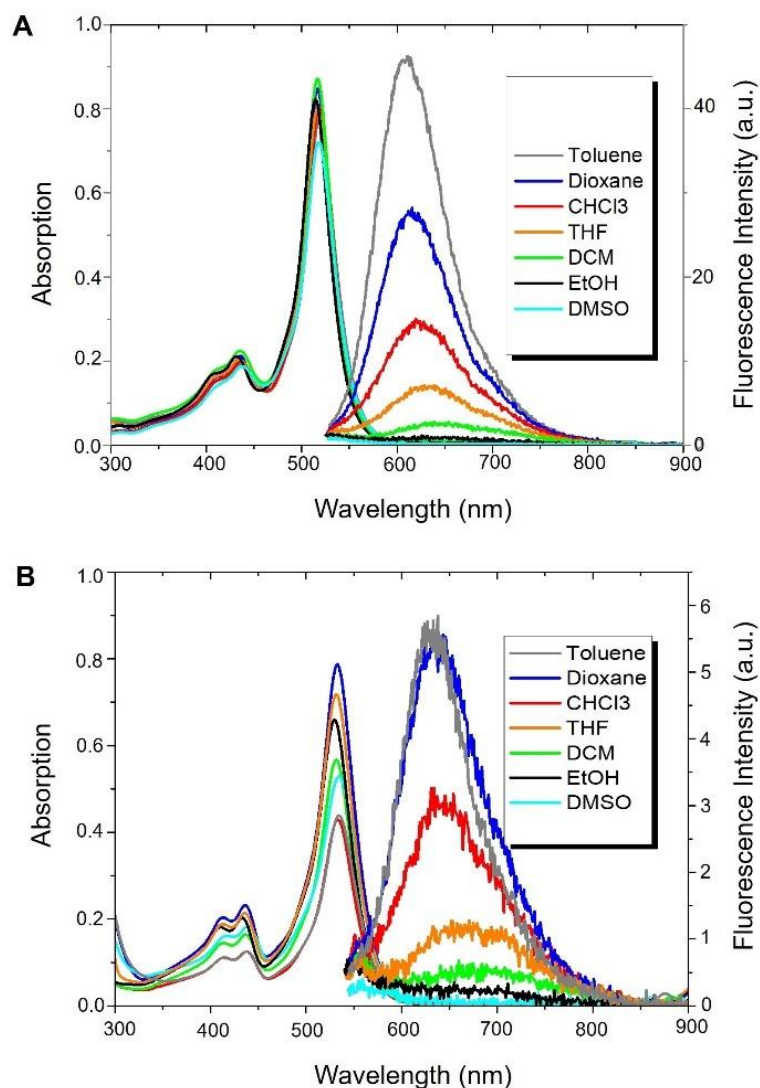


Figure 5.3 Right, Absorption spectra of **(A)** MB2P and **(B)** MB2PI in different solvents at 10 μ M, and, left, their respective emission. Excitation wavelength 532 nm, data recorded with 2.5-2.5 nm slit widths. Courtesy of Dr. Ruben Arturo Arellano-Reyes.

Table 5.1 Photophysical properties of B2P, B2PI, MB2P and MB2PI in 1,4-dioxane and toluene.

	Solvent	λ_{abs} (nm)	λ_{em} (nm)	Stokes Shift (cm ⁻¹)	ϵ (M ⁻¹ cm ⁻¹)	$\Phi_F^{[a]}$	Mol. Bright	τ_1 (ns)	Amp (%)	τ_2 (ns)	Amp (%)	τ_{ave}^b (ns)	$k_r^{[c]}$ (10 ⁷ s ⁻¹)	$k_{nr}^{[c]}$ (10 ⁷ s ⁻¹)
B2P ^[a]	Toluene	518	616	3071	80400	0.21	16884	2.5 $\pm$ 0.0	---	---	---	2.5 $\pm$ 0.01	2.6	79
	Dioxane	515	625	3417	73200	0.09	6588	4.1 $\pm$ 0.2	5.3	1.8 $\pm$ 0.0	94.7	2.0 $\pm$ 0.0	4.6	44
B2PI ^[a]	Toluene	534	636	3003	63300	0.03	1899	2.9 $\pm$ 0.1	7.2	0.7 $\pm$ 0.01	92.8	1.2 $\pm$ 0.0	8.5	32
	Dioxane	531	641	3232	67600	0.03	2028	3.8 $\pm$ 0.1	94.6	0.6 $\pm$ 0.01	5.4	1.4 $\pm$ 0.01	1.8	68
MB2P	Toluene	517	612	3002	89300	0.36	32148	2.8 $\pm$ 0.0	---	---	---	2.8 $\pm$ 0.0	12.9	23
	Dioxane	515	614	3131	89200	0.23	20516	3.1 $\pm$ 0.1	---	---	---	3.1 $\pm$ 0.1	7.4	25
MB2PI	Toluene	534	630	2854	72600	0.09	6534	1.6 $\pm$ 0.1	5.6	0.7 $\pm$ 0.01	94.4	0.8 $\pm$ 0.0	11.2	114
	Dioxane	532	635	3049	72900	0.06	4374	4.0 $\pm$ 0.1	2.5	0.7 $\pm$ 0.01	97.5	1.1 $\pm$ 0.0	5.73	85

^[a] B2P and B2PI data previously published^[26] ^[b] Absolute quantum yields were obtained in an integrating sphere and have a $\pm$ 2% error ^[c] Radiative decay rates calculated using $k_r = \Phi_F / \tau_{ave}$. Non radiative decay estimated from $k_{nr} = (1 - \Phi) / \tau_{ave}$. Intensity weighted average lifetime. Amp = Amplitude.

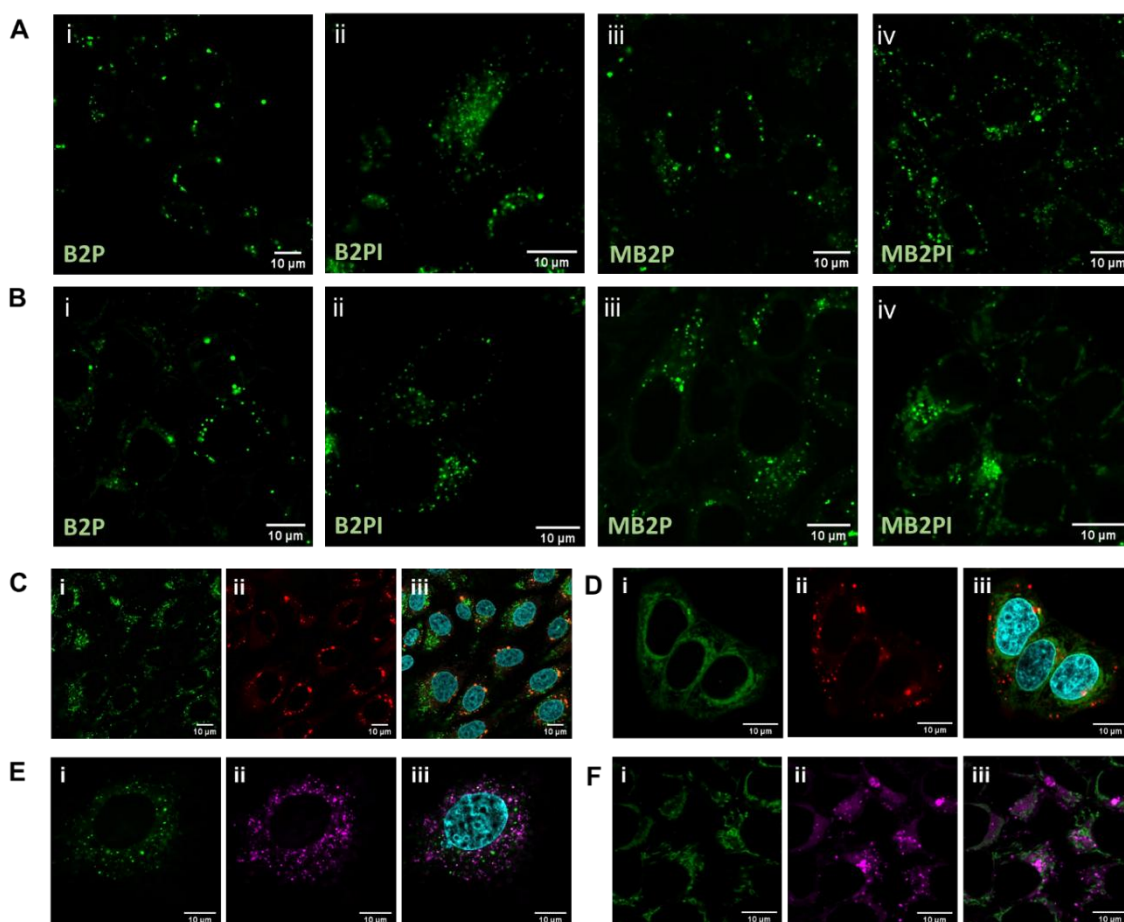


Figure 5.4 Confocal images of **(A)** CHO and **(B)** MCF-7 cells with 10 μ M **(i)** B2P and **(ii)** B2PI and 30 μ M **(iii)** MB2P and **(iv)** MB2PI after a 17 h incubation at 37 $^{\circ}$ C. The λ_{max} of each BODIPY was selected for excitation and emission was collected between 550-700 nm. Co-localisation imaging of BODIPY 493/503 with **(C)** MB2P and **(D)** MB2PI and LysoTracker Deep Red with **(E)** MB2P and **(F)** MB2PI are shown where BODIPY 493/405 is indicated in **(ii)** imaging in red and LysoTracker Deep red is shown in **(ii)** in magenta. Hoechst 33342 indicates the nucleus in C, D and E **(iii)**. Cells were washed twice with PBS and imaged in RPMI (MCF-7) or DMEM/F-12 Ham (CHO) media without phenol red, supplemented with 10% FBS and 1% Penicillin/Streptomycin.

5.3.3 Absorption and Emission Properties

The absorption and emission spectra of MB2P and MB2PI are shown in Figure 5.3, in a range of solvents across polarities. The optical and photophysical data for the complete family of compounds in toluene and dioxane are summarised in Table 5.1. The absorption maxima are centred at approximately 515 nm (MB2P) and 532 nm (MB2PI) in dioxane and are very similar to those of B2P and B2PI, reported previously to exhibit maxima at 515 nm and 531 nm in dioxane. The absorption maxima is largely unaffected by solvent, similar to B2P/B2PI. The extinction coefficient is also modestly affected for MB2P but does vary with solvent for MB2PI, although the change is not correlated with solvent polarity. All compounds also show characteristic perylene absorption features between 400 and 430 nm. Also consistent with this previous study, the emission spectra are strongly solvent dependent. Intensely emissive from non-polar media, MB2P and

MB2PI are minimally emissive in polar (ethanol and DMSO) and aqueous media, (with 10% DMSO for solubilisation). For example, under matched absorption conditions used to generate Figure 5.3, the emission intensity from water was 0.13% (MB2P) and 1.6% (MB2PI) of the emission intensity from toluene (see Figure S5.20).

Based on both DFT calculations reported for B2P/B2PI, and the observation of perylene-BODIPY cation-anion radical pair in femtosecond spectroscopy of these compounds, the absorbance and emission are similarly ascribed to perylene-to-BODIPY charge-transfer transitions, which accounts for the solvent dependent emission behaviour.^[26,38] The compounds also all show large emission Stokes shifts, of approximately 3000 cm⁻¹. While behaviour is very similar across B2P/B2PI and MB2P/MB2PI, the latter show modest blue shifts in their emission maxima, and MB2P/MB2PI show significant increases in quantum yields compared to B2P/B2PI. Correspondingly, as shown in Table 5.1 the molecular brightness, an important parameter in imaging probes, is dramatically improved by methylation of the meso phenyl group and the impact is greatest for the iodinated compounds where mesitylation results in close to 30x increase in molecular brightness for MB2PI compared to B2PI in both toluene and dioxane. The affects are likely attributed to reduced rotational degrees of freedom due to methyl substitution of the meso phenyl group and this is reflected in reduced k_{nr} for MB2P in Table 5.1, but not MB2PI where k_{nr} increases are likely reflective of increased triplet population.

Fluorescence lifetimes are within expected ranges for BODIPY derivatives and are comparable between B2P and MB2P. Notably, as previously reported for B2PI, MB2PI exhibits biexponential fluorescence decays in both dioxane and toluene, whereas MB2P shows monoexponential decay in both media. Deaeration, within experimental error, exerts no influence on the emission lifetimes of the compounds. The short-lived component of the decay at 0.7 ns, dominates for MB2PI in both solvents and while the origin of this component is unclear similar behaviour was noted in B2P. Therein, it was suggested that different rotamers may be at play. In the case of the MB2P derivatives the rotational degrees of freedom are expected to be confined to the perylene moiety as the methylated phenyl group at the meso position is expected to be rotationally locked.^[39,40] Therefore, it is likely, as reported for B2P/B2PI, that the complexity of response for the iodinated species may arise from solvent influence on population and lifetime of the triplet state.^[25]

5.3.4 Cellular Uptake and Localisation

The intracellular uptake of the BODIPY derivatives was assessed in two mammalian cell lines, CHO (non-cancer) and MCF-7 (cancer). Figure 5.4 shows representative confocal microscopy. B2P and B2PI were studied following 4 h and 17 h incubation in MCF-7 cells with 10 μ M of each BODIPY derivative. A 17 h incubation was selected to allow for adequate cell uptake of the complex, ensuring sufficient brightness for confocal imaging while minimising cell toxicity. MB2P and MB2PI were evaluated under the same conditions but interestingly, despite their greater molecular brightness, the emission intensity from within cells from this complex was too low for imaging at a 10 μ M concentration, this may be due to poorer uptake or distribution of the compound into aqueous regions where it does not emit strongly. However, at a higher concentration of 30 μ M used across all subsequent studies the compounds showed strong uptake and emission from within cells.

Across all four compounds uptake of the BODIPYs led to extremely bright and highly punctate staining from within the cells. As all of the BODIPY-perylene probes emit minimally from aqueous media including cell culture media (Figure S5.20) the bright emission is attributed to localisation of the complex to non-aqueous domains, i.e. organelle membranes or to lipid droplets (LDs). To identify which, co-localisation studies with targeted probes were completed. CHO and MCF-7 cells were treated with B2P, B2PI, MB2P or MB2PI and co-stained with specific organelle markers including LD probes; PyLa-C17Cer and BODIPY 493/503, an endoplasmic reticulum stain; ER Tracker Blue-White and a lysosomal stain; LysoTracker Deep Red. PyLa-C17Cer was previously published and localises in both the ER and LDs.^[41] Cellular uptake of B2PI was recently reported in MCF-7 cells, though localisation was not explored.^[25] Here, we found that B2P, B2PI and MB2P localise strongly to the LDs of both test cell lines where the highest Pearson's coefficients were observed for B2P at 0.78 ± 0.09 and 0.69 ± 0.03 with PyLa-C17Cer and BODIPY 493/503 respectively.

MB2PI also accumulates in LDs of MCF-7 cells (Pearson's coefficient = 0.49 PyLa-C17Cer / 0.45 BODIPY 493/503), but its accumulation was comparatively reduced in CHO cells where the Pearson's coefficient for LD probe was <0.3 , it tended instead, as reflected in Table S5.2, to show preference for the ER and lysosome in this cell line. Indeed, all the BODIPY-perylene dyes tested entered the lysosomes of MCF-7 cells to varying extents, however only B2PI, MB2P and MB2PI localise in the lysosomes of CHO cells. Finally, B2P showed no affinity for the ER in either test cell line which is not surprising based on its highly punctate staining. A Pearson's coefficient of ca 0.5 was observed for B2PI, MB2P and MB2PI with ER Tracker in CHO cells and MB2P and MB2PI in MCF-7 cells. The co-localisation results are summarised in Table S5.2.

Lysosomes are rational targets for PDT agents as they play an essential role in cellular homeostasis and photosensitised disruption of their function has been demonstrated to lead to cell death, for example Hu *et al.* reported such an outcome with a lysosomal targeted Aza-BODIPY.^[42] LDs have also been reported as cogent targets for PDT, for example Tabero *et al.* were the first to report targeting LDs for photodynamic theragnosis, a strategy combining bioimaging and PDT.^[43] LDs are closely linked with the ER and emerging evidence indicates that damage to the ER and/or LDs can induce cancer cell death through an ER-stress mediated process. The ER/LD relationship has been exploited by Chauhan *et al.* to design a novel BODIPY-Naphtholimine-BF₂ dyad for treating pancreatic cancer.^[44] Overall, the localisation of the BODIPY-perylene derivatives across the lysosomes, ER and LDs of the test cells is desirable both from an imaging perspective and for PDT.

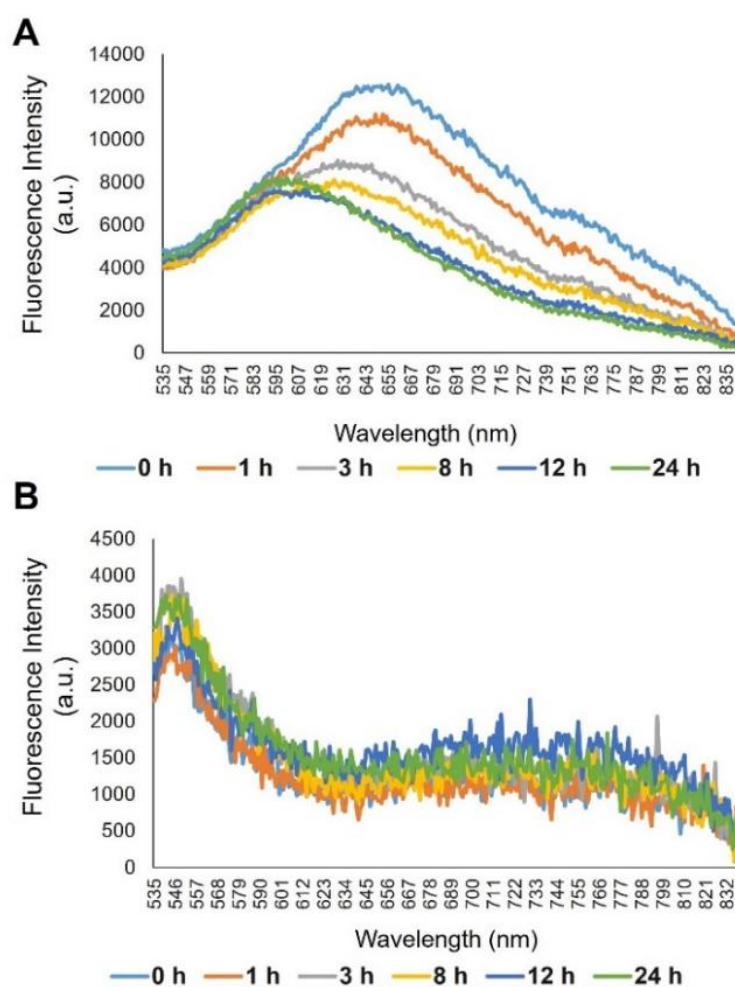


Figure 5.5 Emission spectral response of MB2PI in (A) CHO and (B) MCF-7 cells. At 0 h cells were untreated and at subsequent time points cells were pre-treated with 100 ng/mL TNF- α for 0-24 h prior to the addition of MB2PI (10 μ M, 17 h).

5.3.5 Emission Spectral Response *in cellulo*

Given the different distributions of MB2PI between cell types seen in confocal imaging and the solvent dependence of the probes, we explored the emission spectroscopy of this compound in the live cellular environment and on stimulation of inflammation which is expected to promote lipid droplet formation. Emission spectra were collected from cells incubated for 17 h with MB2PI (after washing and exchanging fresh cell media) and show interesting contrasts in the spectroscopy of MB2PI between the two cell lines. Intense emission was observed centred at 650 nm from MB2PI in CHO with a much weaker emission from MCF7 with peaks evident at approximately 540 and 730 nm profile. The origin of these marked differences is interesting and suggests the sensitivity of the photophysical properties to the cell environment, most likely reflecting the variation in subcellular localisation in each cell type. In CHO cells, MB2PI was localised in lysosomes and the ER, but not in LDs. Whereas in MCF-7 cells, it was found in lysosomes, the ER, and LDs. From solvent studies, in CHO, the data are consistent with a lipophilic environment whereas the emission profile of MCF-7 is similar to that observed in viscous, more protic environments from solvent/viscosity dependent studies. We applied TNF- α as a stimulant for cellular viscosity due to its well-documented role in promoting inflammation and its known effects on cellular processes, including lipid metabolism.^[45,46] TNF- α is known to influence lipid droplet (LD) dynamics by increasing the number and size of lipid droplets within cells. TNF- α incubation times were varied to explore TNF- α -induced changes in LD morphology and this is also expected to influence LD viscosity to which MB2PI may provide a response reflecting inflammatory stimuli. Specifically, cells were pre-treated with 100 ng/mL TNF- α for up to 24 h before adding MB2PI.

MB2PI spectra in CHO cells (Figure 5.5A) showed a slight shift and decreased emission after longer incubations with TNF- α , indicating a potential response to changes in cellular conditions. BODIPY dyes with meso substituents are known to exhibit viscosity-sensitive fluorescence.^[39,47–49] We examined the effect of solvent polarity and viscosity on emission spectroscopy of MB2PI in a toluene-castor oil and glycerol methanol systems as models of highly lipophilic and more protic environments, to see if this might reflect the changes observed on TNF- α treatment *in cellulo*. The mesityl group blocks rotation at the meso position in this compound but the perylene may act as a rotor. In the toluene-castor oil system there was an overall blue shift in emission with increasing viscosity though the changes in intensity were not systematic (Figure S5.21), nonetheless the spectral changes were very similar to TNF- α treated CHO. In the methanol glycerol system (Figure S5.22) the emission profile was notably similar to the emission from MCF-7 cells (Figure 5.5B). Increasing glycerol concentration led to increased emission

intensity but no overall change in emission profile consistent again with the response in MCF7 cells. Therefore, we speculate that the differences in emission profile in each cell type reflect the polarity of the environment the probe is localised to *in cellulo*. As described, MB2PI accumulates in LDs of MCF-7 cells but primarily to the ER and lysosome in CHO. We would expect the compound to penetrate the membraneless LD in the latter case but from the emission spectroscopy it likely resides in the membranes of the lysosome and ER in CHO. The response to TNF- α treatment requires further investigation, however the data suggests that simulation of inflammation affects the viscosity membrane. Overall, the data indicates that the environmental sensitivity of the emission spectroscopy of these probes can facilitate interrogation and dynamic tracking of inflammatory response in live cells.

Table 5.2 IC₅₀ values of CHO and MCF-7 cells (μ M) with and without irradiation

	Cell Line ^a	Dark IC ₅₀ ^b (μ M)	Light IC ₅₀ ^c (μ M)	Photo Index ^d (PI)
B2P	CHO	>50	0.662 $\pm$ 80	>76
	MCF-7	46 $\pm$ 2.38	0.274 $\pm$ 60	>182
	MCF-7 3D		6.91 $\pm$ 0.24	6.6
B2PI	CHO	>50	0.578 $\pm$ 50	>87
	MCF-7	44 $\pm$ 3.84	0.235 $\pm$ 21 ^e	>213
	MCF-7 3D		7.65 $\pm$ 0.23	5.7
MB2P	CHO	>50	0.779 $\pm$ 50	>64
	MCF-7		0.457 $\pm$ 13	>109
	MCF-7 3D		6.26 $\pm$ 2.86	>8
MB2PI	CHO	>50	0.380 $\pm$ 40	>132
	MCF-7		0.229 $\pm$ 7	>218
	MCF-7 3D		5.62 $\pm$ 0.21	>8.9

^a 'MCF-7 3D' indicates MCF-7 spheroids, ^b A maximum of 1% DMSO (50 μ M in 100 μ L cell culture media) was used for toxicity studies ^c Cells were irradiated at 17 $\pm$ 1.64 J/cm² (2.5 mW/cm² for 2 h) and spheroids at 5 $\pm$ 0.54 J/cm² (2.2 mW/cm² for 0.5 h using a 470 nm LED), ^d Phototoxic index (PI) is the ratio of dark IC₅₀ (without irradiation) to light IC₅₀ (irradiated cells), ^e B2PI toxicity in MCF-7 data published previously. ^[25]

5.3.6 Toxicity Studies

The family of BODIPY-perylene probes were found to be minimally toxic in CHO and MCF-7 cells with an IC₅₀ of >50 μ M after a 24 h incubation in the dark at 37 °C. Typical of many BODIPY derivatives, the BODIPY's presented in this work are insoluble in aqueous media, including PBS, and thus the main stock solutions (2-3 mM) were prepared in 100% DMSO and diluted in media immediately before treating the cells. The maximum concentration for dark toxicity was set at 50 μ M to keep the concentration of

DMSO $\leq 1\%$ (v/v) to maintain cell health. As a result, it was not possible to determine an exact dark IC_{50} value as concentrations above 50 μM demanded DMSO concentrations that would affect the viability of the cells. Dietzek-Ivanšić *et al.* previously reported on the effect of DMSO on MCF-7 cell viability, where cell viability remained high (95%) in cells treated with 1% DMSO in cell culture media for 24 h, whereas only 40% of cells remained viable after treatment with 3.8% DMSO. Working stocks in cell culture media remained stable within the incubation times used for cellular studies i.e. up to 24 h.^[25] The phototoxicity of the BODIPY's were assessed to compare the light induced toxicity of the iodinated compounds with their non-iodinated counterparts. CHO and MCF-7 cells were treated with B2P, B2PI, MB2P, and MB2PI in a concentration range from 0.10 to 1 μM for 17 h, the wells were decanted and filled with 100 μL

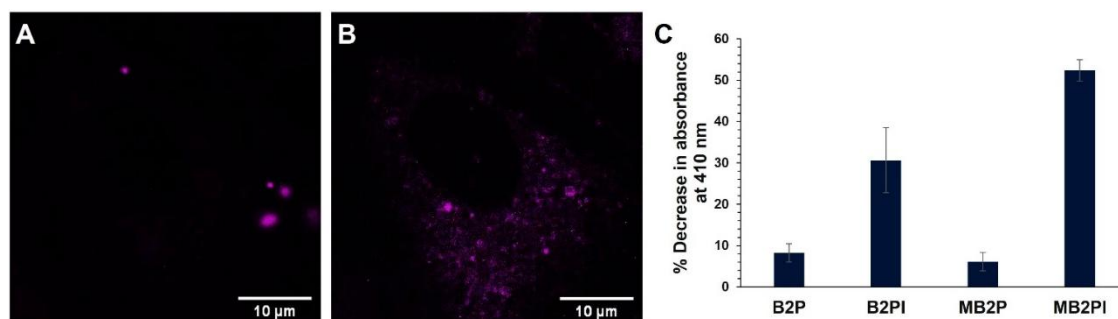


Figure 5.6 ROS dye H_2DCFDA in a live CHO cell **(A)** prior to irradiation and **(B)** after 5 minutes of continuous irradiation confirming ROS generation. Cells were treated with MB2PI prior to irradiation (30 μM , 17 h) and were washed twice with PBS before the addition of H_2DCFDA in cell culture media (RPMI) without phenol red. H_2DCFDA was excited at 494 nm and emission collected between 517-527 nm. A graph of ROS scavenger DPBF response to irradiation of the BODIPY-perylene compounds is shown in **(C)** where a decrease in absorbance at 410 nm confirms $^1O_2$ generation upon photoactivation. Studies were performed with the BODIPY-perylene compounds under absorbance matched conditions. Values shown indicate the % decrease in absorbance values recorded at $t = 0$ and $t = 210$ seconds and is reported as mean $\pm$ standard deviation. Individual results were recorded every 30 seconds from $t = 0$ to 210 seconds and are displayed in Figure S5.32.

phenol red free media prior to irradiation with a 470 nm LED (17 J/cm^2). Cell viability was determined using the alamar blue cell viability assay and % viability was calculated relative to untreated control wells.

All compounds showed excellent phototoxicity, with PI values in CHO cells ranging of >64 (MB2P), >76 (B2P), >87 (B2PI) and >132 (MB2PI). The BODIPY compounds showed slightly higher phototoxicity in MCF-7 cells (IC_{50} values in Table 5.2), however the pattern of least to most phototoxic derivative remained the same where the lowest PI value observed (>109) was in cells treated with MB2P, followed by B2P with a PI value of >182 (B2P). Interestingly, the PI values of B2PI and MB2PI were more similar in MCF-7 cells than CHO at >213 (B2PI) and >218 (MB2PI). A PI value of between 2 and 5 indicates potential phototoxicity and above 5 indicates phototoxicity, and the PI

values reported here surpass this value significantly.^[50] These results indicate that the BODIPY-perylene compounds are potent PSs. Although B2P and MB2P also exhibited high phototoxicity towards the test cells, the substitution of an iodine moiety dramatically enhanced compound phototoxic capacity with B2PI and MB2PI resulting in significantly higher PI values than their non-iodinated counterparts. This was expected since introduction of the iodine group is expected to promote ISC, leading to greater triplet yields and $^1\text{O}_2$ generation. Investigation into the specific molecular interactions and photophysical properties of the BODIPY compounds B2P and B2PI has been published where the findings conclude that the heavy atom effect and SOCT are additive.^[26]

Surprisingly, the addition of a mesityl group to MB2P did not result in enhanced phototoxicity, with higher PI values observed for B2P. Nonetheless, the BODIPY-perylene derivatives reported here, particularly MB2PI, hold promise for potential applications in targeted photodynamic therapy for diseases such as cancer, as exemplified by their effectiveness in MCF-7 and CHO cells.

5.3.7 Mechanism of Phototoxicity

Given the impact of the heavy iodine atom on phototoxicity, to confirm if the origin of toxicity is triplet sensitised formation of $^1\text{O}_2$ we carried out ROS scavenger studies with 1,3-diphenylisobenzofuran (DPBF, Figure 5.6C). Stock solutions of BODIPY-perylene were prepared under absorbance matched conditions and a BODIPY-DPBF solution prepared at concentration of 10 mM DPBF in methanol. The BODIPY-DPBF solution was irradiated (470 nm) from 0-210 seconds and the absorption spectra recorded every 30 seconds. In the presence of $^1\text{O}_2$, DPBF forms an unstable peroxide, endoperoxide 1, that readily decomposes to colourless 1,2-dibenzoylbenzene (DBB).^[51] This change is detected spectrophotometrically with depletion of DPBF absorbance at 410 nm. The DPBF assay results are represented in Figure 5.6C as % decrease in absorbance at 410 nm. The values are determined at $t = 210$ seconds relative to the absorbance values recorded prior to irradiation at $t = 0$. These results correlate well with the phototoxicity data *in cellulo*. As expected, the most phototoxic compound, MB2PI, generates the most $^1\text{O}_2$ upon irradiation with a 470 nm LED with a 52% decrease in DPBF absorbance at 410 nm. This is closely followed by a 31% decrease of DPBF absorbance when irradiated in the presence of B2PI, which also displayed high phototoxicity in the test cells. DPBF is known to be sensitive to light, therefore, to mitigate photodegradation of affecting the assay results, exposure was limited to 3.5 minutes where DPBF absorbance decrease at 410 nm is <10% at this time point. The decrease in absorbance of DPBF alone upon irradiation by the 470 nm LED suggests that B2P and MB2P are

not generating ROS after 3.5 minutes of irradiation, rather the DPBF itself is degrading as a decrease in absorbance of only 8% (B2P) and 6% (MB2P) were observed for these derivatives. Due to the photo-vulnerability of DPBF under visible irradiation, this study is limited by a short experimental window and ignores any ROS generation over longer irradiation periods. It is important to note that although DPBF is highly sensitive to $^1\text{O}_2$, it can also react with other ROS, for example, superoxide anions or hydroxyl radicals.^[51] To study the production of ROS in a live cell environment a commercial ROS dye, H_2DCFDA was used. CHO and MCF-7 cells were incubated with MB2PI (30 μM , 17 h), washed with PBS and co-stained with H_2DCFDA (5 μM , 30 min). To ensure no photoactivation of MB2PI, emission from H_2DCFDA was collected alone prior to the excitation of the BODIPY. The complex was then irradiated continuously at 488 nm (0.38 mW/cm^2) for 5 minutes. As shown in Figure 5.6, irradiating MB2PI results in increased production of ROS in CHO cells with extensive cytoplasmic staining, though no ROS production was observed in the nucleus. Weak emission of H_2DCFDA was observed prior to irradiation, consistent with the low cytotoxicity of the complex. However, after irradiation, the fluorescence intensity of the ROS dye increases dramatically indicating ROS production, consistent with the high phototoxicity of MB2PI in both test cell lines. The higher phototoxicity of MB2PI in MCF-7 cells restricted the ROS imaging study to CHO cells as the MCF-7 cells began to die rapidly upon irradiation. Controls completed by adding H_2DCFDA in test cells without MB2PI confirmed no ROS production with or without irradiation at 488 nm.

As MB2PI was found to be the most phototoxic BODIPY under normoxic conditions, it was selected for further analysis under chemically induced hypoxia to determine the role of oxygen in the phototoxicity of the BODIPYs. Cobalt chloride (CoCl_2), a popular chemical hypoxia inducer, was added to the cells (after incubation with B2PI or MB2PI) at a total concentration of 300 μM CoCl_2 in

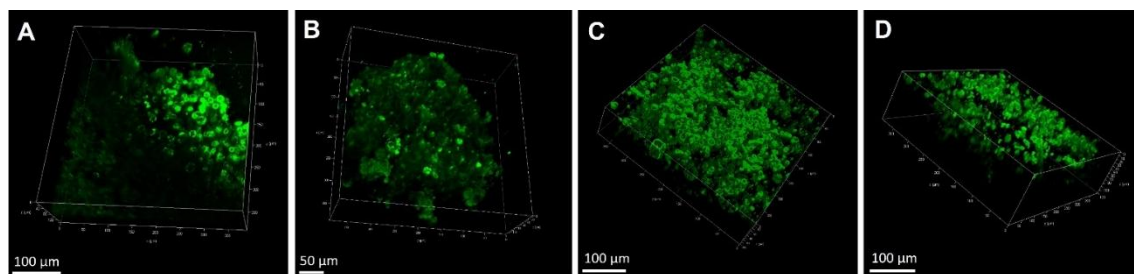


Figure 5.7 3D reconstruction of live MCF-7 spheroids treated with (A) B2P, (B) B2PI, (C) MB2P or (D) MB2PI (50 μM / 17 h). Spheroids were imaged in RPMI media without phenol red, supplemented with 10% FBS and 1% penicillin/streptomycin. Z-stack images were captured from below to above the spheroids and combined to produce the 3D reconstructions. Scale bars read 100 μm (A, C, D) and 50 μm (B).

media and incubated for 4 h to mimic hypoxic conditions. In comparison to low-oxygen induced hypoxia, this method stabilises hypoxia inducible factor 1 alpha subunit (HIF-

1 α) for some hours after treatment, allowing a greater window for the phototoxicity studies.^[52] We have previously confirmed the chemical induction of hypoxia with CoCl₂ using an enzyme linked immunosorbent assay to detect HIF-1 α in cell lysates.^[53] The cells were irradiated immediately upon removal of the media/CoCl₂ solution as per the normoxic phototoxicity protocol. As expected, phototoxicity was significantly reduced under hypoxic conditions, with good cell viability maintained up to our maximum condition of 1 μ M i.e. hypoxic IC₅₀ is >1 μ M, in contrast to the light IC₅₀ values of <0.6 μ M and ca. 0.2 μ M for the BODIPYs tested under normoxic conditions in CHO and MCF-7 cells respectively, confirming the pathway is likely Type II.

5.3.8 3D Cell Studies

To investigate if the uptake of the BODIPY probes carried from cell monolayers to 3D cell models, the distribution of B2P, B2PI, MB2P and MB2PI were assessed in a tumour-like environment. Multicellular tumour spheroids are a popular choice of 3D cell model and provide a more physiologically relevant model to study potential PSs. Spheroids are a superior model of *in vivo* tumour compared to cell monolayers. They comprise a layered organisation with an outer proliferative zone, a senescent or quiescent zone and often a hypoxic or necrotic core which is analogous particularly to metastatic tumours.^[54] Additionally, there are increased cell-to-cell and cell-to-extracellular matrix interactions that produce physical barriers, better reflecting potential drug uptake in a real tumour.^[55] To assess uptake of the BODIPYs in MCF-7 spheroids, the BODIPY-perylene compound were incubated with the spheroids at 50 μ M for 17 h and imaged using a confocal microscope. All the BODIPY derivatives were found to deeply penetrate MCF-7 spheroids as shown in representative imaging in Figure 5.7, where there is widespread distribution of B2P and B2PI throughout the depth of the spheroid. Interestingly, consistent with 2D cell culture, MB2P and MB2PI were not taken up throughout the spheroid as effectively as B2P and B2PI. Consistent also with 2D studies all compounds were nuclear excluded and evident from punctate features widespread in the cytoplasm. Notably, the dyes exhibited strong fluorescence in the spheroids, requiring a lower laser intensity for imaging than cell monolayers. In spite of the evidently lower uptake, phototoxicity studies confirmed MB2PI is the most phototoxic BODIPY-perylene derivative in MCF-7 spheroids, with a PI of >8.9. It is important to note that studies in spheroids were performed at a lower irradiation dose of 5 J/cm², as spheroid viability was affected at 17 J/cm². Generally, PI values are significantly lower in spheroids in comparison to studies in cell monolayers.^[53,56] Nonetheless, the PI values reported are notable and indicate effective phototoxicity in a tumour-like environment by these

compounds. The data also underscore the need for 3D models as an intermediate between studies in cell monolayers and *in vivo* studies. Given that spheroids are an underexplored model for BODIPY applications, the difference in phototoxicity of the BODIPY-perylene derivatives observed in cell monolayers compared to spheroids highlights the importance of further investigation into their use for more accurate and clinically relevant assessments of novel BODIPY-based PSs for PDT.

5.4 Conclusions

The uptake and phototoxicity of a family of BODIPY-perylene charge-transfer compounds was studied. The de-novo synthesis of BODIPY-perylene containing mesitylated units; MB2P and MB2PI is reported, and the crystal structure of MB2P is defined. The photophysics of these compounds, their cellular uptake and phototoxicity in 2D and 3D *in vitro* cell models were studied. All compounds are readily permeable to CHO and MCF-7 cells and localise mainly in the LDs and lysosomes, common localisation for BODIPY derivatives, that is associated with their lipophilicity. All compounds showed excellent phototoxicity. Under 17 J/cm² irradiation (470 nm LED) B2P, B2PI, MB2P and MB2PI provide impressive PI values of >109 (MB2P), >182 (B2P), >213 (B2PI) and >218 (MB2PI) in MCF-7 cells and >64 (MB2P), >76 (B2P), >87 (B2PI) and >132 (MB2PI) in CHO cells. Correlations between the DPBF assays and phototoxicity indices indicate that ROS, most likely ¹O₂ formation, is responsible for this effect in B2PI and MB2PI since functionalisation with iodine increased phototoxicity in both test cell lines. The most toxic complex, MB2PI, with a PI value of >132 and >218 in CHO and MCF-7 cells respectively was almost twice that of its non-iodinated counterpart. These results confirm the combination of charge-transfer and iodination can be employed to promote triplet state formation effectively enhancing phototoxic capacity. Furthermore, phototoxicity and ROS generation by the MB2PI is superior to that of B2PI, likely attributed to the greater fluorescent yield and lifetime of the mesitylated compound. Notably, increased phototoxicity was not associated with increased dark toxicity up to 50 µM, with the IC₅₀ of our iodinated BODIPY's remaining >50 µM.

The ROS generating capability of MB2PI was confirmed *in cellulo* by imaging the cells with ROS dye H₂DCFDA prior and post irradiation. Photoirradiation of MB2PI in cells under hypoxic conditions further confirmed this with a marked decrease in phototoxicity, confirming phototoxicity is oxygen dependent. It is important to note that the reported PI values are likely much higher than calculated as the exact dark IC₅₀ could not be established due to solubility limitations of the dyes in water. Nonetheless, these results highlight the promise of the BODIPY-perylene derivatives presented here as powerful

tools for PDT. Confocal imaging of MCF-7 spheroids confirmed the BODIPY probes can penetrate a 3D tumour model, though MB2P and MB2PI fail to penetrate throughout the entire spheroid. Despite this, nonetheless impressive PI values >8.9 were observed in MCF-7 spheroids, where MB2PI was again found to be the most phototoxic with a PI value of >8.9.

5.5 References

- [1] G. Ulrich, R. Ziessel, A. Harriman, *Angew. Chem. Int. Ed Engl.* **2008**, *47*, 1184–1201.
- [2] T. Kowada, H. Maeda, K. Kikuchi, *Chem. Soc. Rev.* **2015**, *44*, 4953–4972.
- [3] W. Miao, C. Yu, E. Hao, L. Jiao, *Front. Chem.* **2019**, *7*, 825.
- [4] M. M. Ogle, A. D. Smith McWilliams, M. J. Ware, S. A. Curley, S. J. Corr, A. A. Martí, *J. Phys. Chem. B* **2019**, *123*, 7282–7289.
- [5] M. K. Kuimova, G. Yahioglu, J. A. Levitt, K. Suhling, *J. Am. Chem. Soc.* **2008**, *130*, 6672–6673.
- [6] C. Dolan, A. Byrne, C. Long, K. Czamara, A. Kaczor, M. Baranska, T. E. Keyes, *RSC Adv.* **2017**, *7*, 43743–43754.
- [7] P. Kaur, K. Singh, *J. Mater. Chem. C* **2019**, *7*, 11361–11405.
- [8] I. W. Badon, C. Kim, J. M. Lim, D. K. Mai, T. P. Vales, D. Kang, S. Cho, J. Lee, H.-J. Kim, J. Yang, *J. Mater. Chem. B* **2022**, *10*, 1196–1209.
- [9] R. Mori, M. Asakura, Y. Kobayashi, H. Mashimo, M. Roppongi, S. Ito, *Tetrahedron Lett.* **2022**, *96*, 153759.
- [10] D. Pfeifer, A. Russegger, I. Klimant, S. M. Borisov, *Sens. Actuators B Chem.* **2020**, *304*, 127312.
- [11] M. Strobl, T. Rappitsch, S. M. Borisov, T. Mayr, I. Klimant, *Analyst* **2015**, *140*, 7150–7153.
- [12] P. Shrestha, D. Kand, R. Weinstein, A. H. Winter, *J. Am. Chem. Soc.* **2023**, *145*, 17497–17514.
- [13] P. Wu, Y. Zhu, S. Liu, H. Xiong, *ACS Cent. Sci.* **2021**, *7*, 2039–2048.
- [14] S. Das, S. Dey, S. Patra, A. Bera, T. Ghosh, B. Prasad, K. D. Sayala, K. Maji, A. Bedi, S. Debnath, *Biomolecules* **2023**, *13*, 1723.
- [15] M. C. Malacarne, M. B. Gariboldi, E. Caruso, *Int. J. Mol. Sci.* **2022**, *23*, 10198.
- [16] M. A. Filatov, *Org. Biomol. Chem.* **2019**, *18*, 10–27.
- [17] A. Kamkaew, S. H. Lim, H. B. Lee, L. V. Kiew, L. Y. Chung, K. Burgess, *Chem. Soc. Rev.* **2013**, *42*, 10.1039/c2cs35216h.

- [18] H. Liang, S. Sun, M. Zafar, Z. Yuan, Y. Dong, S. Ji, Y. Huo, M.-D. Li, J. Zhao, *Dyes Pigments* **2020**, 173, 108003.
- [19] J. Zhao, K. Xu, W. Yang, Z. Wang, F. Zhong, *Chem. Soc. Rev.* **2015**, 44, 8904–8939.
- [20] A. Prieto-Castañeda, F. García-Garrido, C. Díaz-Norambuena, B. Escriche-Navarro, A. García-Fernández, J. Bañuelos, E. Rebollar, I. García-Moreno, R. Martínez-Máñez, S. de la Moya, A. R. Agarrabeitia, M. J. Ortiz, *Org. Lett.* **2022**, 24, 3636–3641.
- [21] M. Lv, Y. Yu, M. E. Sandoval-Salinas, J. Xu, Z. Lei, D. Casanova, Y. Yang, J. Chen, *Angew. Chem. Int. Ed.* **2020**, 59, 22179–22184.
- [22] Z. Zhang, R. M. Edkins, J. Nitsch, K. Fücke, A. Steffen, L. E. Longobardi, D. W. Stephan, C. Lambert, T. B. Marder, *Chem. Sci.* **2014**, 6, 308–321.
- [23] J. Strahan, B. C. Popere, P. Khomein, C. A. Pointer, S. M. Martin, A. N. Oldacre, S. Thayumanavan, E. R. Young, *Dalton Trans.* **2019**, 48, 8488–8501.
- [24] Z. Wang, M. Ivanov, Y. Gao, L. Bussotti, P. Foggi, H. Zhang, N. Russo, B. Dick, J. Zhao, M. Di Donato, G. Mazzone, L. Luo, M. Fedin, *Chem. – Eur. J.* **2020**, 26, 1091–1102.
- [25] T. Yang, R. A. Arellano-Reyes, R. C. Curley, K. K. Jha, A. Chettri, T. E. Keyes, B. Dietzek-Ivanšić, *Chem. – Eur. J.* **2023**, 29, e202300224.
- [26] R. A. Arellano-Reyes, A. Prabhakaran, R. C. E. Sia, J. Guthmuller, K. K. Jha, T. Yang, B. Dietzek-Ivanšić, V. McKee, T. E. Keyes, *Chem. – Eur. J.* **2023**, 29, e202300239.
- [27] J. T. Ly, K. F. Presley, T. M. Cooper, L. A. Baldwin, M. J. Dalton, T. A. Grusenmeyer, *Phys. Chem. Chem. Phys.* **2021**, 23, 12033–12044.
- [28] M. J. Ortiz, A. R. Agarrabeitia, G. Duran-Sampedro, J. Bañuelos Prieto, T. A. Lopez, W. A. Massad, H. A. Montejano, N. A. García, I. Lopez Arbeloa, *Tetrahedron* **2012**, 68, 1153–1162.
- [29] T. Yogo, Y. Urano, Y. Ishitsuka, F. Maniwa, T. Nagano, *J. Am. Chem. Soc.* **2005**, 127, 12162–12163.
- [30] M. Liu, C. Wang, Y. Qian, *New J. Chem.* **2021**, 45, 18082–18089.
- [31] J. Song, Y. Xue, S. Wang, Z. Zhang, S. Yang, H. Yang, C. Qin, *Mol. Phys.* **n.d.**, e2371554.
- [32] C. A. Lipinski, F. Lombardo, B. W. Dominy, P. J. Feeney, *Adv. Drug Deliv. Rev.* **2001**, 46, 3–26.
- [33] M. Wachowska, A. Muchowicz, M. Firczuk, M. Gabrysiak, M. Winiarska, M. Wańczyk, K. Bojarczuk, J. Golab, *Molecules* **2011**, 16, 4140–4164.

- [34] P. M. R. Pereira, N. Berisha, N. V. S. D. K. Bhupathiraju, R. Fernandes, J. P. C. Tomé, C. M. Drain, *PLoS ONE* **2017**, *12*, e0177737.
- [35] J. W. Campbell, M. T. Tung, R. M. Diaz-Rodriguez, K. N. Robertson, A. A. Beharry, A. Thompson, *ACS Med. Chem. Lett.* **2021**, *12*, 1925–1931.
- [36] M. Ballestri, E. Marras, E. Caruso, F. Bolognese, M. C. Malacarne, E. Martella, M. Tubertini, M. B. Gariboldi, G. Varchi, *Cancers* **2022**, *15*, 92.
- [37] L. Schneider, M. Kalt, S. Koch, S. Sithamparanathan, V. Villiger, J. Mattiat, F. Kradolfer, E. Slyshkina, S. Lubner, M. Bonmarin, C. Maake, B. Spingler, *J. Am. Chem. Soc.* **2023**, *145*, 4534–4544.
- [38] K. Kumar Jha, A. Prabhakaran, R. Cane Sia, R. A. Arellano Reyes, N. Kumar Sarangi, T. Yang, K. Kumar, S. Kupfer, J. Guthmuller, T. E. Keyes, B. Dietzek-Ivanšić, *ChemPhotoChem* **2023**, *7*, e202300073.
- [39] A. Vyšniauskas, I. López-Duarte, N. Duchemin, T.-T. Vu, Y. Wu, E. M. Budynina, Y. A. Volkova, E. P. Cabrera, D. E. Ramírez-Ornelas, M. K. Kuimova, *Phys. Chem. Chem. Phys.* **2017**, *19*, 25252–25259.
- [40] T. Truc Vu, R. Méallet-Renault, G. Clavier, B. A. Trofimov, M. K. Kuimova, *J. Mater. Chem. C* **2016**, *4*, 2828–2833.
- [41] D. O Connor, A. Byrne, G. B. Berselli, C. Long, T. E. Keyes, *The Analyst* **2019**, *144*, 1608–1621.
- [42] M. Hu, X. Dong, W. Zhao, *Bioorg. Med. Chem.* **2024**, *99*, 117583.
- [43] A. Tabero, F. García-Garrido, A. Prieto-Castañeda, E. Palao, A. R. Agarrabeitia, I. García-Moreno, A. Villanueva, S. de la Moya, M. J. Ortiz, *Chem. Commun.* **2020**, *56*, 940–943.
- [44] N. Chauhan, M. Koli, R. Ghosh, A. G. Majumdar, A. Ghosh, T. K. Ghanty, S. Mula, B. S. Patro, *JACS Au* **2024**, jacsau.3c00539.
- [45] K. Czamara, K. Majzner, A. Selmi, M. Baranska, Y. Ozaki, A. Kaczor, *Sci. Rep.* **2017**, *7*, 40889.
- [46] X. Chen, K. Xun, L. Chen, Y. Wang, *Cell Biochem. Funct.* **2009**, *27*, 407–416.
- [47] E. M. Woodcock, P. Girvan, J. Eckert, I. Lopez-Duarte, M. Kubánková, J. J. W. A. van Loon, N. J. Brooks, M. K. Kuimova, *Biophys. J.* **2019**, *116*, 1984–1993.
- [48] R. S. Ehrlich, S. Dasgupta, R. E. Jessup, K. L. Teppang, A. L. Shiao, K. Y. Jeoung, X. Su, A. Shivkumar, E. A. Theodorakis, F. Paesani, J. Yang, *J. Phys. Chem. B* **2024**, *128*, 3946–3952.
- [49] X. Liu, W. Chi, Q. Qiao, S. V. Kokate, E. P. Cabrera, Z. Xu, X. Liu, Y.-T. Chang, *ACS Sens.* **2020**, *5*, 731–739.
- [50] **2018**.
- [51] T. Entradas, S. Waldron, M. Volk, *J. Photochem. Photobiol. B* **2020**, *204*, 111787.

- [52] J. Muñoz-Sánchez, M. E. Cháñez-Cárdenas, *J. Appl. Toxicol.* **2019**, 39, 556–570.
- [53] L. Holden+, R. C. Curley+, G. Avella, C. Long, Keyes, Tia, *Angew. Chem. Int. Ed.* **2024** e202408581.
- [54] B. Pinto, A. C. Henriques, P. M. A. Silva, H. Bousbaa, *Pharmaceutics* **2020**, 12, 1186.
- [55] K. Poornima, A. P. Francis, M. Hoda, M. A. Eladl, S. Subramanian, V. P. Veeraraghavan, M. El-Sherbiny, S. M. Asseri, A. B. A. Hussamuldin, K. M. Surapaneni, U. Mony, R. Rajagopalan, *Front. Oncol.* **2022**, 12, 891673.
- [56] R. C. Curley, C. S. Burke, K. S. Gkika, S. Noorani, N. Walsh, T. E. Keyes, *Inorg. Chem.* **2023**, 62, 13089–13102.
- [57] Rigaku Oxford Diffraction, **2023**, CrysAlisPro Software system, version 1.171.43.101a, Rigaku Corporation, Oxford, UK.
- [58] C. B. Hübschle, G. M. Sheldrick, B. Dittrich, *J. Appl. Crystallogr.* **2011**, 44, 1281–1284.
- [59] O. V. Dolomanov, L. J. Bourhis, R. J. Gildea, J. a. K. Howard, H. Puschmann, *J. Appl. Crystallogr.* **2009**, 42, 339–341.
- [60] G. M. Sheldrick, *Acta Crystallogr. Sect. A Found. Adv.* **2015**, 71, 3–8.
- [61] G. M. Sheldrick, *Acta Crystallogr. Sect. C Struct. Chem.* **2015**, 71, 3–8.

Chapter 6 : Conclusions and Future Perspectives

The charge-transfer compounds presented in this thesis offer a foundation for future investigation into new and improved theranostic probes. A primary objective of this work was to assess the potential of novel multi-modal photosensitisers that could harness the benefits associated with charge-transfer processes *in cellulo* for applications in cellular imaging and phototherapy including photodynamic therapy and photoactivated chemotherapy.

Confocal microscopy studies confirmed the complexes were readily taken up into live cells, localising in the cytoplasm and within specific cellular organelles. For example, Chapter 2 reported a family of ruthenium complexes taken up rapidly into cells by passive diffusion, resulting in cytoplasmic staining. The Ru-RHAU complex described in Chapter 3 was taken up into lysosomes and stress granules (SGs), Ru-TAP-PDC3 (Chapter 4) targeted the mitochondria and the BODIPY-perylene compounds in Chapter 5 were taken up across lipid droplets, lysosomes and the endoplasmic reticulum. Throughout the past decade, luminescent metal complexes have gained attention as imaging probes for super-resolution microscopy including stimulated emission depletion (STED) microscopy and the Ru-TAP-PDC3 complex described in Chapter 4 was studied as a STED probe, where enhanced resolution was observed in comparison to confocal.

The phototoxicity of all the charge-transfer compounds reported in this thesis were studied in cell monolayers and multicellular tumour spheroids, with the exception of Ru-RHAU which was tested in monolayers only with a focus on imaging rather than therapeutic potential. All of the test compounds were found to be highly phototoxic under normoxic conditions. Chapter 2 presented a ruthenium parent complex Ru-bqp-ester and its peptide conjugates Ru-bqp-MPP and Ru-bqp-R8 which exhibited exceptional phototoxicity in HPAC cell monolayers. Ru-bqp-ester was particularly impressive with a PI value of 760 under 17 J/cm² irradiation. The PI values reported in Chapter 2 are significant in comparison to many Ru(II) polypyridyl complexes reported in cell monolayers in the literature.

The phototoxicity of a novel ruthenium probe, Ru-TAP-PDC3 was reported in Chapter 4. This is the first ruthenium TAP probe reported to target mitochondrial G4s and showed great phototherapeutic potential under both normoxic and hypoxic conditions. At an irradiation dose of 15 J/cm² normoxic and hypoxic PI values of >170 and >13 respectively were observed after a 24 h incubation with the complex. Probes with functionality under normoxic and hypoxic conditions can address the challenge of

phototherapeutic efficacy in hypoxic tumour environments, where traditional PDT is less effective. However, as mentioned in Chapter 1, the development of novel PACT agents that are solely PACT agents and not a combination of PDT/PACT are essential for the future.

Additionally, Chapter 5 focuses on the combination of charge-transfer and heavy atom effects to enhance the phototoxicity of BODIPY-perylene dyads. A distinct increase in the PI values of the iodinated BODIPYs in comparison to their non-iodinated counterparts in both CHO and MCF-7 cells confirmed that iodination can be used to enhance the phototherapeutic capacity of a BODIPY-based photosensitiser. Though both charge-transfer and the heavy atom effects have been reported separately, research has been limited into the combination of the two for enhanced phototoxicity and the results reported in Chapter 5 report the benefit of a joint approach.

The toxicity studies performed in multicellular tumour spheroids in Chapter 2, 4 and 5 highlighted a consistent decrease in PI values when compared to cell monolayers of the same cell line (notwithstanding a lower irradiation dose in some cases). The results reflect the importance of incorporating an intermediate cell model before moving to studies *in vivo* to confirm the phototherapeutic potential of a novel compound carries through from cell monolayers to more physiologically relevant cellular models. 3D cellular models are becoming increasingly popular and future research will likely prioritise their use to bridge the gap between cell monolayer and *in vivo* animal studies.

The entry of the first Ru(II) complex, TLD-1433, into clinical trials for PDT has encouraged further efforts to design and develop charge-transfer compounds as phototherapeutics. As reported in Chapter 3, bioconjugation to a RHAU peptide can transport a ruthenium complex into SGs. The link between RNA G4s (rG4s) and SGs is an underexplored area of research and rG4 targeting presents a promising option for studying SG dynamics in live cells with the potential to provide a better understanding of how SGs contribute to therapeutic resistance. Moreover, the preliminary studies reported in Chapter 3 indicate SGs could be a useful target for PDT. In addition to their link to cancer progression and drug resistance, SGs have also been reported to play a role in neurological disorders such as Alzheimer's disease. The Ru-RHAU complex reported in Chapter 3 could prove to be an effective probe for studying SGs in live neuronal cells to better understand their dynamics in this context.

Further applications of these compounds are in cellular sensing. This thesis briefly touches on the viscosity sensing potential of BODIPY molecular rotors. This is an emerging area of research and the development of multi-modal photosensitisers with the

ability to sense viscosity changes in real-time could advance diagnostic or treatment strategies for PDT patients.

The future work to emerge from this thesis will focus on precision targeted charge-transfer compounds for cellular imaging, sensing and phototherapy. As described in Chapter 1, an area which has received attention in recent years is the development of multi-modal probes with enhanced specificity through conjugation with targeting peptides. A promising direction is the development of probes that can selectively bind to guanine quadruplexes (G4s), by bioconjugation to G4-targeting peptides. Another avenue to precision targeted theranostics involves conjugation of novel probes to peptides that target cancer-specific biomarkers, such as CD44 or fatty acid synthase (FASN), which are often overexpressed in cancer cells.

In conclusion, the synthetic versatility and multi-modal applications of luminescent transition metal complexes and BODIPY-based charge-transfer compounds, along with their desirable photophysical properties, position them as promising candidates for the development of novel phototherapeutics in the future.

Appendix A: Supplementary Information - Chapter 2

S2.1 Experimental

S2.1.1 Materials

All chemicals and reagents, DMEM/F-12 Ham, RPMI and DMEM cell culture media, and corresponding components were purchased from Sigma-Aldrich and were used as received. Phenol red free media (Gibco), fetal bovine serum (FBS, heat-inactivated, Gibco), alamarBLUE reagent (Invitrogen) and co-localising dyes were purchased from ThermoFisher Scientific. For synthesis, Anion metathesis using tetrabutylammonium chloride in acetone provided the chloride salts from their hexafluorophosphate salts. Peptides were purchased from Celtek Peptides (USA) at >95 % purity. All other materials were obtained from Sigma-Aldrich (Merck) or Fluorochem (UK) and were used without further purification.

S2.1.2 Synthesis

The syntheses of bqpCOOEt , $\text{mer-[Ru(bqp)(CH}_3\text{CN)}_3\text{](PF}_6\text{)}_2$, $\text{mer-[Ru(bqp)(bqpCOOH)](PF}_6\text{)}_2$ and $\text{mer-[Ru(bqp)(bqpCOOEt)](PF}_6\text{)}_2$ have been described previously (references 57-59, chapter 2). In this work, an alternate route to $\text{mer-[Ru(bqp)(bqpCOOH)](PF}_6\text{)}_2$ and $\text{mer-[Ru(bqp)(bqpCOOEt)](PF}_6\text{)}_2$ was developed (see below). Structure and purity were confirmed by NMR and Mass Spectra data. ^1H NMR spectra were recorded at 400 or 600 MHz as indicated using Bruker spectrometers and deuterated solvent for homo-nuclear lock. The spectra were processed using Bruker Topspin software and were calibrated against solvent peaks according to published values. High Resolution Mass Spectrometry (HR-MS) was performed at the HR-MS facility, Trinity College Dublin (parent complexes); or the Mass Spectrometry Facility, NUI Maynooth (conjugates).

Synthesis of $\text{mer-[Ru(bqp)(bqp-COOH)](PF}_6\text{)}_2$ (Ru-bqp-acid)

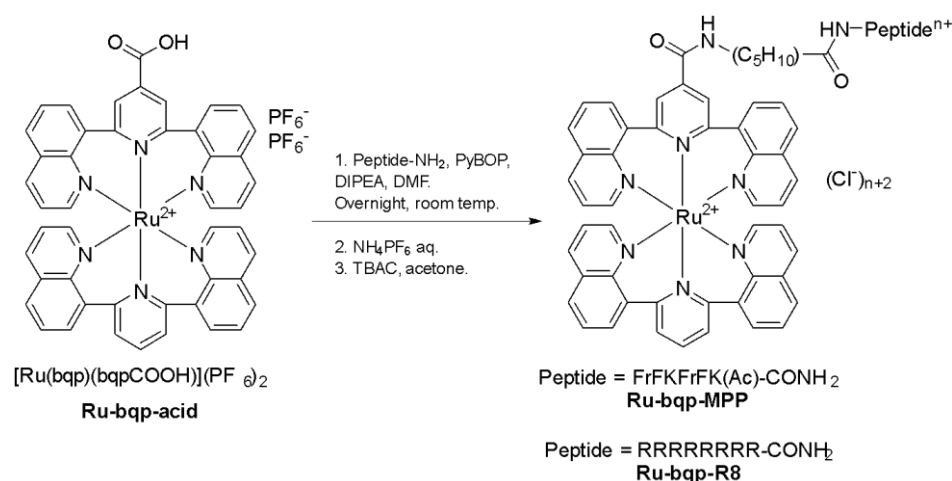
Synthesis of the ruthenium complexes presented in this chapter was completed by Dr. Christopher Burke. $\text{mer-[Ru(bqp)(CH}_3\text{CN)}_3\text{](PF}_6\text{)}_2$ (250 mg, 0.29 mmol) and bqpCOOEt (132 mg, 0.33 mmol) were heated at reflux in ethylene glycol (15 mL) for 72 hours. The deep-red reaction mixture was cooled and poured on a stirring solution of NH_4PF_6 to precipitate crude $[\text{Ru(bqp)(bqp-‘ester’)}](\text{PF}_6)_2$ which was filtered and washed with water. The Ru-ester material was partially purified by dissolution in acetone (30 mL), filtering out insoluble solids, and evaporation of the red filtrate to provide a dry residue. Next, dried crude $[\text{Ru(bqp)(bqp-‘ester’)}](\text{PF}_6)_2$ was suspended at room temperature in a mixture of 4:1 THF: CH_3OH (50 mL) and was treated with a solution of $\text{LiOH}\cdot\text{H}_2\text{O}$ (126 mg, 3.0 mmol) in 10 mL water. The combined reaction mixture was stirred overnight in

the dark. Upon completion, the reaction volume was concentrated and a red solid was isolated by filtration after the addition of NH_4PF_6 in 1 M HCl (aq.) (50 mL). The crude Ru-acid material was purified by column chromatography on silica gel using 90/10/1 $\text{CH}_3\text{CN}/\text{H}_2\text{O}/\text{KNO}_3$ (20% aq.) as eluent. $\text{mer-}[\text{Ru}(\text{bqp})(\text{bqpCOOH})](\text{PF}_6)_2$ was isolated from the dried deep-red product band by twice precipitating a solid from acetone solutions using NH_4PF_6 in 1 M HCl (aq.). Yield: red solid, 146 mg (45 %). ^1H NMR (600 MHz, CD_3CN) δ (ppm): 7.01 (dd, 2H); 7.06 (dd, 2H); 7.44 (q, 4H); 7.65 (2d, 4H); 7.70 (d, 2H); 7.80 (d, 2H); 7.87 (d, 2H); 8.03 – 8.05 (m, 6H); 8.08 (d, 2H); 8.15 (t, 1H); 8.30 (s, 2H). ^{13}C NMR (CD_3CN) δ (ppm): 122.69, 123.07, 127.52, 127.58, 127.65, 127.91, 128.61, 128.81, 131.06, 131.51, 132.77, 133.32, 133.82, 133.94, 138.37, 138.43, 138.96, 147.49, 147.57, 157.22, 157.81, 159.19, 159.62, 166.48. HR-MS (ESI(+)-qTOF) m/z : 812.1470 $[\text{M} - 2\text{PF}_6]^{+}$; Calculated for $\text{C}_{47}\text{H}_{30}\text{N}_6\text{O}_2\text{Ru}$: 812.1474.

Synthesis of $\text{mer-}[\text{Ru}(\text{bqp})(\text{bqp-COOEt})](\text{PF}_6)_2$ (Ru-bqp-ester)

Purified $\text{mer-}[\text{Ru}(\text{bqp})(\text{bqpCOOH})](\text{PF}_6)_2$ (100 mg) was suspended in 10 mL of ethanol and treated with three drops of concentrated sulfuric acid. The mixture was heated at reflux overnight, then cooled and poured onto 100 mL of an aqueous NH_4PF_6 solution to precipitate $\text{mer-}[\text{Ru}(\text{bqp})(\text{bqpCOOEt})](\text{PF}_6)_2$ in quantitative yield. ^1H NMR (600 MHz, Acetone- d_6) δ (ppm): 8.46 (d, 2H), 8.43 (d, 2 H), 8.38 (s, 2 H), 8.35 (t, 1 H), 8.31 (d, 4 H), 8.15 (d, 2 H), 8.03 (d, 2 H), 7.98 (d, 2 H), 7.89 (dd, 4 H), 7.61 (m, 4 H), 7.27 (q, 4 H), 4.47 (m, 2 H), 1.39 (t, 3 H).

Synthesis of Ru-bqp Peptide Conjugates



Scheme S2.1 Peptide conjugation to Ru-bqp-acid to provide Ru-bqp-MPP and Ru-bqp-R8. Peptide conjugation was carried out using PyBOP coupling chemistry. Specifically, Ru-bqp-acid (16 mg, 1 eq.), peptide-NH₂ (40mg, about 2eq.), PyBOP (30 mg, 3 eq.) and DIPEA (20 μL), were stirred overnight in DMF (1 mL) in a glass vial. Next, the mixture was added dropwise to a stirring aqueous solution of NH₄PF₆ to precipitate the conjugate. The solid was filtered, washed well with water and allowed to dry. The crude material was dissolved in minimum acetone, filtered, and added dropwise to a stirring solution of tetrabutylammonium chloride in acetone. The precipitated chloride salt of the conjugate was collected by filtration and washed well with acetone.

[Ru-bqp-MPP], [Ru(bqp)(bqp-CONH-ahx-FrFKFrFK(Ac)-CONH₂)]⁵⁺.

¹H NMR (600 MHz, CD₃OD/D₂O) δ (ppm): 8.28 (m, 3H), 8.17 (m, 4H); 8.12 (m, 4H); 8.02 (m, 2H); 7.96 (m, 2H); 7.87 (d, 2H); 7.78 (m, 4H); 7.54 (m, 4H); 7.00 – 7.33 (m, 24H); 4.51 (m, 4H, peptide α -H); 3.80 – 4.30 (m, 4H, peptide α -H); 2.80 – 3.25 (m, 17H, peptide-H); 0.80 – 2.05 (m, 37H, peptide-H).

¹³C NMR (CD₃OD/D₂O) δ (ppm): 122.69, 123.07, 127.52, 127.58, 127.65, 127.91, 128.61, 128.81, 131.06, 131.51, 132.77, 133.32, 133.82, 133.94, 138.37, 138.43, 138.96, 147.49, 147.57, 157.22, 157.81, 159.19, 159.62, 166.48.

HRMS (Q-Exactive, ESI(+)-qTOF, CH₃OH/TFA), m/z (Calculated, Assignment): 1175.9556 (1175.9559, [M - H⁺ + 2TFA]²⁺); 746.3077 (746.3087, [M - H⁺ + TFA]³⁺); 531.4844 (531.4852, [M - H⁺]⁴⁺); 425.3891 (425.3896, [M]⁵⁺).

[Ru-bqp-R8], [Ru(bqp)(bqp-CONH-ahx-RRRRRRRRR-CONH₂)]¹⁰⁺.

¹H NMR (600 MHz, CD₃OD/D₂O); very broad peaks, 8.17 – 8.42 (11 H), 8.03 - 8.14 (4 H), 7.90 - 7.97 (2 H), 7.80 – 7.89 (4 H), 7.58 – 7.67 (4 H), 7.18 – 7.28 (4 H), 4.41 (peptide α -H, 8 H), 3.53 (3 H), 0.95 – 2.50 (47 H, peptide-H).

HRMS (Q-Exactive, ESI(+)-qTOF, CH₃OH/TFA) m/z (Calculated, Assignment): 714.7584 (714.7567, [M + 6TFA]⁴⁺); 435.2167 (435.2154, [M - 5H⁺]⁵⁺); 362.8484 (363.8474, [M - 4H⁺]⁶⁺); 311.1567 (311.1559, [M - 3H⁺]⁷⁺).

S2.1.3 Photophysical Measurements

Absorbance measurements of the Ru(II) complexes at 20 μ M were performed using a Jasco V670 Spectrophotometer. Emission spectra were collected using a Varian Cary Eclipse fluorescence spectrophotometer and luminescent lifetime measurements were performed on a PicoQuant FluoTime 100 FLS TCSPC system using a 450 nm pulsed laser and an external pulse generator. Lifetime decay curves were analysed using the PicoQuant Fluofit software with fitting criteria; $0.9 < X^2 < 1.1$. For O₂ sensitivity studies, the emission spectrum and luminescent lifetime of each complex (10 μ M) were recorded under O₂ saturation, and after N₂ purge for 20 minutes. All measurements were performed in triplicate and reported as the mean $\pm$ SD.

S2.1.4 Cell Monolayer and 3D Spheroid Cell Culture

Three cell lines were selected for cell monolayer studies: a lung carcinoma cancer cell line (A549), a human pancreatic cancer cell line (HPAC) and a non-cancer cell line derived from Chinese hamster ovaries (CHO-K1). A 50% Dulbecco's Modified Eagle's medium (DMEM) and F-12 Nutrient Mixture Ham (F-12 Ham) media was used to culture

CHO cells, DMEM for A549 cells and Rosewell Park Memorial Institute (RPMI-1640) medium for HPAC. Both CHO and A549 media were supplemented with 10 % FBS and 1 % penicillin-streptomycin, and HPAC media supplemented with 5% FBS. The media type and FBS concentrations used for each cell line were selected based on those commonly reported in the literature to support optimal cell growth and proliferation, as well as the recommendations from reputable vendors such as ATCC. All cells were grown at 37 °C with 5 % CO₂ and sub-cultured at 80-90 % confluency. To prepare 3D multicellular tumour spheroids (MCTS), HPAC cells were seeded at 2×10^4 cells/well in clear U-bottom 96 well plates coated with polyHEMA (Poly(2-hydroxyethyl methacrylate), Sigma), centrifuged at 700 rpm for 10 minutes at 37 °C and allowed to grow and compact for 96 hours prior to treatment. A partial media change was performed after 48 hours.

S2.1.5 Dark Toxicity Studies

The alamar blue assay (Invitrogen) was used to assess the viability of A549, CHO and HPAC cell monolayers treated with the Ru(II) probes, Ru-bqp-ester, Ru-bqp-MPP, Ru-bqp-R8. CHO, A549 and 2D HPAC cells were seeded in a 96-well plate (Nunc, flat bottom cell culture treated) at 1×10^4 cells per well i.e., in 100 µL media for 24 h at 37 °C with 5 % CO₂. Ru-bqp-ester, Ru-bqp-R8 and Ru-bqp-MPP were added for 24 h at 37 °C and 5 % CO₂ in the absence of light. The alamar blue (Resazurin) assay was performed to measure cell viability by replacing the test solution with 100 µL of a 10% v/v media/resazurin solution and incubating for 5 h at 37 °C in the absence of light. Absorbance was measured using a BMG Labtech ClarioStar (plus) plate reader at 570 nm and 600 nm (corrected for background subtraction). Cell viability is presented as a percentage (%) compared to untreated control wells.

S2.1.6 Phototoxicity Studies

All cell monolayers were prepared as described above for the phototoxicity assay. A549 and CHO cells were treated at varying concentrations of the complexes and incubated for 30 minutes in the absence of light, HPAC were incubated for 2 hours to allow uptake. A 470 nm blue light LED array was used to irradiate the cells in the wells at a total dose of 17 ± 1.64 J/cm² (2 h at 2.37 ± 0.23 mW/cm²). A dark control plate was performed alongside all phototoxicity plates. Irradiation conditions were tested on untreated cells to confirm cells remained viable at this dose before any experiments were performed and each phototoxicity plate included untreated test wells for cell viability comparison. All viability assays were performed in triplicate.

S2.1.7 *In vitro* Singlet Oxygen Detection

To assess singlet oxygen ($^1\text{O}_2$) production and confirm the phototoxicity of our Ru(II) complexes was caused by the generation of reactive oxygen species (ROS), a $^1\text{O}_2$ scavenger study was completed using 1,3-diphenylisobenzofuran (DPBF). As DPBF has poor solubility in aqueous solutions, a stock solution of 10 mM DPBF and 20 μM of the parent complex Ru-bqp-ester was prepared in Ethanol with 1% DMSO. Aliquots of 200 μL of this solution were added to a 96 well plate and irradiated with a 470 nm LED at $2.37 \pm 0.23 \text{ mW/cm}^2$ in 1-minute intervals for a total of 6 minutes and a platereader was used to measure the absorbance of the solution following each irradiation step. The % change in absorbance was calculated using the formula $100 - ((X/A) \times 100)$ where A = the absorbance of Ru-DPBF at 410 nm where $t = 0$ and X = the absorbance at 410 nm at $t = 1, 2, 3, 4, 5$ or 6 minutes.

S2.1.8 Confocal Laser Scanning Microscopy (CLSM)

Uptake studies were carried out for A549, HPAC and CHO cell lines. Cells were seeded in 35 mm glass bottom dishes or 4-chamber slides (Ibidi, Germany) at 1×10^5 cells per mL for 24 h at 37 °C at 5 % CO_2 . A final concentration of 30 μM Ru-bqp-ester, Ru-bqp-R8 and Ru-bqp-MPP in growth media were added to the cells and incubated for 1 h in A549 and CHO cells and 4 h in HPAC cells. The dye/media solution was removed, and the cells were washed twice with 1X PBS. Cells were imaged in supplemented PBS (MgCl_2 and CaCl_2) or phenol red free media. Cells were imaged using a Leica TCS DMI8 inverted confocal microscope with a 63x oil immersion lens and heated stage at 37 °C. The Ru(II) complexes were excited using a 490 nm white light laser and the emission collected at 580-730 nm unless stated otherwise. DRAQ7 (3 μM) was added to the cells during initial uptake studies to detect cell death or damage. DRAQ7 is a nuclear dye that is taken up by damaged or dead cells and was excited at 633 nm with emission collected at 680-750 nm.^[60] Temperature dependent uptake studies were performed using the same protocol described for routine uptake studies at 37 °C, however the 1 h incubation was at 4 °C.

Further studies were performed to assess potential efflux of the Ru(II) complexes in cells. A549 and CHO cells were incubated with Ru-bqp-ester, Ru-bqp-MPP and Ru-bqp-R8 for 3 hours and HPAC cells for 4 h prior to confocal imaging. DRAQ7, was added immediately before imaging to reveal damaged/dead cells.

Co-localisation studies were carried out to determine the localisation of the complexes at 30 μM . MitoTracker Deep Red (100 nM) was used to selectively stain mitochondria and assess co-localisation with the Ru(II) peptide conjugates. MitoTracker Deep Red

was selected as a co-localisation dye as Ru-bqp-MPP was expected to target the mitochondria and was excited at 644 nm with emission collected between 730-820 nm. Pearson's co-efficient values were calculated using ImageJ software. Briefly, co-localisation studies of Ru-bqp-MPP were also completed with LysoTracker Green DND-26 (50 nM) and Rab-7a-GFP, late endosomal stain. We selected just one of the Ru(II) complexes for further co-localisation analysis due to the similarity in uptake of the three complexes, with punctate appearance and no uptake in the nucleus across all test cell lines. LysoTracker Green DND-26 was excited at 504 nm and emission collected between 500-540 nm and Rab7a-GFP was excited at 488 nm and emission collected between 500-540 nm.

S2.1.9 Confocal Imaging of HPAC Spheroids

HPAC cells were seeded in U-bottom 96 well plates pre-treated with 0.5% polyHEMA (in 95% Ethanol) at 2×10^4 cells per well. The plate was then centrifuged at 700 rpm at 37 °C using a BMG Labtech ClarioStar (plus) platereader for 10 minutes. The cells were incubated for 96 hours to allow spheroid formation before any dye was added for imaging studies. Ru-bqp-ester, Ru-bqp-MPP and Ru-bqp-R8 were added to the spheroids at 30 and 100 μ M in the 96 well plate and after a 24 h incubation the spheroids were carefully transferred to an 8-chamber slide (ibidi), with a single spheroid per chamber, and directly imaged using a Leica TCS DMI8 confocal microscope (40x oil immersion objective). Hoechst 33342 nuclear stain (1 μ g/mL) was added to the spheroids for 45 minutes as a contrast agent and excited using a 405 nm laser with emission collected between 425 and 475 nm. The Ru(II) complexes were excited using a white light laser at 490 nm and emission collected between 580-730 nm. Spheroid images were acquired using z-scanning across the z-axis of the samples. On average, 40-50 images were acquired per z-scan and used to obtain 3D spheroid reconstructions using Leica Application Suite X (LAS X) software.

S2.1.10 Evaluation of Spheroid Viability

Spheroid viability was evaluated using a CellTiter-Glo 3D assay. Although the alamar blue assay is a robust and reliable option for 2D cell viability, the CellTiter-Glo 3D assay is specifically designed to penetrate deeper into 3D cell culture, and thus offers better specificity for these studies. Cells were seeded in round bottom 96 well plates at 2×10^4 cells per well and grown for 96 hours. The cells were treated for 24 hours and a media change performed. A 470 nm LED was used to irradiate the spheroids at 4.27 ± 0.41 J/cm² (30 min at 2.37 ± 0.23 mW/cm²) and a dark control plate was performed simultaneously. Irradiation conditions were tested on untreated spheroids to confirm

viability remained unaffected at this irradiation dosage before any experiments were performed. The spheroids were allowed to recover and the CellTiter-Glo 3D assay performed to determine cell viability as per the manufacturer's protocol. Percentage cell viability was determined relative to untreated control spheroids.

S2.2 Characterisation Data (Courtesy of Dr. Christopher S. Burke)

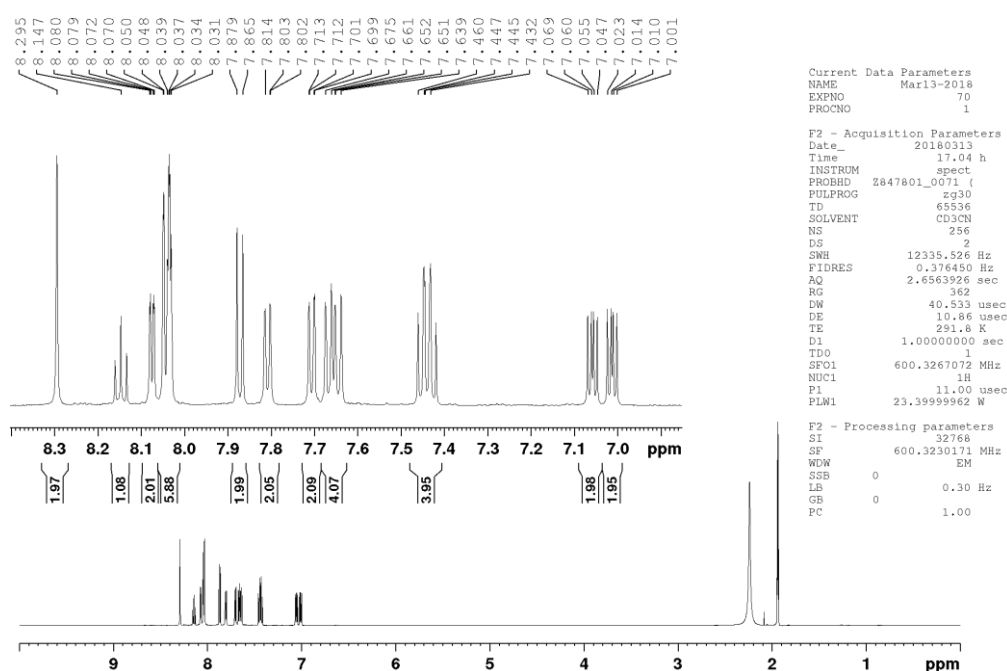


Figure S2.1 ^1H NMR Spectrum (600 MHz, CD_3CN) for $\text{mer-}[\text{Ru}(\text{bqp})(\text{bqpCOOH})](\text{PF}_6)_2$ with insets to show regions of interest above the full spectrum.

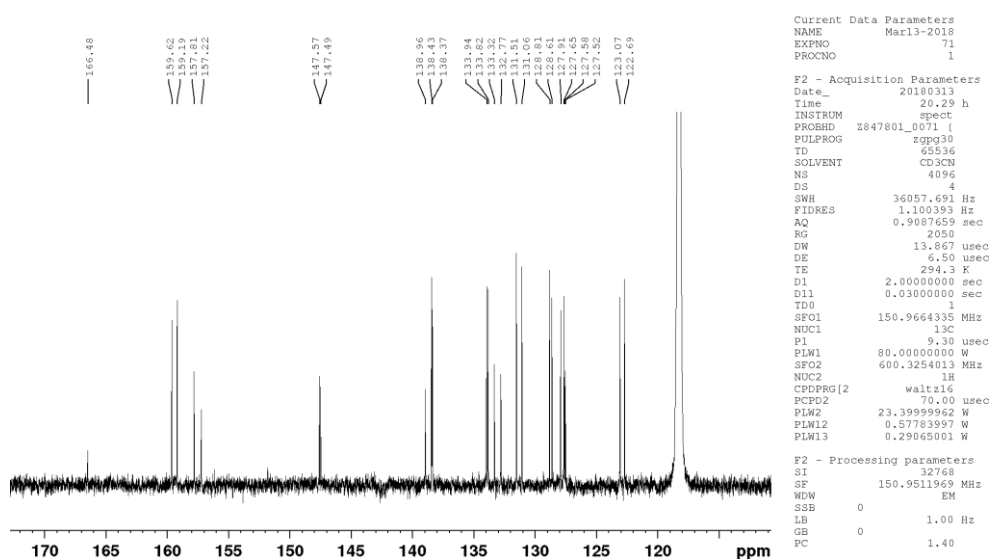


Figure S2.2 ^{13}C NMR Spectrum (150 MHz, CD_3CN) for $\text{mer-}[\text{Ru}(\text{bqp})(\text{bqpCOOH})](\text{PF}_6)_2$ to show region of interest.

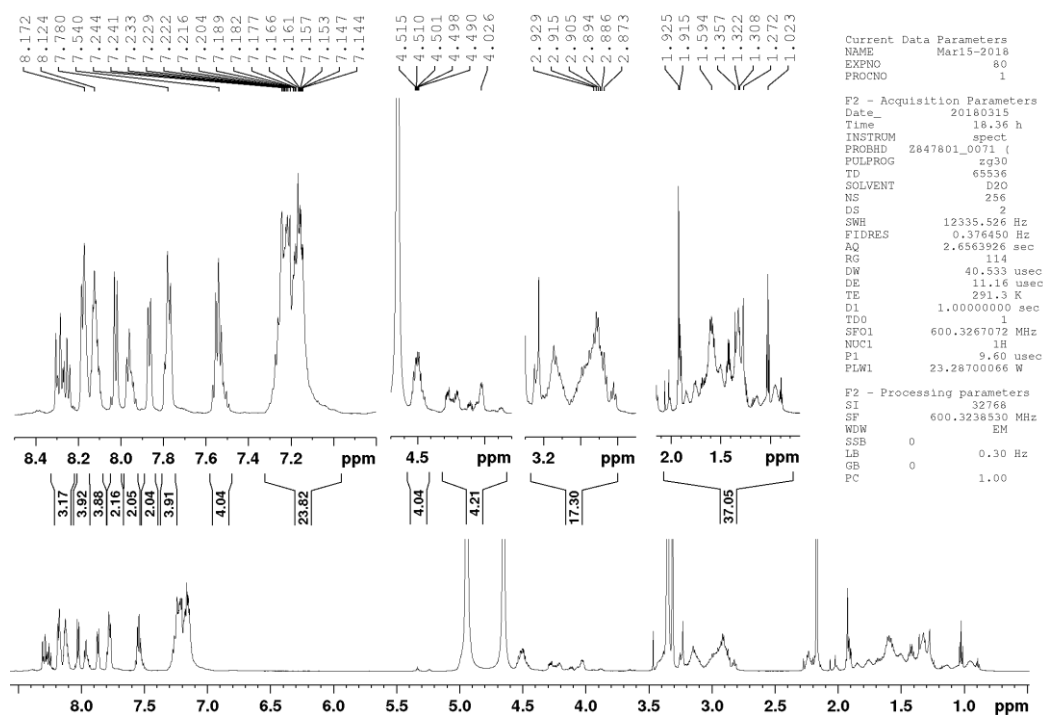


Figure S2.3 ^1H NMR Spectrum (600 MHz, $\text{CD}_3\text{OD}/\text{D}_2\text{O}$) for Ru-bqp-MPP with insets to show regions of interest above the full spectrum.

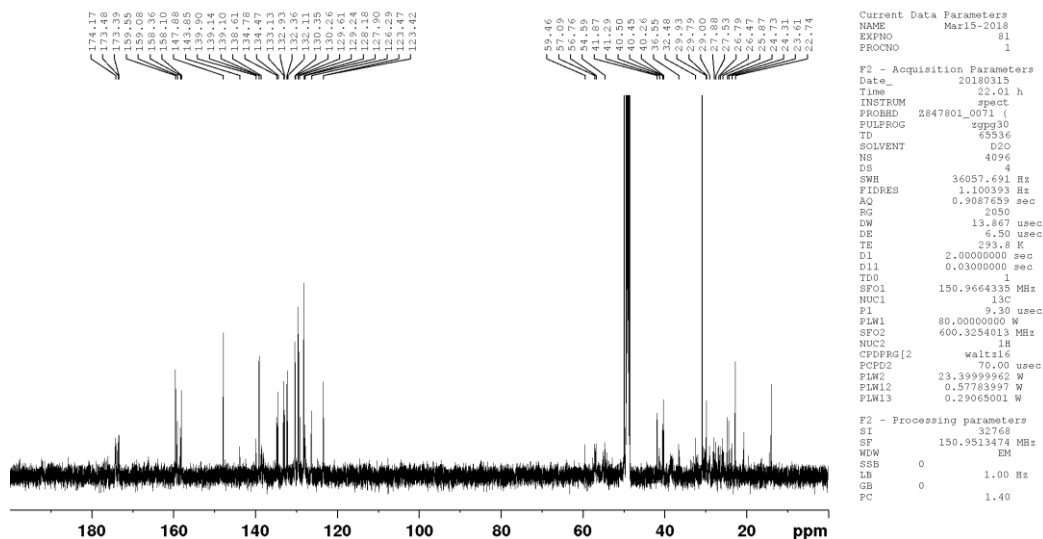


Figure S2.4 ^{13}C NMR Spectrum (150 MHz, $\text{CD}_3\text{OD}/\text{D}_2\text{O}$) for Ru-bqp-MPP.

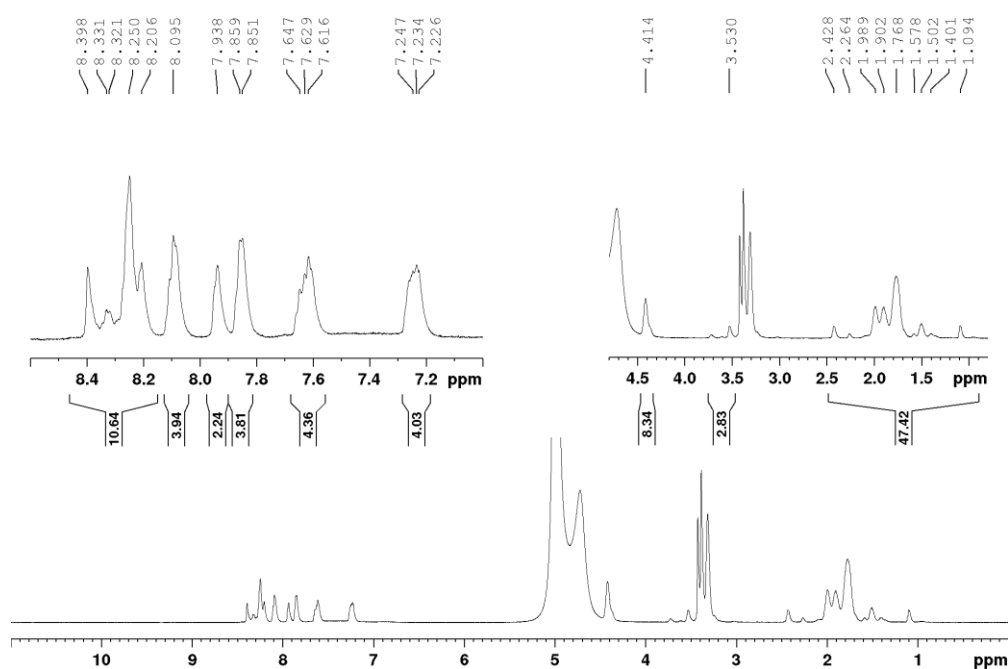


Figure S2.5 ^1H NMR Spectrum (600 MHz, $\text{CD}_3\text{OD}/\text{D}_2\text{O}$) for Ru-bqp-R8 with insets to show regions of interest above the full spectrum.

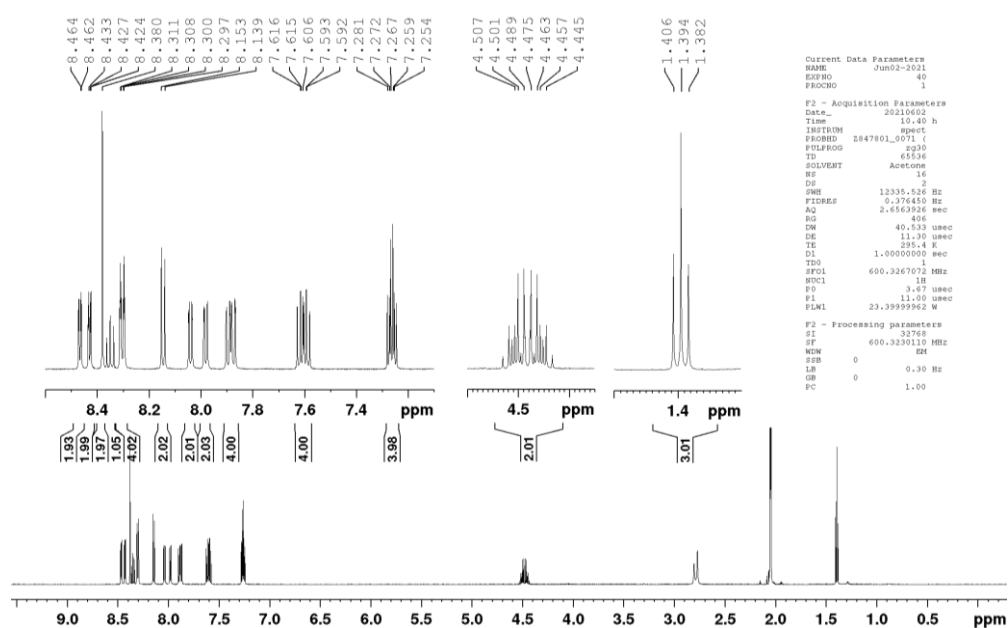


Figure S2.6 ^1H NMR Spectrum (600 MHz, Acetone- d_6) for mer-[Ru(bqp)(bqpCOOEt)](PF_6) $_2$ with insets to show regions of interest above the full spectrum.

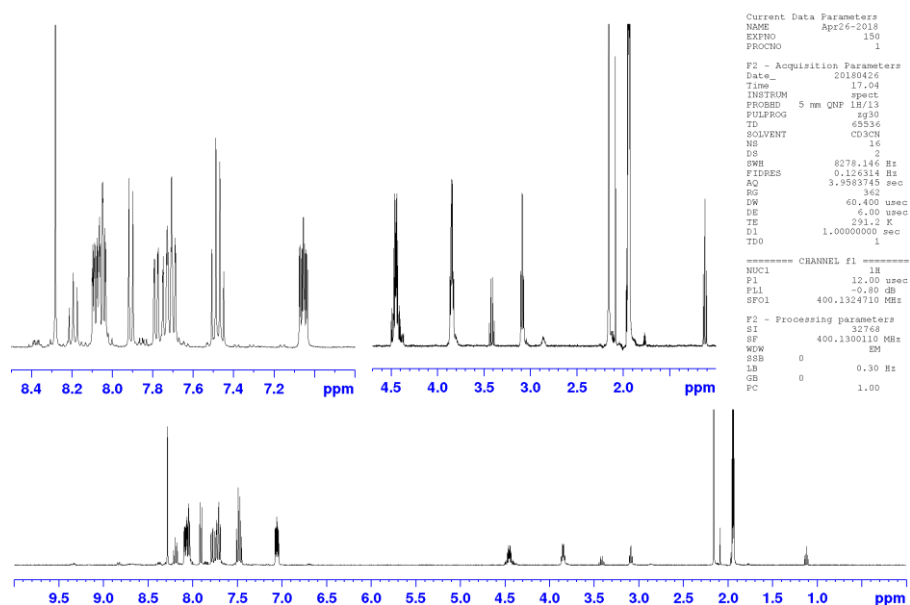


Figure S2.7 ^1H NMR Spectrum (400 MHz, CD_3CN) for the mixed ester mer-[Ru(bqp)(bqp-‘ester’)](PF_6) $_2$ product obtained from long reflux in ethylene glycol with insets to show regions of interest above the full spectrum.

Single Mass Analysis

Tolerance = 50.0 PPM / DBE: min = -1.5, max = 200.0

Element prediction: Off

Number of isotope peaks used for i-FIT = 5

Monoisotopic Mass, Odd and Even Electron Ions

75 formula(e) evaluated with 1 results within limits (up to 10 best isotopic matches for each mass)

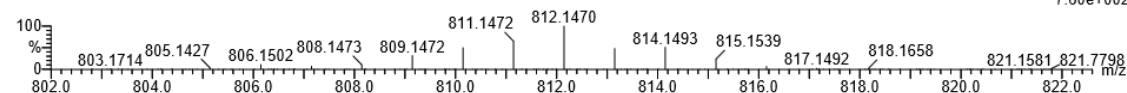
Elements Used:

C: 0-47 H: 0-30 N: 0-6 O: 0-2 Ru: 0-2

Christopher Burke (TK), CBTK0603

Q-TOF20180618MF020 47 (1.125) AM (Cen,6, 80.00, Ht,10000.0,1570.68,0.70); Sm (SG, 2x3.00); Sb (15,10.00); Cm (7:87-46:78)

TOF MS LD+
7.60e+002



Minimum:

Maximum:

Mass Calc. Mass mDa PPM DBE i-FIT i-FIT (Norm) Formula

812.1470 812.1474 -0.4 -0.5 36.0 106.1 0.0 C47 H30 N6 O2 Ru

Figure S2.8 HR-MS (ESI-qTOF, MS+) spectrum for Ru-bqp-COOH to show region of interest and single mass analysis.

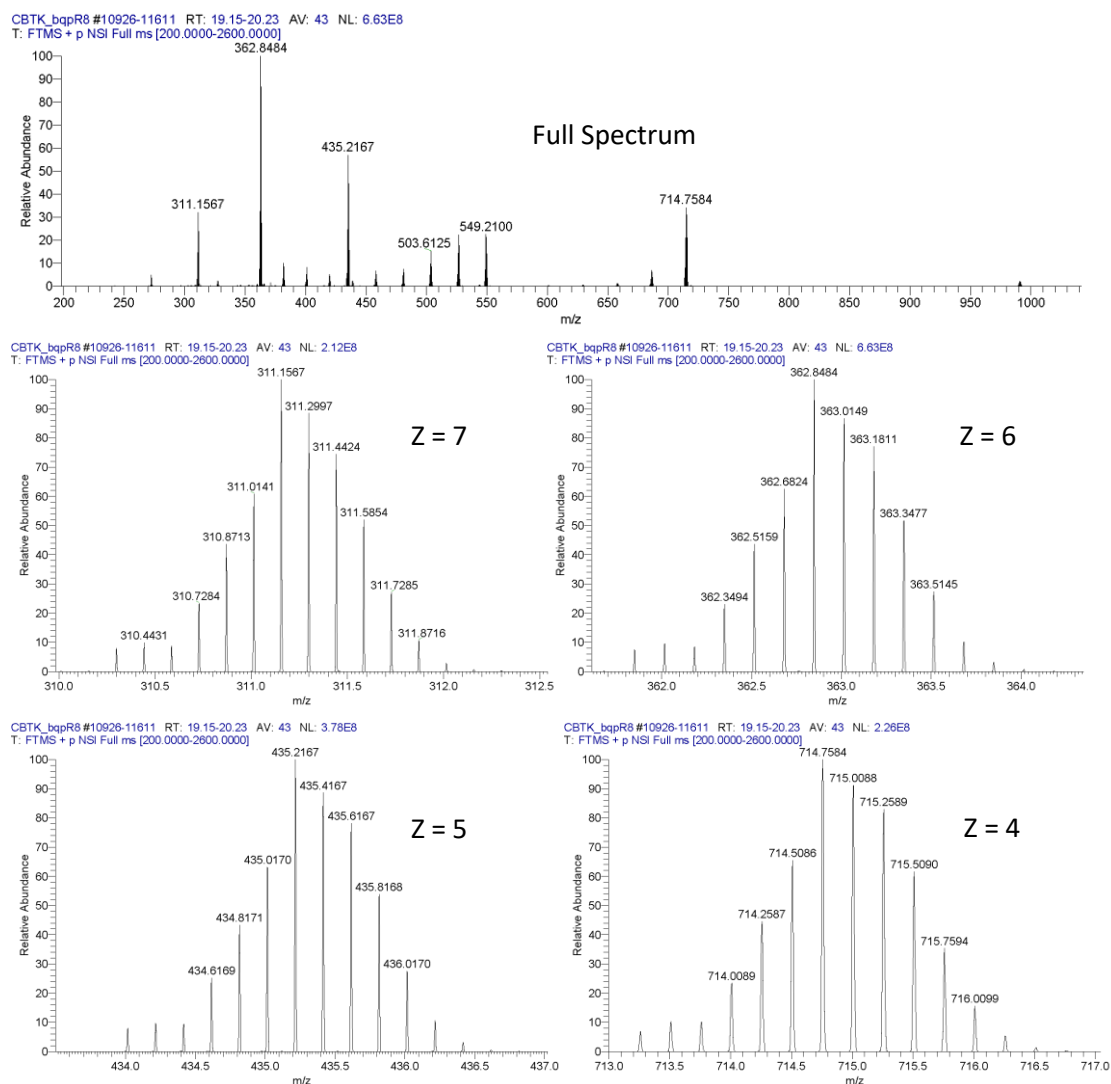
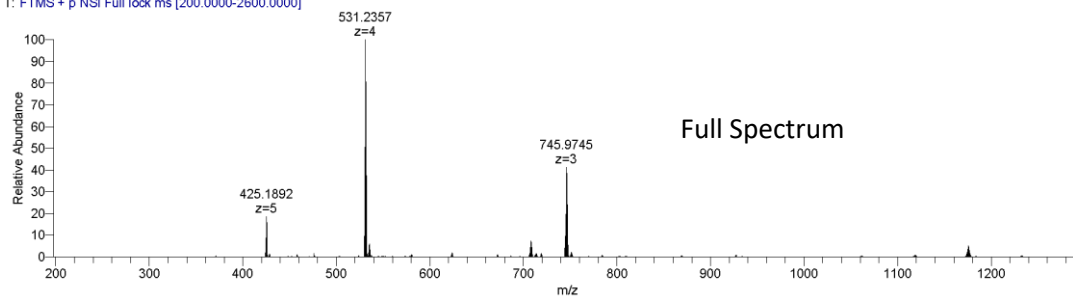
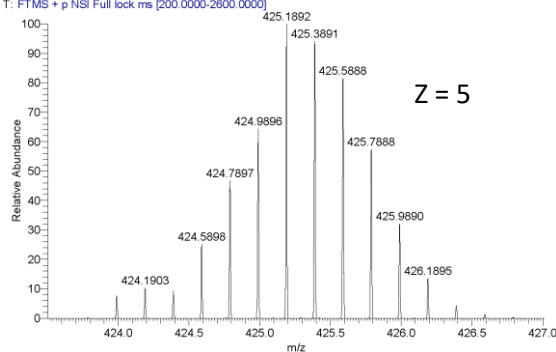


Figure S2.9 HR-MS analysis (Q-Exactive, Ion-Trap MS+, MeOH/TFA) for Ru-bqp-R8 to show the full spectrum at top and a close-up of individual major peaks with the associated ion calculated charge, Z .

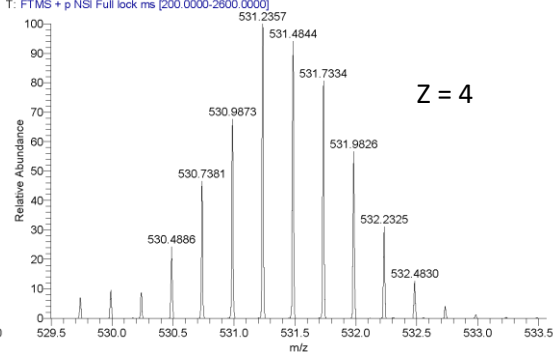
CBTK_bqpMPP#14513-15435 RT: 25.39-26.89 AV: 58 NL: 1.71E9
T: FTMS + p NSI Full lock ms [200.0000-2600.0000]



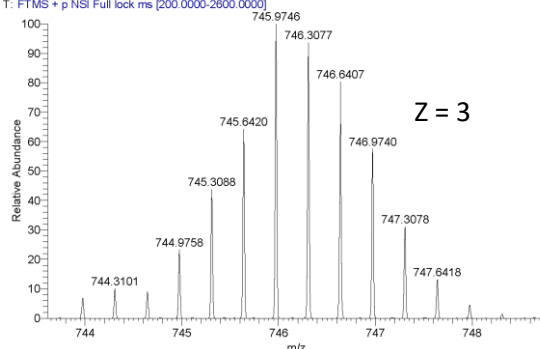
CBTK_bqpMPP#14513-15435 RT: 25.39-26.89 AV: 58 NL: 3.20E8
T: FTMS + p NSI Full lock ms [200.0000-2600.0000]



CBTK_bqpMPP#14513-15435 RT: 25.39-26.89 AV: 58 NL: 1.71E9
T: FTMS + p NSI Full lock ms [200.0000-2600.0000]



CBTK_bqpMPP#14648-15227 RT: 25.62-26.55 AV: 36 NL: 9.78E8
T: FTMS + p NSI Full lock ms [200.0000-2600.0000]



CBTK_bqpMPP#14648-15227 RT: 25.62-26.55 AV: 36 NL: 1.28E8
T: FTMS + p NSI Full lock ms [200.0000-2600.0000]

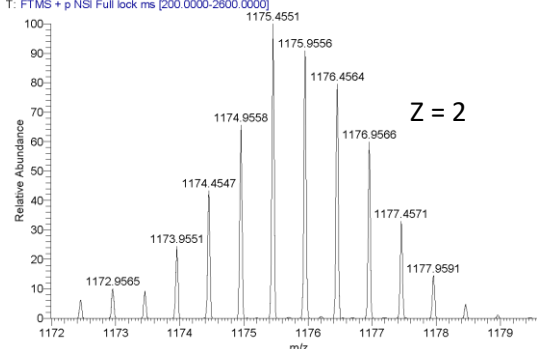


Figure S2.10 HR-MS analysis (Q-Exacte, Ion-Trap MS+, MeOH/TFA) for Ru-bqp-MPP to show the full spectrum at top and a close-up of individual major peaks with the associated ion calculated charge, Z.

S2.3 Photophysical Studies

S2.3.1 O₂ Sensitivity Studies

Oxygen sensitivity study results using Ru-bqp-MPP as an example:

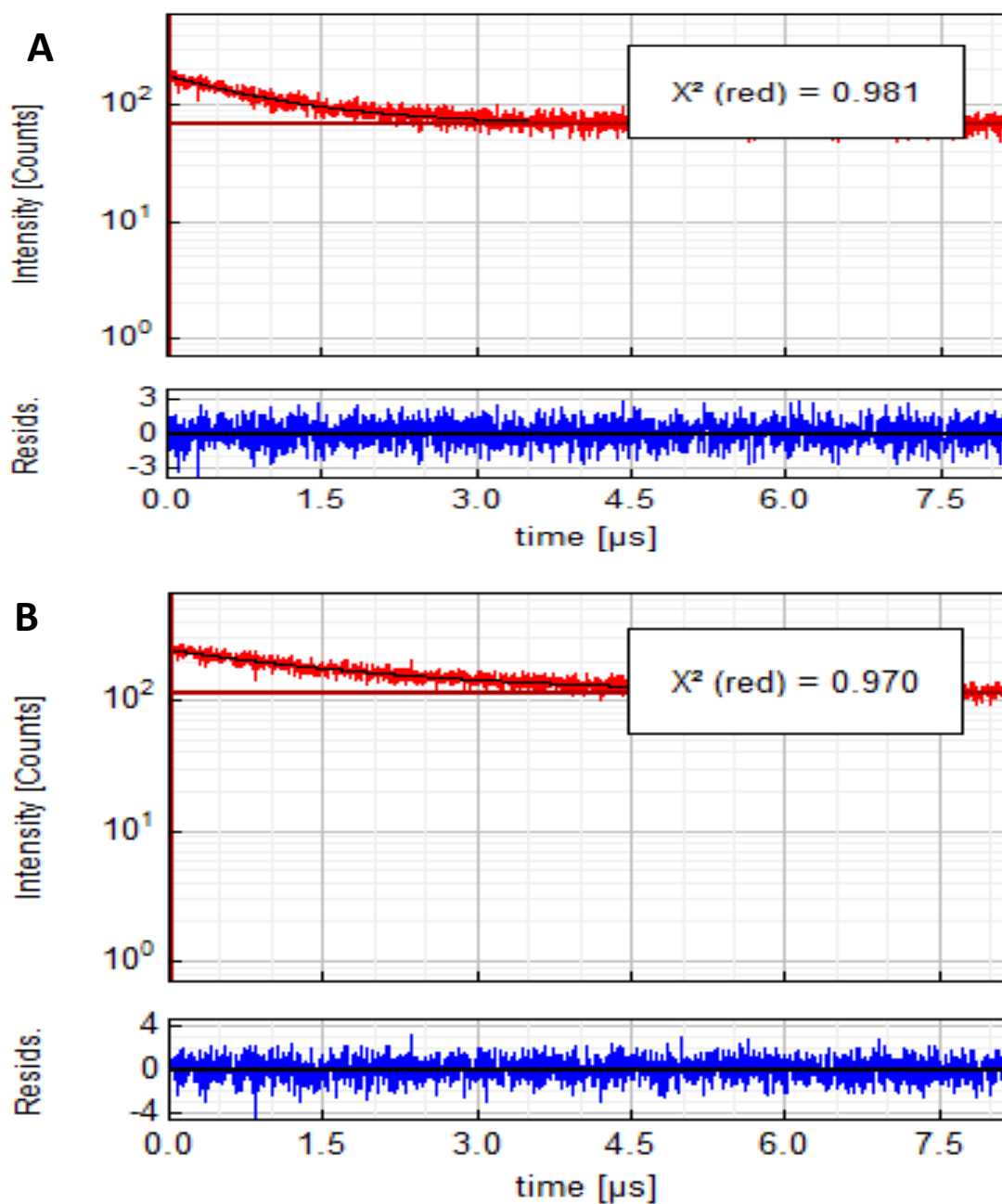


Figure S2.11 Emission decays of Ru-bqp-MPP (10 μ M in PBS, pH 7.4) in **(A)** aerated and **(B)** de-aerated conditions. Aerated lifetime of 1.077 ± 0.033 μ s and de-aerated lifetime 2.011 ± 0.021 μ s exactly. Residual plots for the exponential fitting of the curves are shown below each plot.

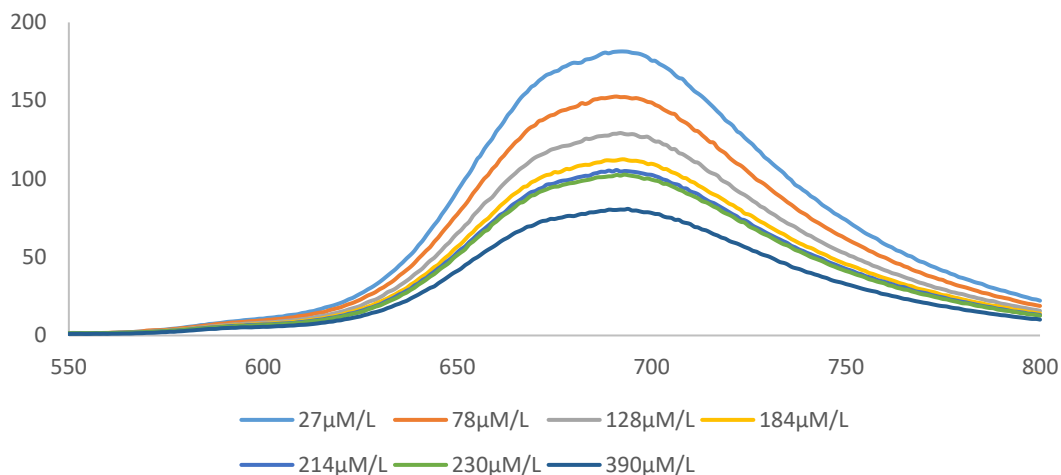


Figure S2.12 Ru-bqp-MPP emission intensity response to changing O₂ concentration (μM/L). Ru-bqp-MPP was excited at 494 nm with excitation and emission slit width of 10. O₂ concentration of 10 μM Ru-bqp-MPP in PBS (pH 7.4) was measured at room temperature.

S2.4 Cell Studies

S2.4.1 Confocal Laser Scanning Microscopy (CLSM)

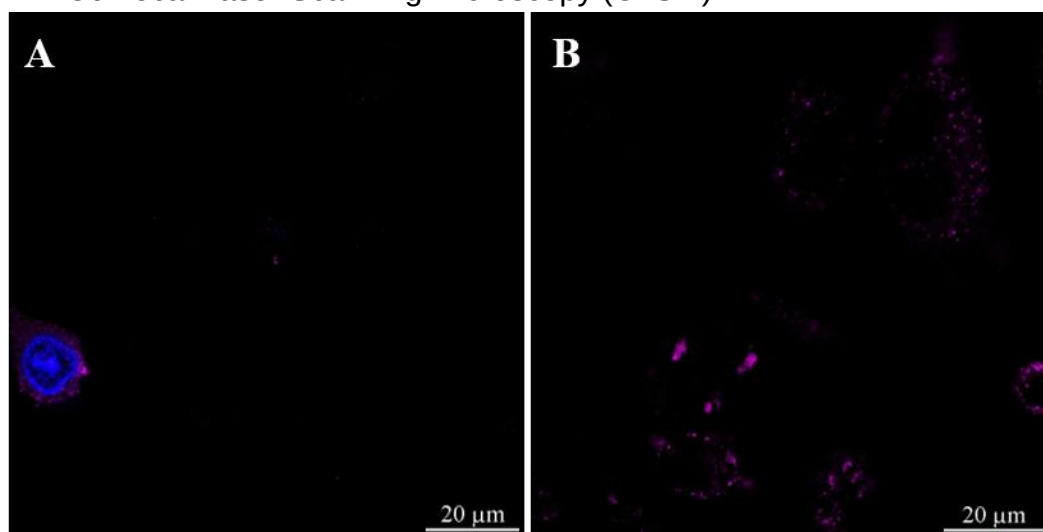


Figure S2.13 Ru-bqp-MPP (30 μM) at 3 h incubation in **(A)** A549 and **(B)** CHO cells. DRAQ7 (3 μM) was added to the cells before imaging to detect cell death. Ru-bqp-MPP is only seen in dead cells stained with DRAQ7 but remains in CHO under our regular imaging conditions including laser intensity.

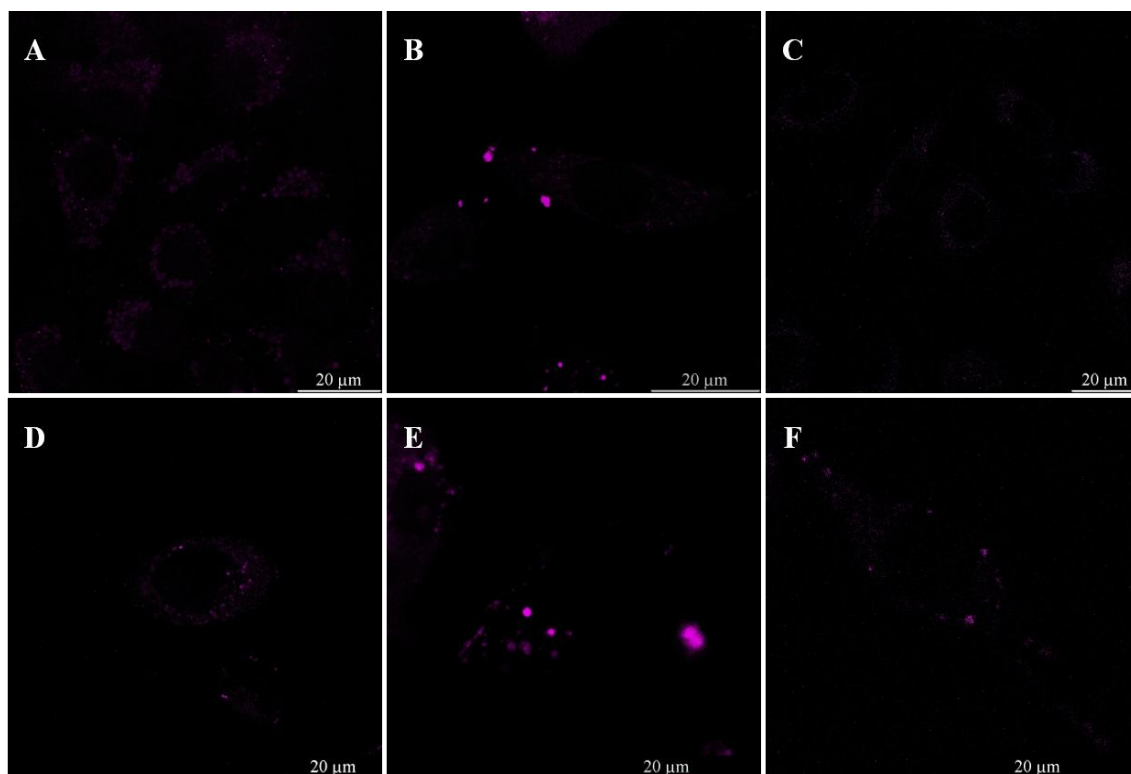


Figure S2.14 Cellular uptake of Ru-bqp-MPP in **(A)** A549 and **(B)** CHO cells, Ru-bqp-R8 in **(B)** A549 and **(E)** CHO cells and Ru-bqp-ester in **(C)** A549 and **(F)** CHO cells. 30 μ M and 1 h incubation with Ex 490 and Em 580-730 nm.

S2.4.2 Localisation Studies in 2D Cells

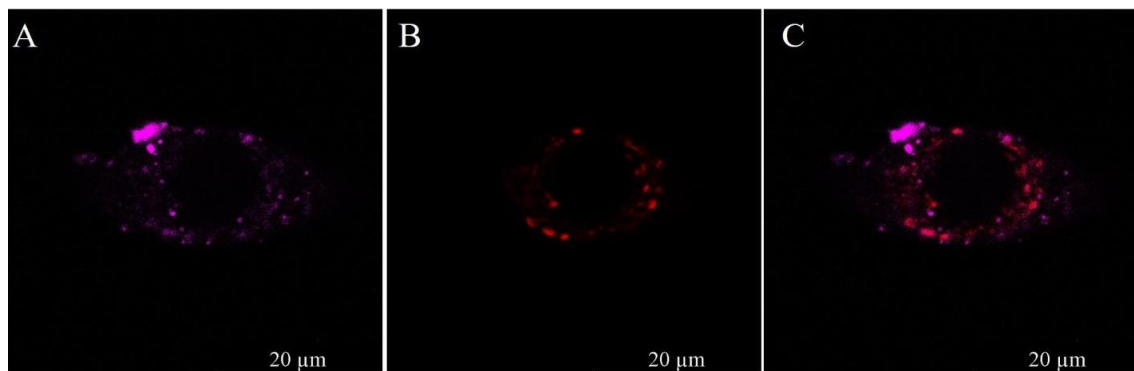


Figure S2.15 Cellular uptake and localisation studies of Ru-bqp-R8 (30 μ M) in live A549 cells. Cells were incubated for 1 h in the dark with **(A)** Ru-bqp-R8 and co-stained with **(B)** MitoTracker Deep Red (100 nM) for 30 minutes. Pearson's coefficient = 0.163 ± 0.02 . A 470 nm white light laser was used to excite Ru-bqp-R8 and emission was collected between 580 and 700 nm. The MitoTracker Deep Red dye was excited at 644 nm and emission collected between 730-820 nm.

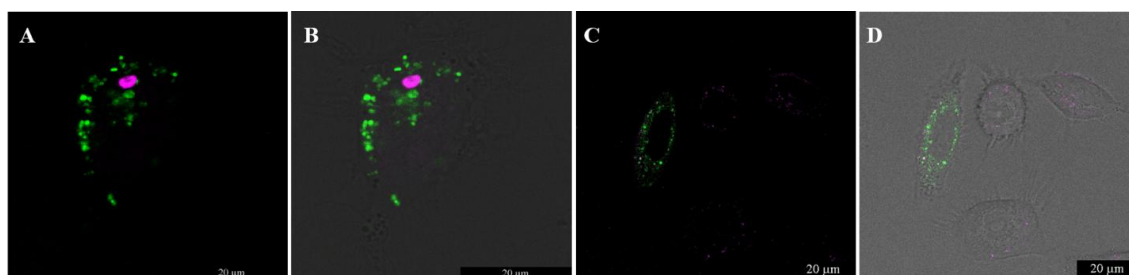


Figure S2.0.16 Co-localisation studies of Ru-bqp-MPP (30 μ M, 1 h) in live A549 (**A-B**) and CHO (**C-D**) cells. Co-localisation with LysoTracker Green (Ex 504 nm Em 500-540 nm) is shown in (**A**) with brightfield overlay (**B**) and co-localisation with the late endosomal dye Rab7a-GFP (**C**) with brightfield overlay (**D**). Co-localisation values were obtained using specific regions of interest, as not all cells were stained with Rab7a-GFP.

S2.4.3 Toxicity

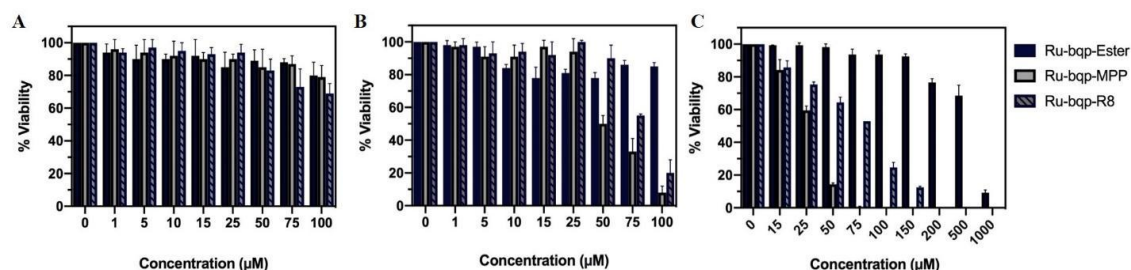


Figure S2.17 Cell viability of (**A**) A549, (**B**) CHO and (**C**) HPAC cells at 24 h exposure to varying concentrations of Ru-bqp-ester, Ru-bqp-R8 and Ru-bqp-MPP in the absence of light. Cell viability was assessed using the alamar blue assay. Viability is determined as a percentage of untreated control cells incubated in media only. (N=3). Maximum DMSO concentration is <0.003%.

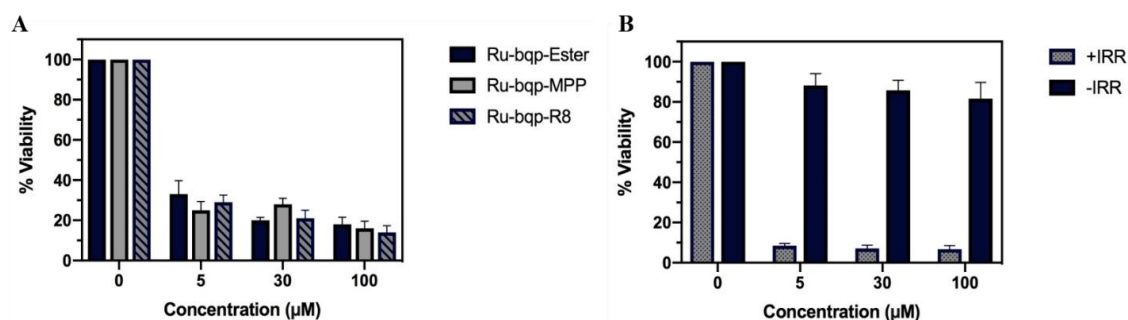


Figure S2.18 Cell viability of **(A)** A549 cells after a 30 minute incubation with Ru-bqp-ester, Ru-bqp-MPP and Ru-bqp-R8 at 5, 30 and 100 μM followed by irradiation with a 470 nm blue light LED at 17 J/cm² (A549 dark control not graphed, cell viability >80% up to 100 μM) and **(B)** CHO cells after 30 minute incubation with Ru-bqp-ester at 5, 30 and 100 μM and irradiation (+IRR) at 17 J/cm² including CHO dark control (-IRR). Cell viability was assessed as per the cytotoxicity protocol described in experimental. Maximum DMSO concentration <0.003%.

Table S2.1 Singlet oxygen (¹O₂) scavenger assay results. The decrease in absorbance at 410 nm is displayed as a percentage compared to the absorbance of the solution prior to irradiation (t=0) with a 470 nm LED.

Irradiation Time (minutes)	DPBF (10 mM)	DPBF (10 mM) with Ru-bqp-ester (20 μM)	
	OD at 410 nm (DPBF only)	OD at 410 nm (Ru-DPBF)	% Decrease (compared to t=0)
0	3.118	3.047	0
1	2.995	2.916	4.30
2	2.916	2.572	15.59
3	2.867	0.965	68.33
4	3.035	0.947	68.92
5	2.847	0.613	79.88
6	2.194	0.630	79.32

S2.4.4 3D Cell Studies

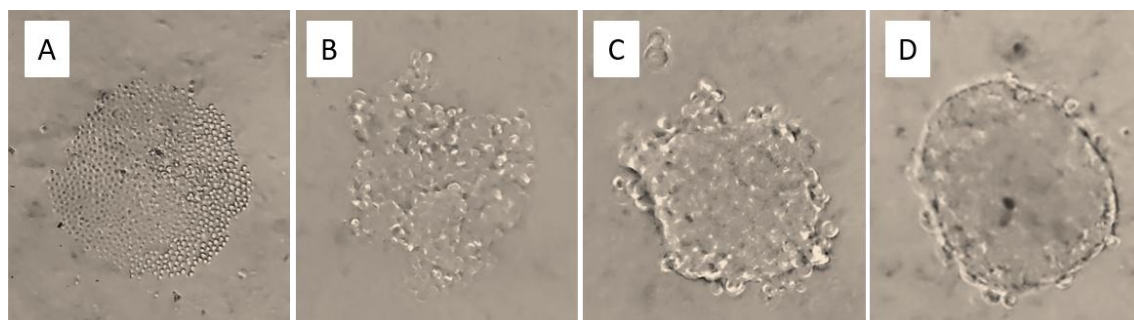


Figure S2.19 HPAC spheroid growth over 96 hours, imaged through an inverted Nikon (TMS) phase contrast light microscope focused using a 20x lens with 1x android camera magnification.

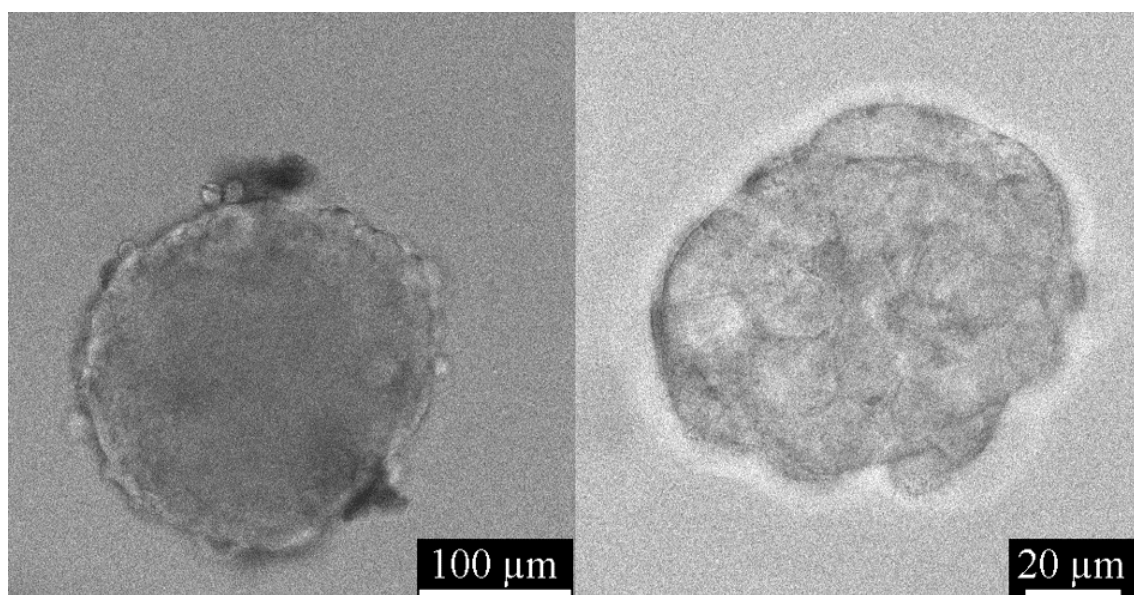


Figure S2.20 Live HPAC spheroids imaged using a confocal microscope (40x lens) after 96 hours growth.

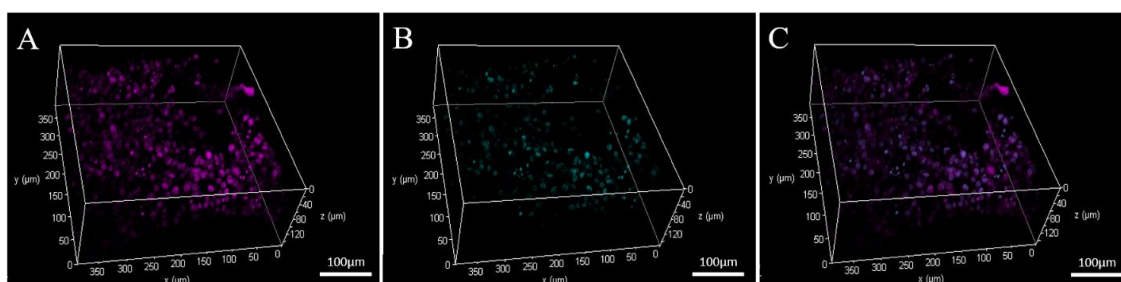


Figure S2.21 3D reconstruction of a live HPAC spheroid with 100 μM Ru-bqp-MPP at 24 h and 1 $\mu\text{g/mL}$ Hoechst 33342 nuclear stain added 45 minutes prior to imaging. Hoechst was excited using a 405 nm laser and emission collected between 425-475 nm, Ru-bqp-MPP was excited using a white light laser at 490 nm with emission 580-800 nm. Scale bar reads 100 μm .

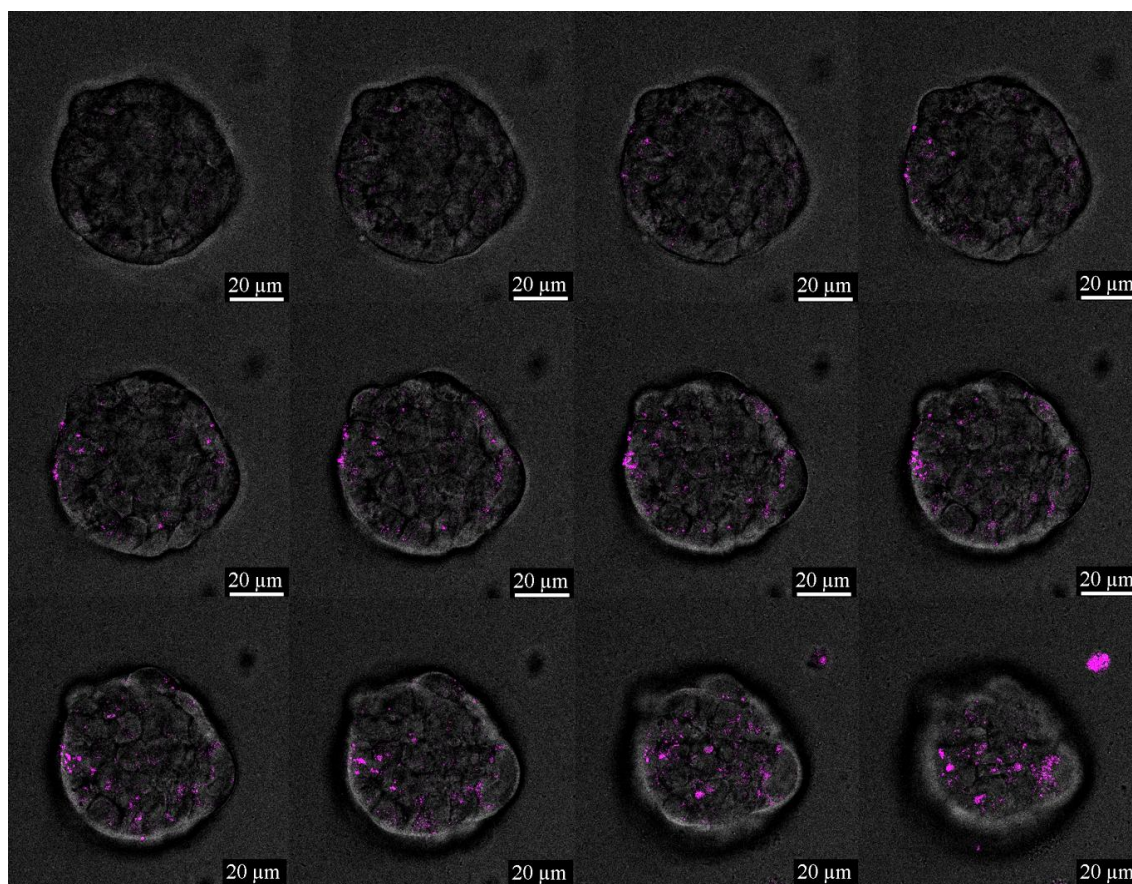


Figure S2.22 Z-stack images of Ru-bqp-MPP uptake in a HPAC spheroid (100 μM , 24 h) with brightfield contrast image overlay. Ex 490 nm & Em 580-800 nm (40x oil immersion lens).

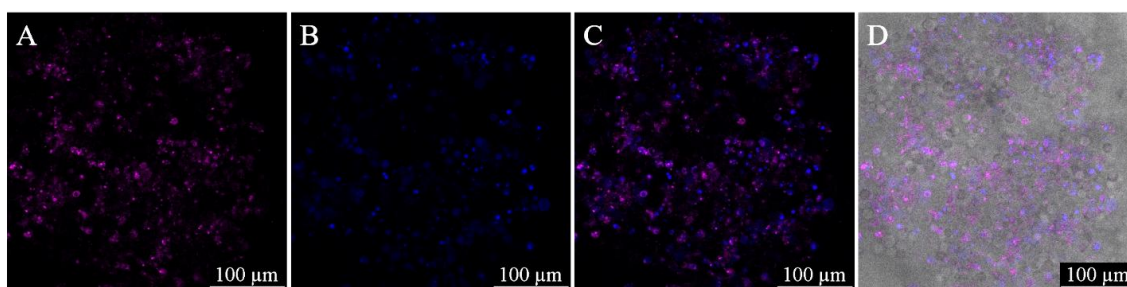


Figure S2.23 Individual z-stack image of the centre of a live HPAC spheroid with **(A)** 100 μM Ru-bqp-MPP, **(B)** 3 μM DRAQ7, **(C)** an overlay of B & C and **(D)** overlay with brightfield contrast image of the spheroid, imaged immediately after irradiation with a 470 nm LED ($4.27 \pm 0.41 \text{ J/cm}^2$). DRAQ7 uptake is linked to cellular death, but also cell damage. Spheroids were incubated overnight after irradiation to allow for cell recovery from radiation damage, control spheroids were shown to recover well.

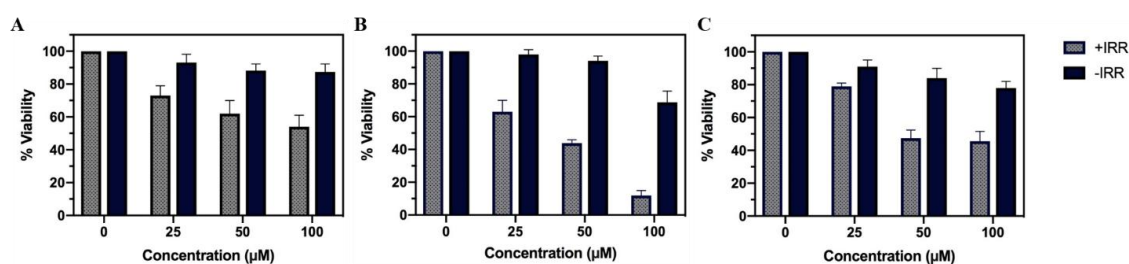


Figure S2.24 Dark and phototoxicity of our Ru(II) complexes in HPAC spheroids. The spheroids were incubated with **(A)** Ru-bqp-ester, **(B)** Ru-bqp-MPP and **(C)** Ru-bqp-R8 at 25, 50 and 100 μM for 24 h and irradiated with a 470 nm blue light LED at $4.27 \pm 0.41 \text{ J/cm}^2$. Dark control plates were performed alongside the phototoxicity assay and the dark and light IC_{50} values used to determine the PI values. Cell viability was assessed using the CellTiter-Glo 3D assay (N=3).

Appendix B: Supplementary Information – Chapter 3

S3.1 Synthesis and Characterisation (Performed by Dr. Lorcan Holden)

S3.1.1 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.

S3.1.2 [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. 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).

S3.1.3 Pic-COOH

To synthesise Pic-COOH the method developed previously by the Keyes group was used. 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).

S3.1.4 [Ru(bpy)₂(Pic-COOH)]²⁺

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 KNO₂) 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 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).

S3.1.5 Ru-RHAU

Ru-RHAU was synthesized using conjugation methods previously reported in our group. $[\text{Ru}(\text{bpy})_2(\text{Pic}-\text{COOH})]^{2+}$, 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 approximately 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, $\text{MeOD}/\text{D}_2\text{O}$) 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). m/z calculated = 3382.6494, found = 3382.5083.

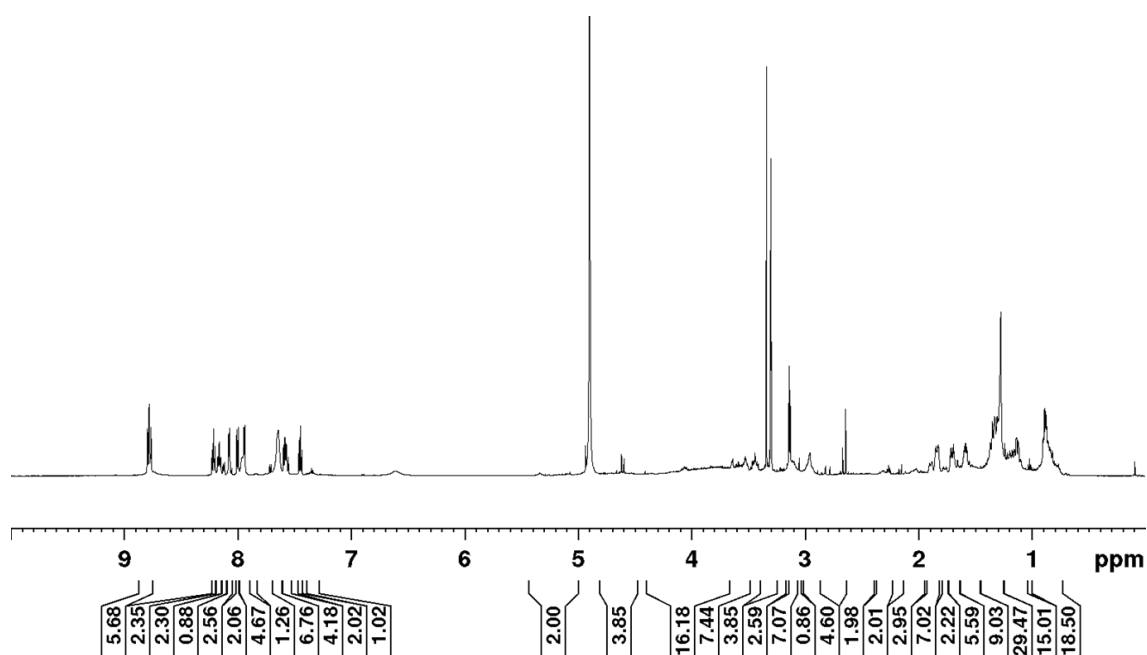


Figure S3.1 ^1H NMR of Ru-RHAU (CD_3OD , D_2O).

S3.1.6 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 Figure S3.2. Peak detection was recorded at 280 nm and 450 nm using a photodiode array detector. Figure S3.2 shows the peaks obtained and their respective retention times at 280 nm. UV-Vis spectra of each peak was also recorded.

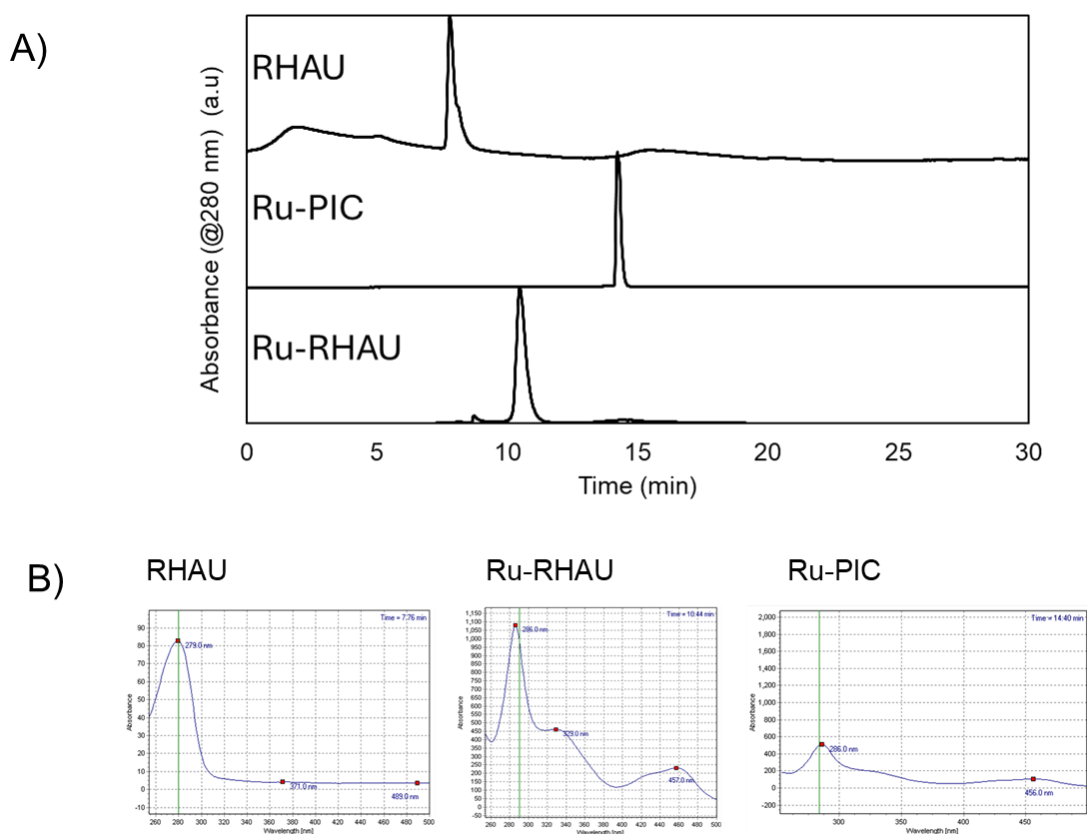


Figure S3.2 (A) HPLC chromatograms of RHAU, Ru-PIC and Ru-RHAU. **(B)** Absorbance spectra gathered from the PDAD from 280 to 500 nm.

Table S3.1 Panel of oligonucleotides selected for testing.

Name	Secondary structure	Sequence 5'→3'
22AG	Mixed hybrid	AGGGTTAGGGTTAGGGTTAGGG
KRAS	Parallel	AGGGCGGTGTGGGATAGGGAA
CMYC	Parallel	TGGGGAGGGTGGGGAGGGTGGGGAAGG
Pu24T	Parallel	TGAGGGTGGTGTAGGGTGGGGAAGG
NRAS	Parallel	UGUGGGAGGGGCGGGUCUGGGUGC
ds26	Double Stranded	CAATCGGATCGAATTCGATCCGATTG
ctDNA	Double stranded	

S3.1.7 Spectroscopic Studies

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 deionised 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 fluorescence and absorbance titration experiments were performed in tandem using a stock concentration of 1 mM 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 µM 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.

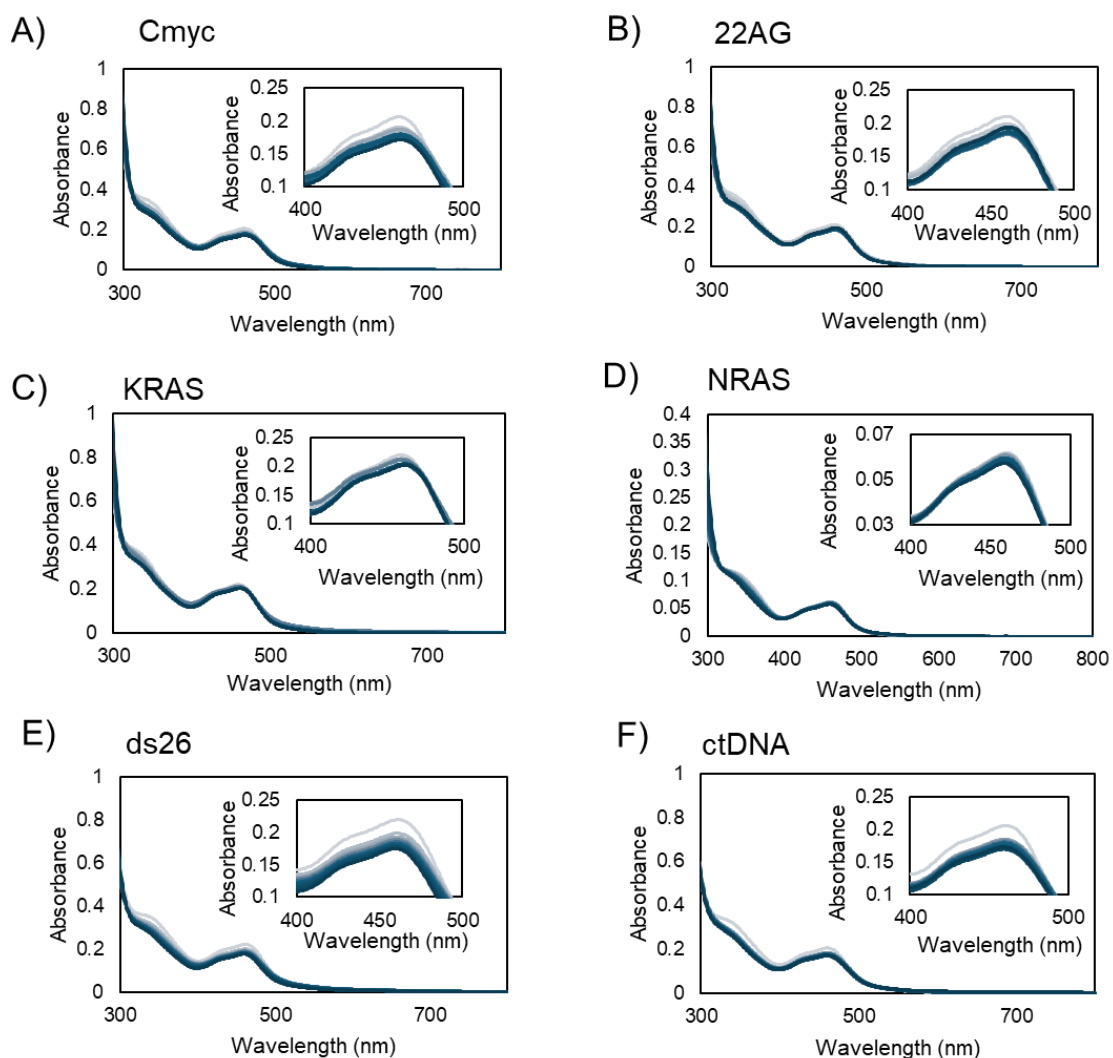


Figure S3.3 Representative absorption spectra obtained from a series of DNA titrations. Ru-RHAU (10 μ M) was titrated with the DNA G4s CMYC, KRAS, 22AG (0- 30 μ M) and the duplex DNA ds26 and ctDNA (0-50 μ M base pairs). Ru-RHAU (2 μ M) titration with the rG4 NRAS (0-6 μ M) in RNase free buffer. Insets of all graphs show the expanded MLCT region from 400 to 500 nm.

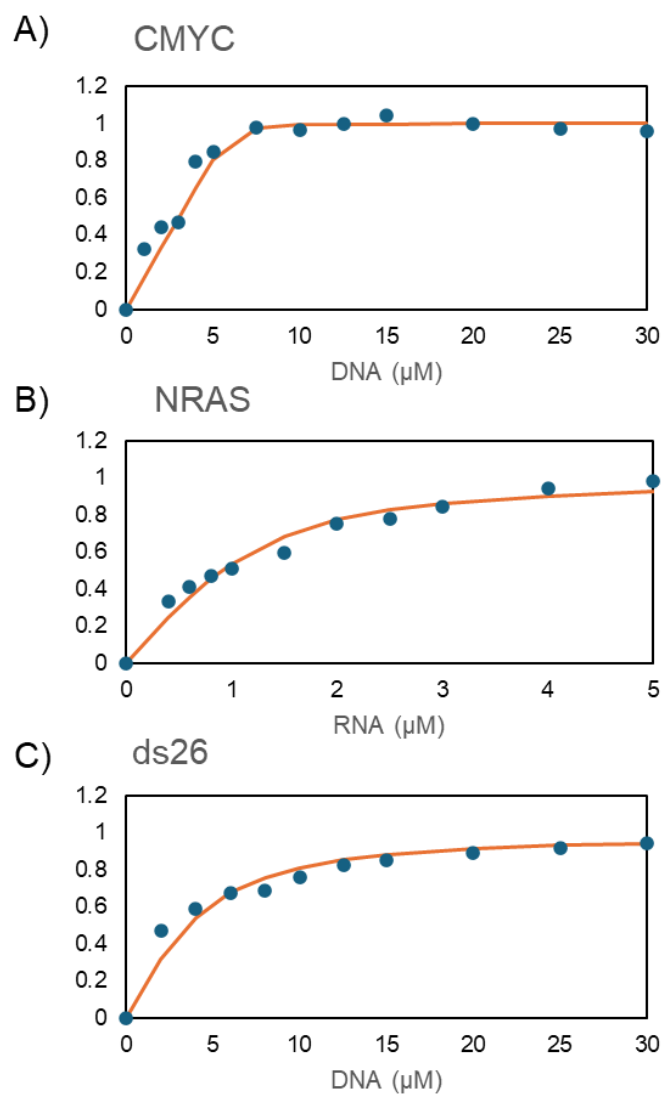


Figure S3.4 Representative absorption titrations of Ru-RHAU with CMYC, NRAS and ds26 and their respective binding curves.

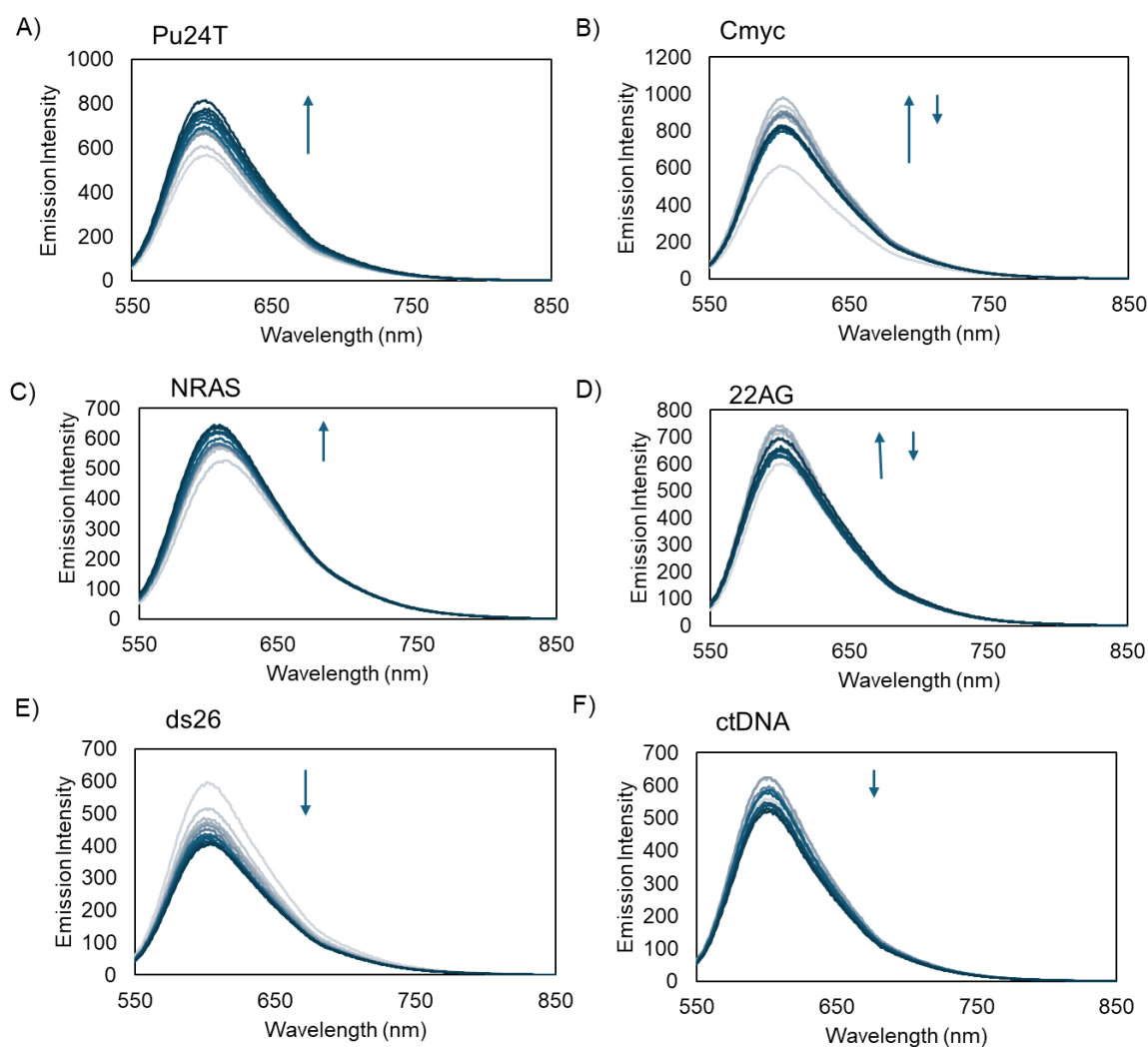


Figure S3.5 Representative emission spectra obtained from a series of DNA titrations. Ru-RHAU (10 μ M) was titrated with the DNA G4s CMYC, KRAS, 22AG (0- 30 μ M) and the duplex DNA ds26 and ctDNA (0-50 μ M base pairs). Ru-RHAU (2 μ M) titration with the rG4 NRAS (0-6 μ M) in RNase free buffer. Ru-RHAU was excited at 490 nm. Arrows indicate the increase or decrease in emission intensity upon addition of oligonucleotides. A second arrow indicates a small decrease in intensity that occurs after reaching an emission maximum with the further addition of oligonucleotides.

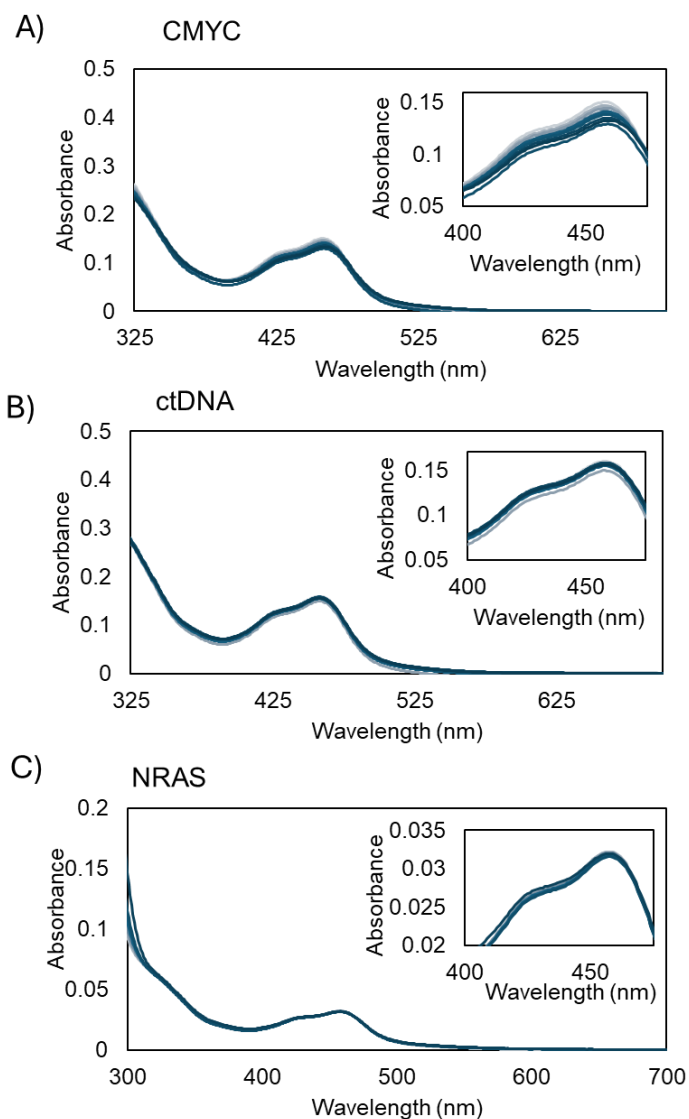


Figure S3.6 Representative absorption spectra of Ru-PIC (10 μM) when titrated with **(A)** CMYC (0-30 μM) and **(B)** ctDNA (0-50 μM). **(C)** Representative luminescence spectra of Ru-PIC (2 μM) when titrated with NRAS (0-6 μM). The inset figures show expanded MLCT regions from 400 to 500 nm.

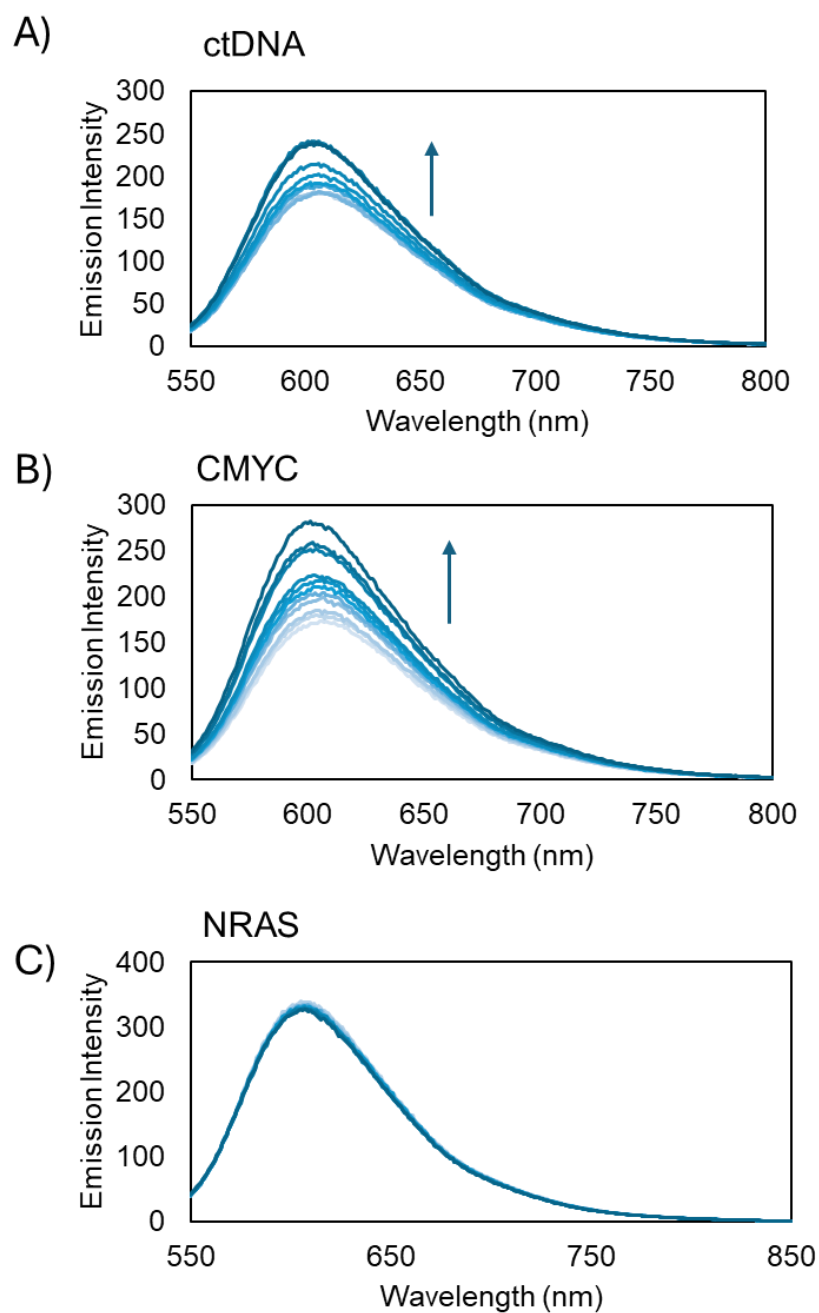


Figure S3.7 Representative luminescence spectra of Ru-PIC (10 μ M) when titrated with **(A)** ctDNA (0-50 μ M) and **(B)** CMYC (0-30 μ M). Excitation at 490 nm (Slit widths 5 nm). **(C)** Representative luminescence spectra of Ru-PIC (2 μ M) when titrated with NRAS (0-6 μ M) (Slit widths 10 nm).

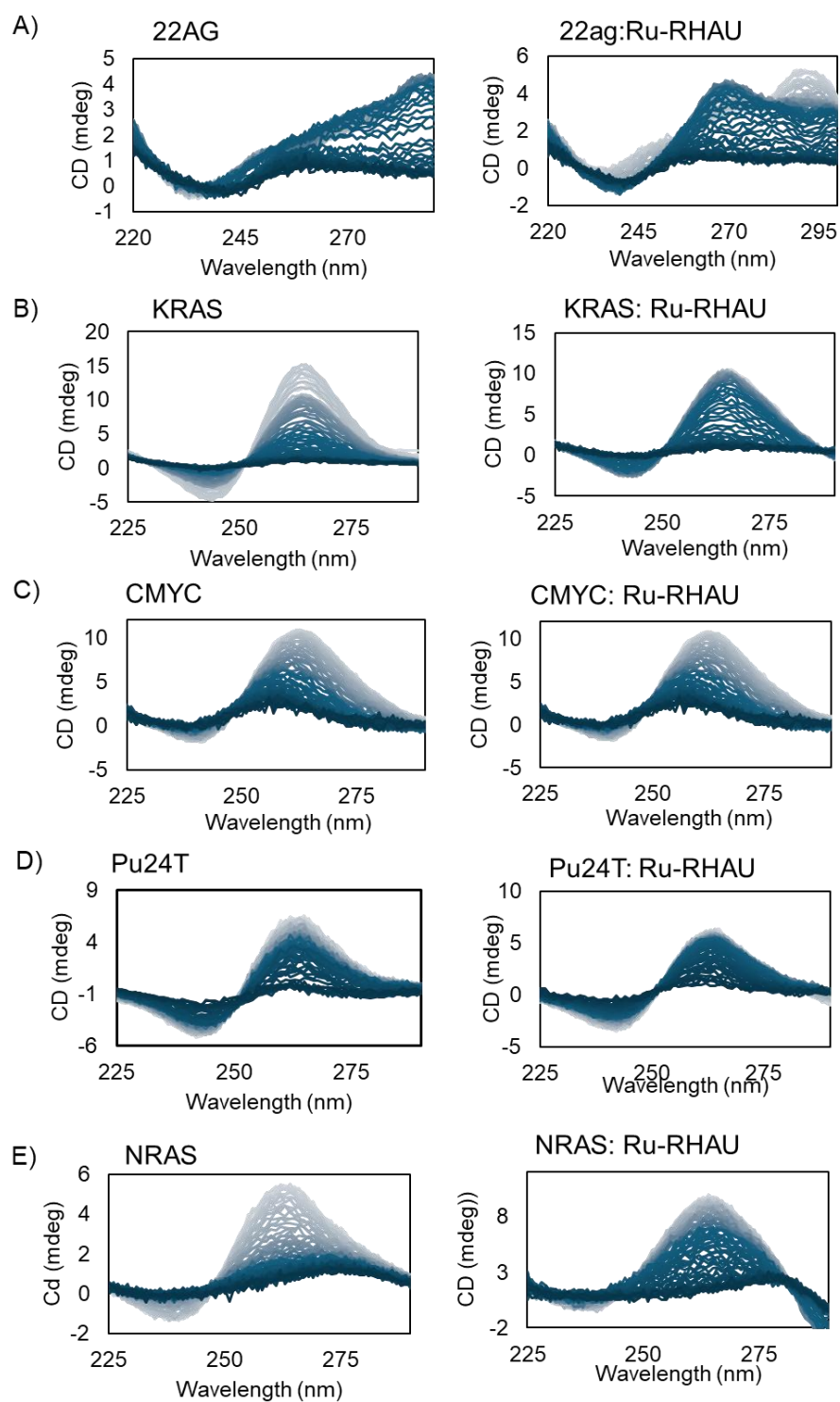


Figure S3.8 Melting curves of G4s at (5 μ M) and when bound to Ru-RHAU (10 μ M), **(A)** 22AG, **(B)** KRAS, **(C)** CMYC, **(D)** PU24T and **(E)** NRAS. Spectra are measured for every increase in 1 $^{\circ}$ C from 20 $^{\circ}$ C to 94 $^{\circ}$ C across a window of 225 nm to 290 nm.

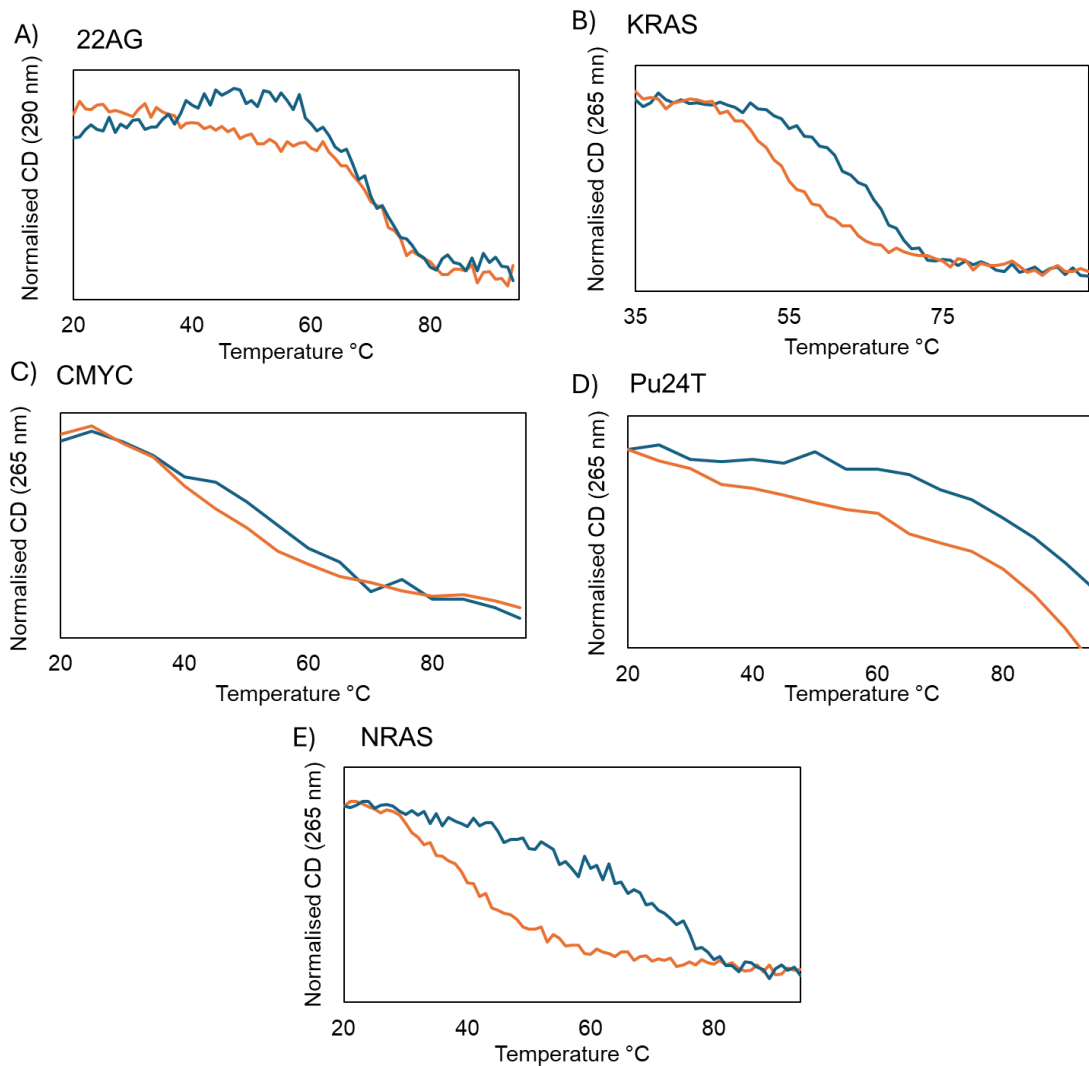


Figure S3.9 Comparison of thermal stability of G4s (5 μ M) when unbound (orange) and bound (blue) to Ru-RHAU (10 μ M).

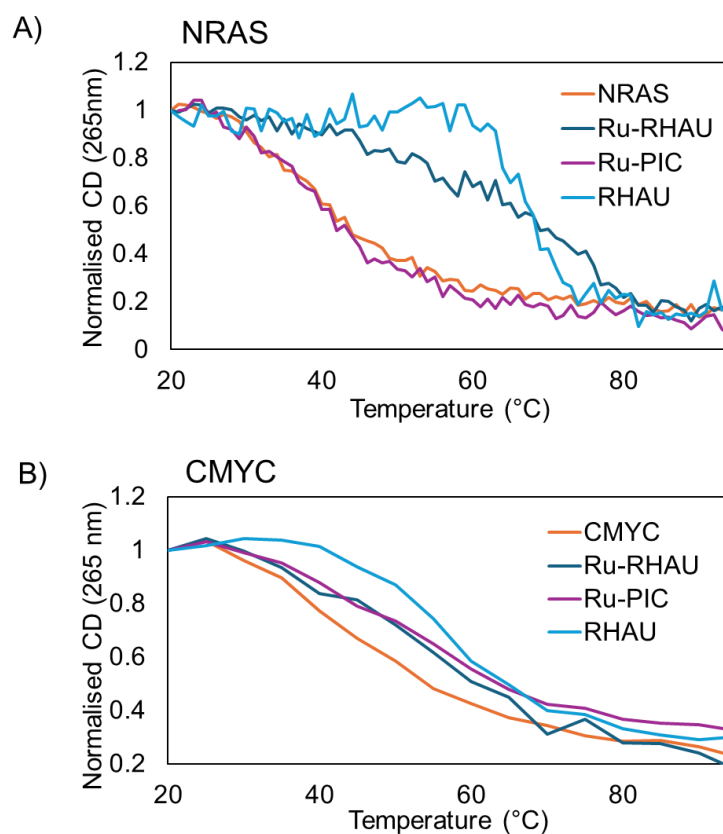


Figure S3.10 (A) Normalised changes in the CD signal of NRAS (μM) at 265 nm when bound to 2 equivalents of RHAU, Ru-PIC or Ru-RHAU ($10\ \mu\text{M}$). **(B)** Normalised changes in the CD signal of CMYC (μM) at 265 nm when bound to 2 equivalents of RHAU, Ru-PIC or Ru-RHAU ($10\ \mu\text{M}$). Increasing temperature from 20 °C to 94 °C at a rate of 1 °C/min.

S3.2 Cellular Studies

S3.2.1 Cellular Uptake and SG Assembly

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-RHAU, 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 L-glutamine and 10% FBS. Cells were imaged using a Leica TCS SP8 DMI8 confocal microscope (63× or 100x oil immersion objective lens) with a heated stage at 37 °C. Ru-RHAU 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-RHAU under the following conditions: 10, 15, 25, 30, 35, 40, 50 and 100 μM at 6 h incubation, 25, 30, 35, 40 μM for 8 h incubation and 10, 15, 25, 30, 40, 50 μM for 24 h. DRAQ7 (3 μM) was added each well prior to imaging to determine if the cells were damaged under the imaging conditions being tested. MCF-7 cells were seeded at 1×10^5 cells/mL in 8 chamber slides and allowed to grow for 48 h at 37 °C with 5% CO₂ and treated with Ru-RHAU at 40 μM (6 h) or 25 μM (24 h).

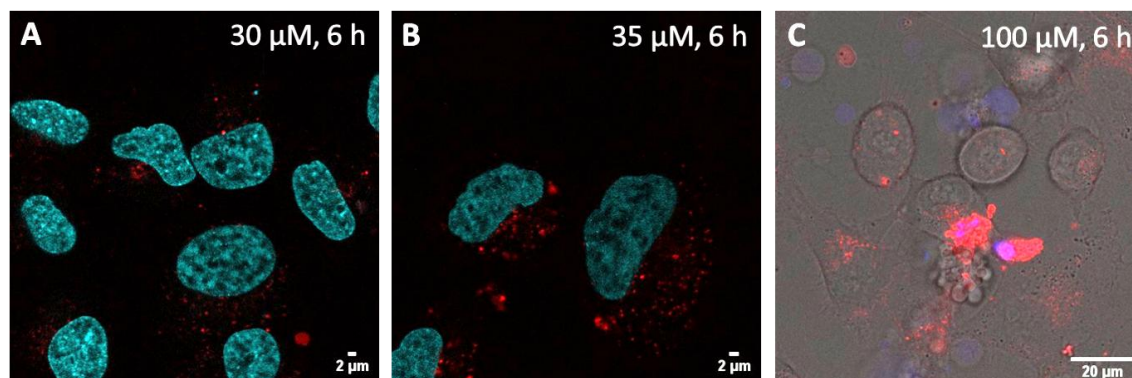


Figure S3.11 Confocal images of **HeLa** cells after a 6 h incubation with Ru-RHAU at **(A)** 30 μM, **(B)** 35 μM and **(C)** 100 μM.

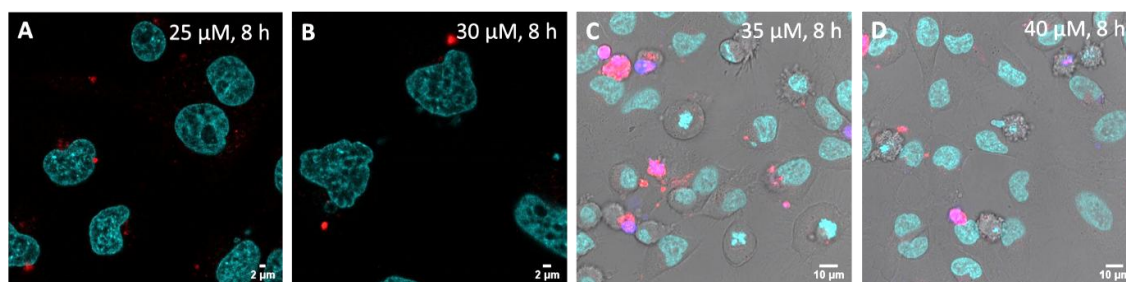


Figure S3.12 Confocal images of **HeLa** cells after an 8 h incubation with Ru-RHAU at (A) 25 μM, (B) 30 μM and (C) 35 μM and (D) 40 μM. 63x oil lens.

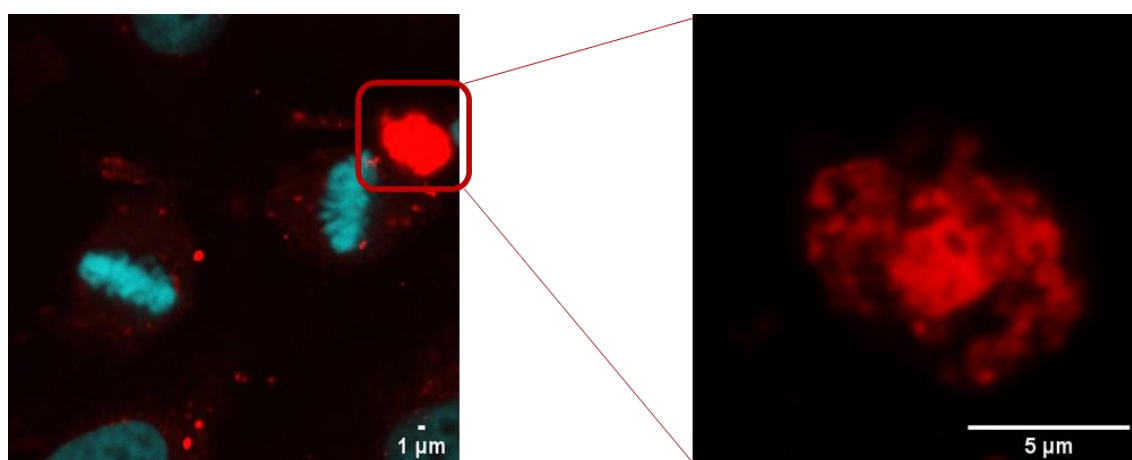


Figure S3.13 Confocal image of **HeLa** cells in telophase with corresponding zoomed image of large SG imaged at lower intensity to allow for better visualisation of the SG contents.

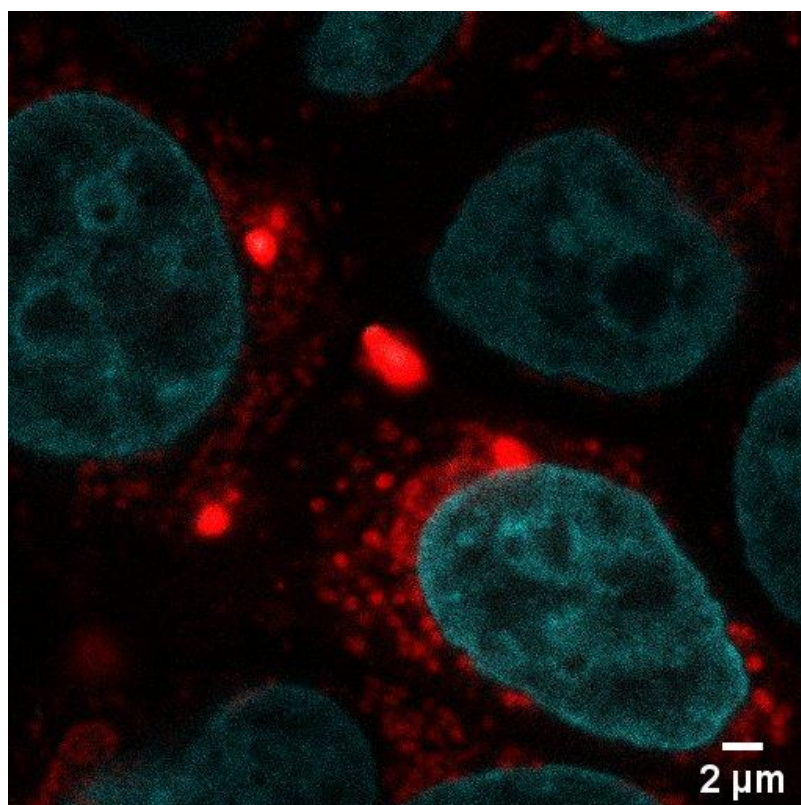


Figure S3.14 Confocal image of Ru-RHAU (40 μ M, 6 h) in live **MCF-7** cells. Hoechst 33342 (cyan) indicates the nucleus. 100x oil.

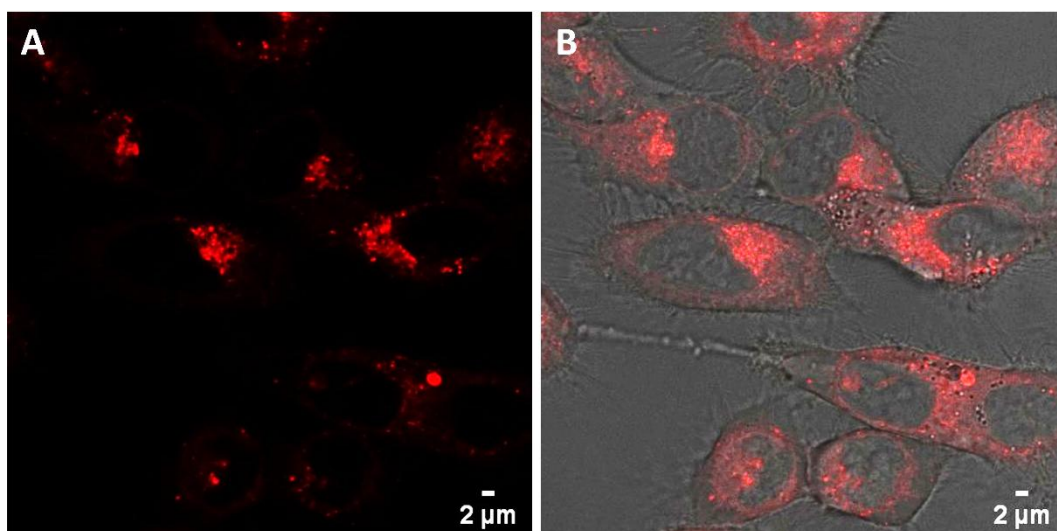


Figure S3.15 (A) Live **HeLa** cells pre-treated with Oligomycin and 2DG prior to Ru-RHAU (25 μ M, 24 h) and **(B)** the overlay with phase contrast channel.

S3.2.2 Localisation in Live HeLa Cells

Co-localisation studies were performed in HeLa cells with LysoTracker Deep Red lysosomal dye (100 nM, 1 h) with a WLL at 647 nm and emission collected between 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-RHAU at 40 μ M for 6 h or 25 μ M for 24 h. After the incubation period 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-RHAU was excited at 488 nm and emission collected between 580-645 nm. Co-localisation analysis was performed using ImageJ software.

Table S3.2 Co-localisation results of Ru-RHAU with LysoTracker Deep Red in live HeLa cells.

Concentration of Ru-RHAU (μ M)	Incubation time (h)	Commercial Dye	Organelle	Pearson's Coefficient r =
25	24	LysoTracker	Lysosomes	0.52 ± 0.08
40	6	Deep Red		0.47 ± 0.06

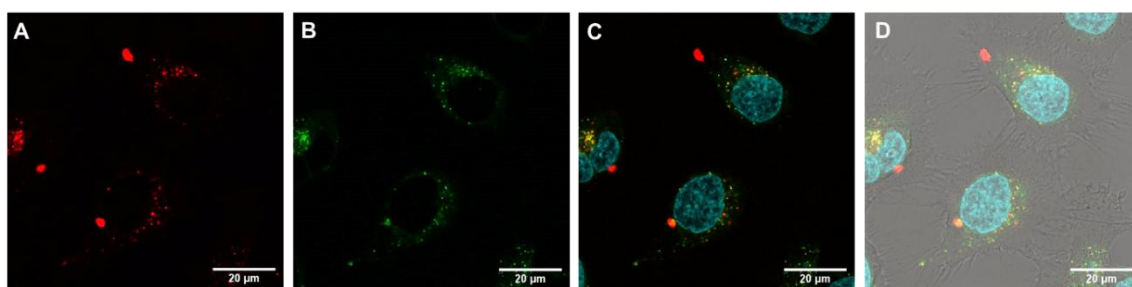


Figure S3.16 Confocal imaging of **(A)** Ru-RHAU (25 μ M, 24 h), **(B)** LysoTracker Deep Red (100 nM, 45 min) and **(C)** overlay of A and B with Hoechst 33342 nuclear stain (1 μ g/mL, 15 min) and **(D)** phase contrast in live **HeLa** cells. 100x oil.

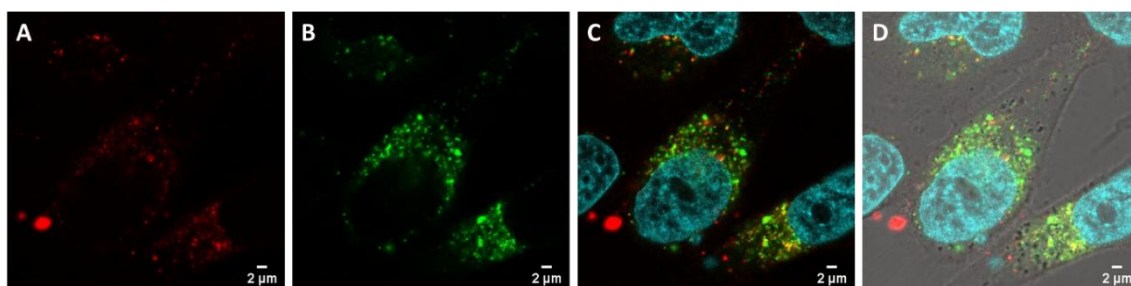


Figure S3.17 Confocal imaging of **(A)** Ru-RHAU (40 μ M, 6 h), **(B)** LysoTracker Deep Red (100 nM, 45 min) and **(C)** overlay of A and B with Hoechst 33342 nuclear stain (1 μ g/mL, 15 min) and **(D)** phase contrast in live **HeLa** cells. 100x oil.

S3.2.3 Super-Resolution Imaging

Live and fixed HeLa cells were prepared as per routine cell imaging studies for super-resolution imaging using either the Zeiss LSM 980, Airyscan 2 (Figure 3.5) and for routine confocal imaging using the Leica DMI8 confocal microscope to depict Ru-RHAU intensity in SGs as a comparison to the Airyscan 2 imaging.

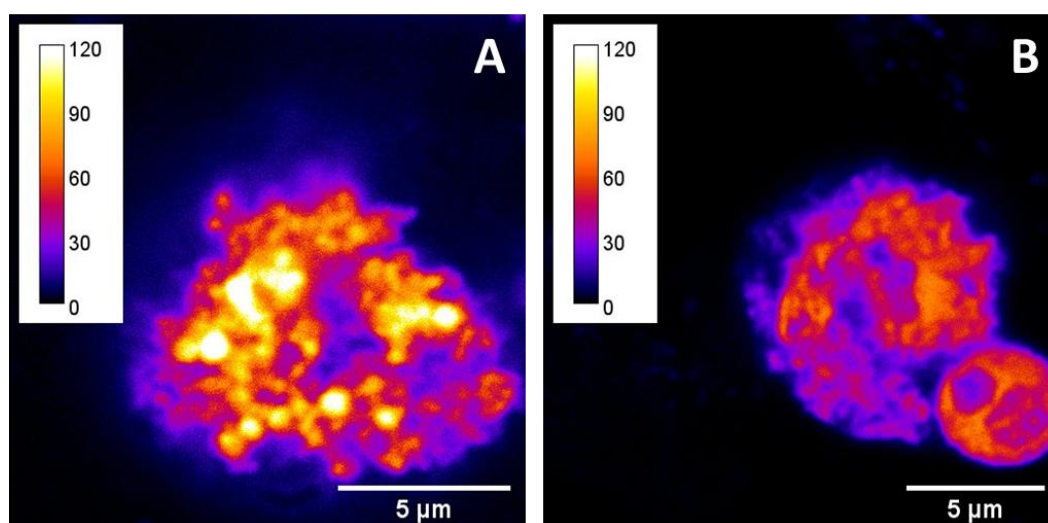


Figure S3.18 (A) and **(B)** depict confocal images of Ru-RHAU (25 μ M, 24 h) in live **HeLa** cells, showing Ru-RHAU intensity throughout the SGs. These images indicate a similar intensity pattern as in the LSM airyscan super-resolution imaging shown in Figure 3.5 and were acquired using the Leica DMI8 confocal microscope.

S3.2.4 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, Ø: 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-RHAU at 40 µM for 6 h or 25 µM for 24 h before fixation.

Cells were fixed with 4% paraformaldehyde (BioLegend) for 15 minutes at room temperature (RT), and subsequently permeabilised 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 RT in the dark and sealed prior to confocal imaging.

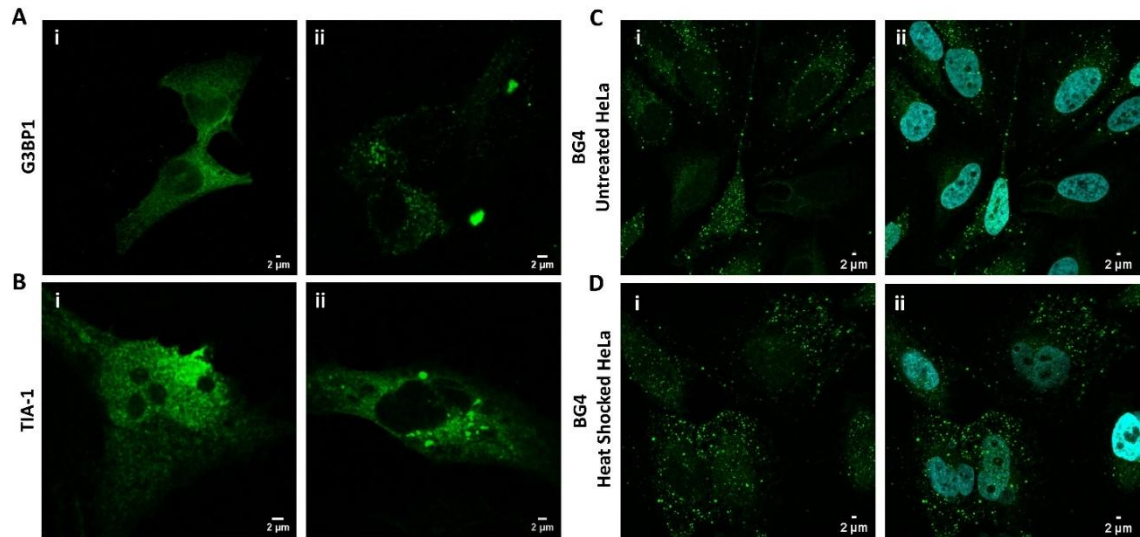


Figure S3.19 Immunostaining controls in fixed **HeLa** cells showing **(A)** G3BP1 and **(B)** TIA-1 in **(i)** untreated cells and **(ii)** cells treated by heat shock (42 °C, 1 h) prior to fixation and **(C)** untreated control cells with BG4 or cells treated by heat shock prior to fixation and BG4 staining (with **(i)** and without **(ii)** Hoechst 33342 nuclear staining).

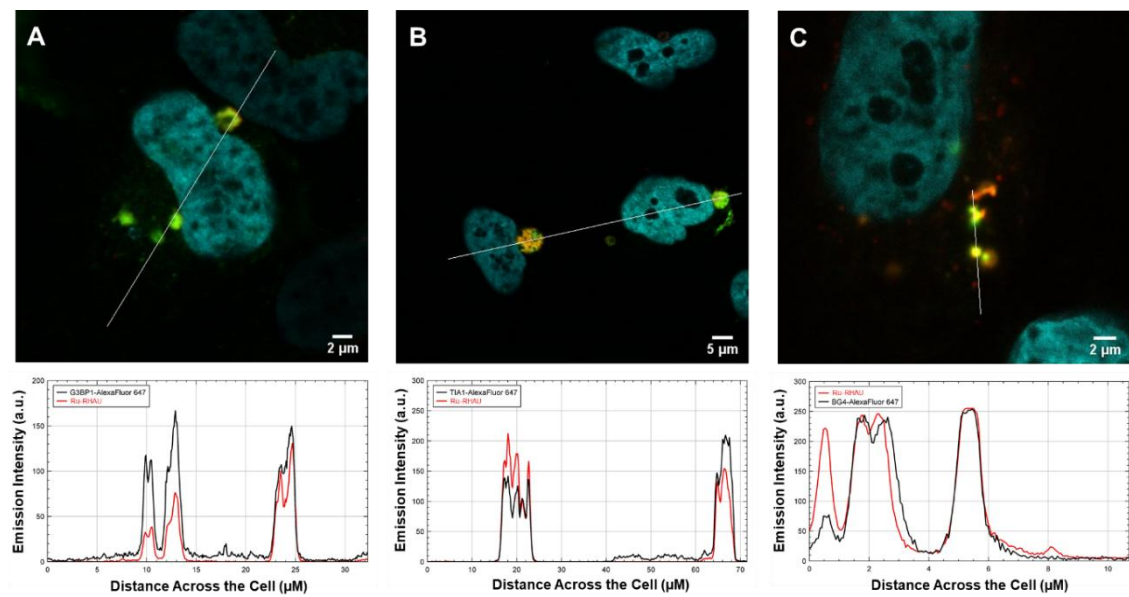


Figure S3.20 Co-localisation of **HeLa** cells with Ru-RHAU (25 μM, 24 h) and **(A)** G3BP1, **(B)** TIA-1 or **(C)** BG4. The ROI used to generate the co-localisation graphs is indicated by the white lines in each image.

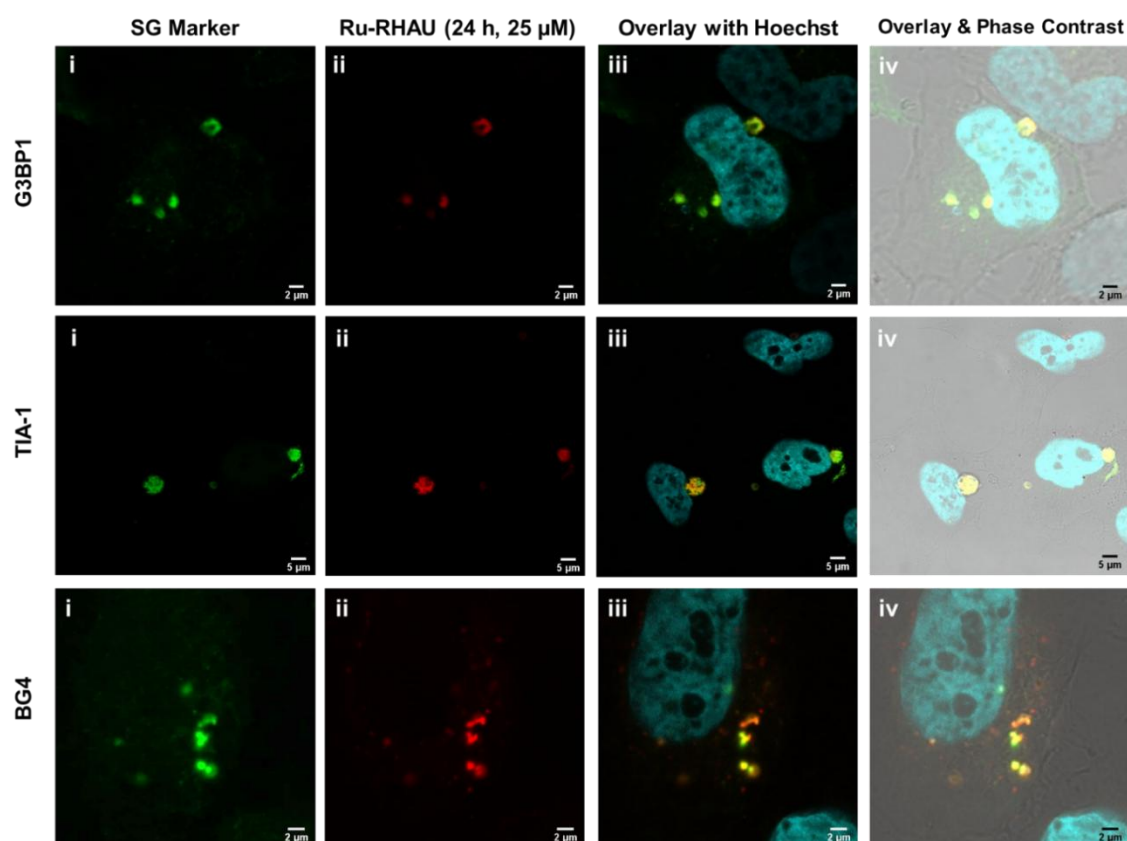


Figure S3.21 Confocal imaging of immunostaining of the individual channels in Figure S3.20, where **(i)** depicts antibody staining with AlexaFluor 647, **(ii)** shows Ru-RHAU staining and **(iii-iv)** is the overlay of **(i)** and **(ii)** with Hoechst 33342 nuclear stain (2 μ g/mL, 7 min) and the corresponding phase contrast image. 100x oil.

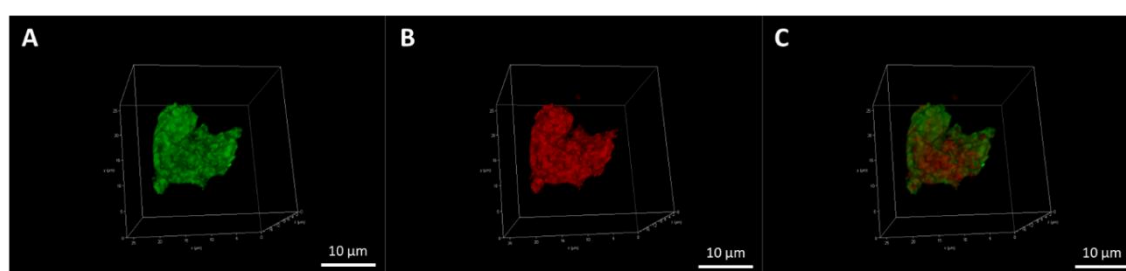


Figure S3.22 3D rendering of a SG in a HeLa cell stained with **(A)** TIA-1 (1:50), **(B)** Ru-RHAU (25 μ M, 24 h) and **(C)** the overlay. 100x oil.

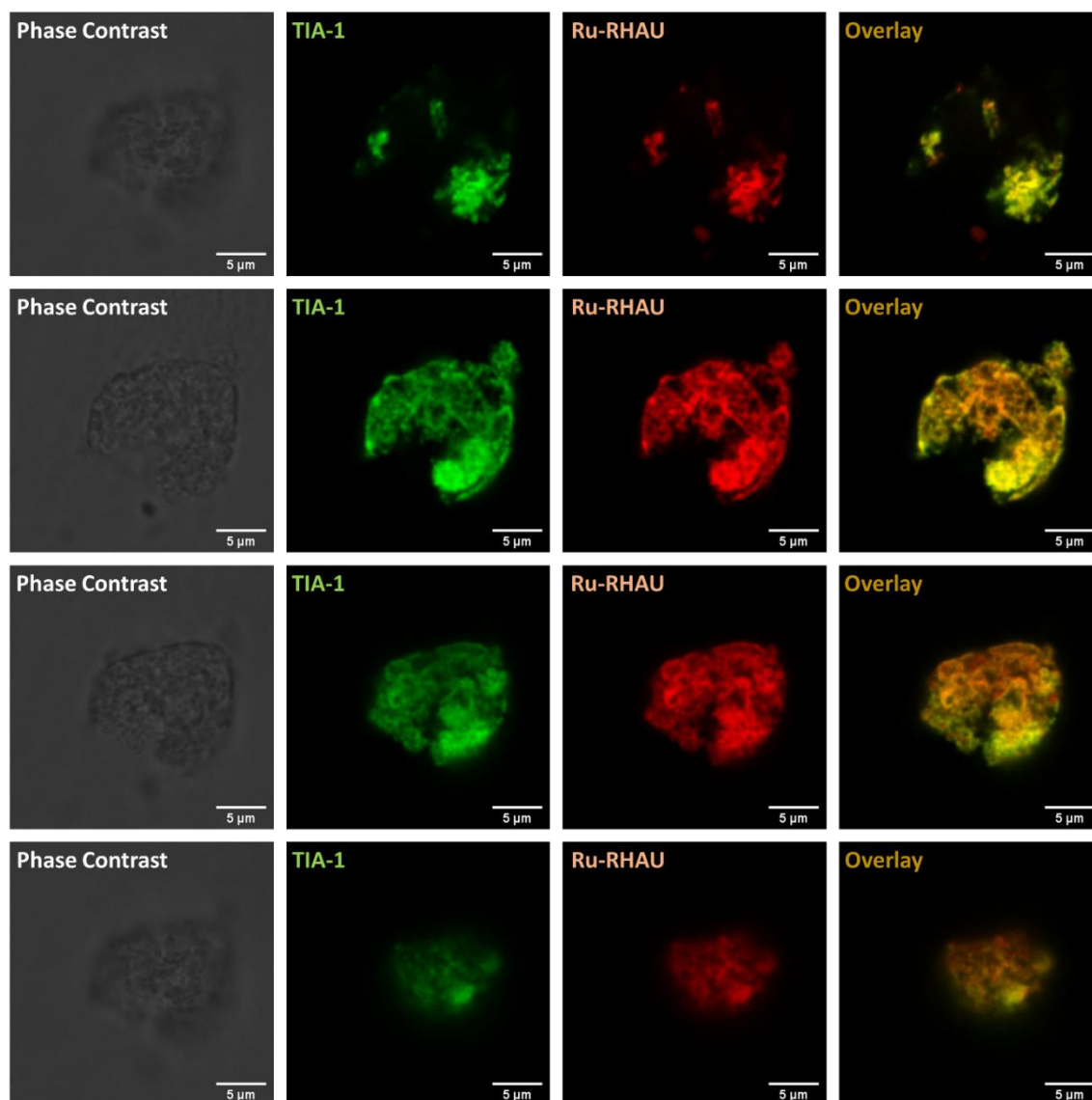


Figure S3.23 Confocal imaging of individual z's (TIA-1, Ru-RHAU, the overlay and corresponding phase contrast channel) used to create a 3D reconstruction image in Figure S3.22.

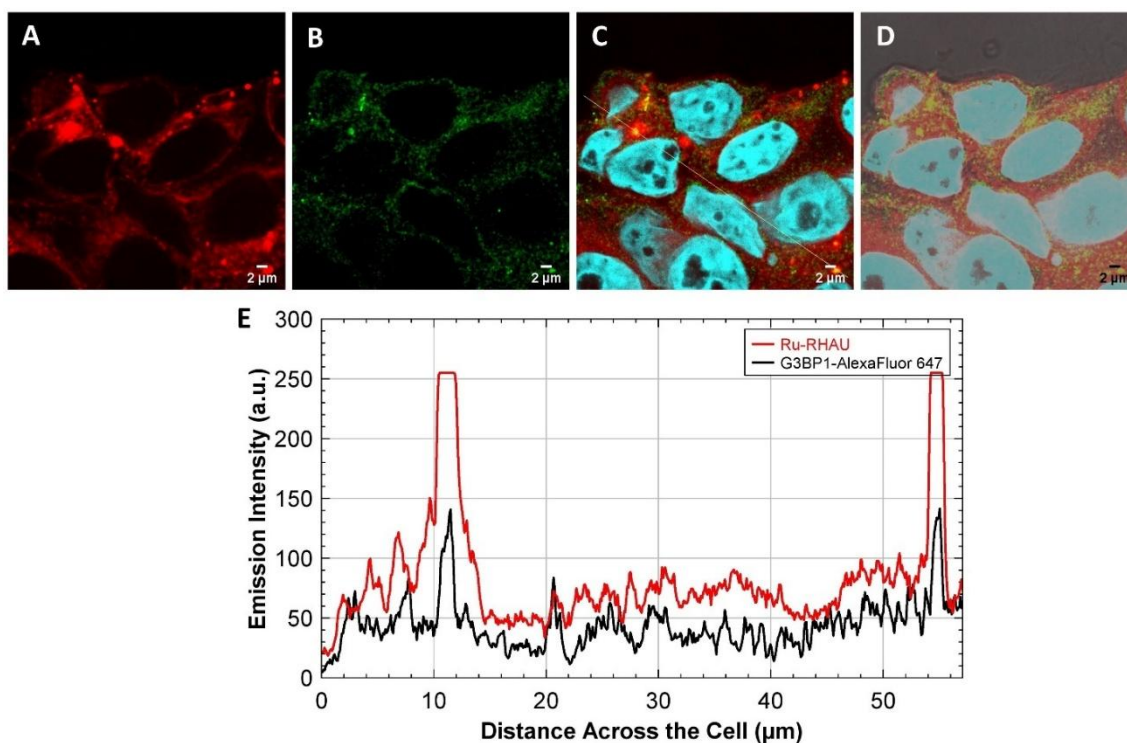


Figure S3.24 Confocal imaging of **MCF-7** cells treated with **(A)** Ru-RHAU (40 μM, 6 h) and immunostained with **(B)** G3BP1 where **(C)** is the overlay of the channels with Hoechst 33342 nuclear stain (2 μg/mL, 7 min) and **(D)** with the corresponding phase contrast image. The plot profile **(E)** was generated using the ROI indicated by the white line in (C). 100x oil.

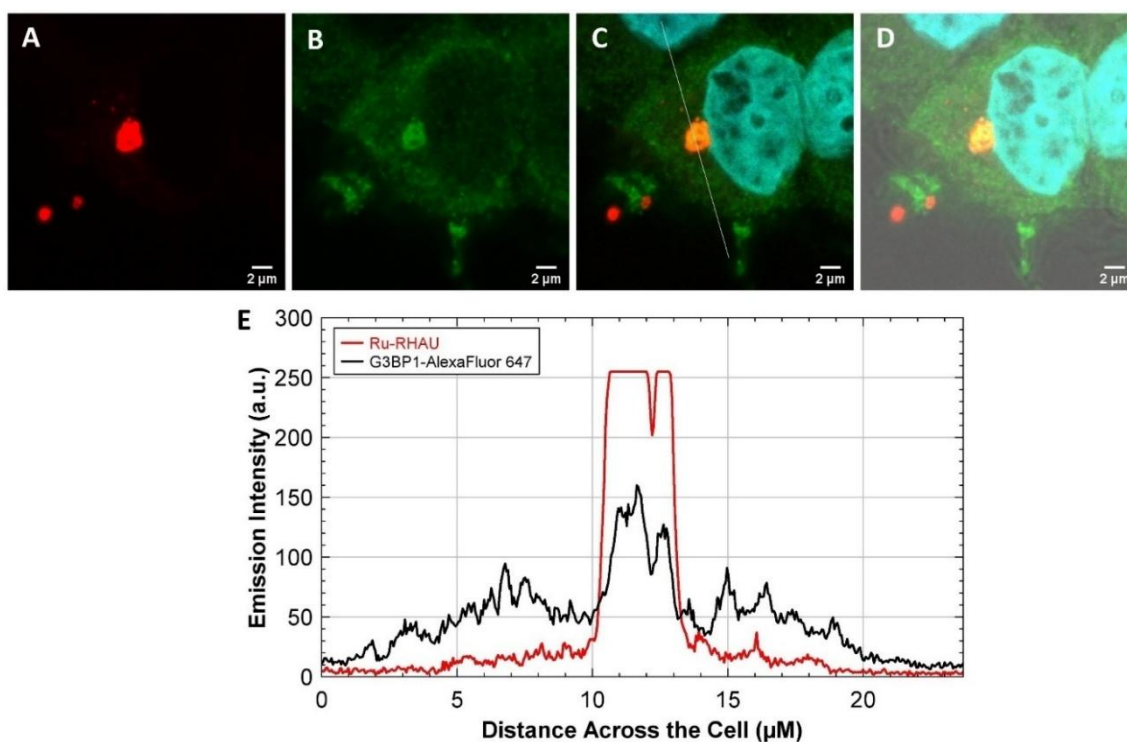


Figure S3.25 Confocal imaging of **MCF-7** cells treated with **(A)** Ru-RHAU (25 μM , 24 h) and immunostained with **(B)** G3BP1 where **(C)** is the overlay of the channels with Hoechst 33342 nuclear stain (2 $\mu\text{g/mL}$, 7 min) and **(D)** with the corresponding phase contrast image. The plot profile **(E)** was generated using the ROI indicated by the white line in **(C)**. 100x oil.

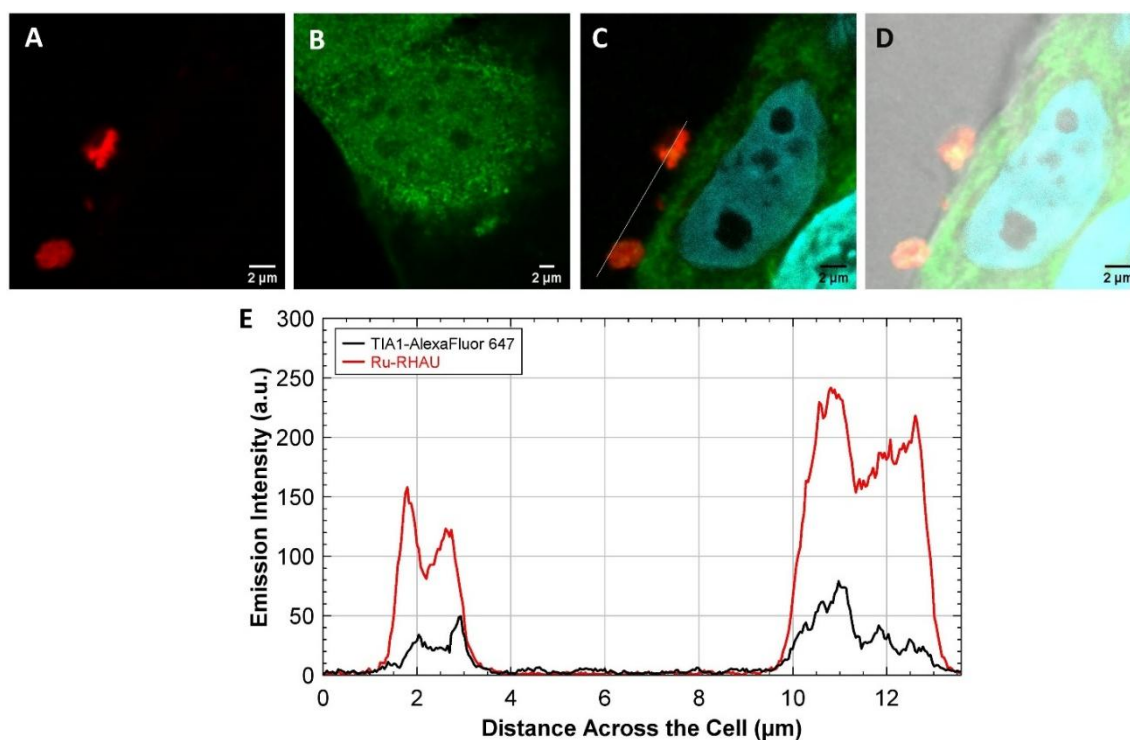


Figure S3.26 Confocal imaging of **MCF-7** cells treated with **(A)** Ru-RHAU (40 μM, 6 h) and immunostained with **(B)** TIA-1 where **(C)** is the overlay of the channels with Hoechst 33342 nuclear stain (2 μg/mL, 7 min) and **(D)** with the corresponding phase contrast image. The plot profile **(E)** was generated using the ROI indicated by the white line in **(C)**. 100x oil.

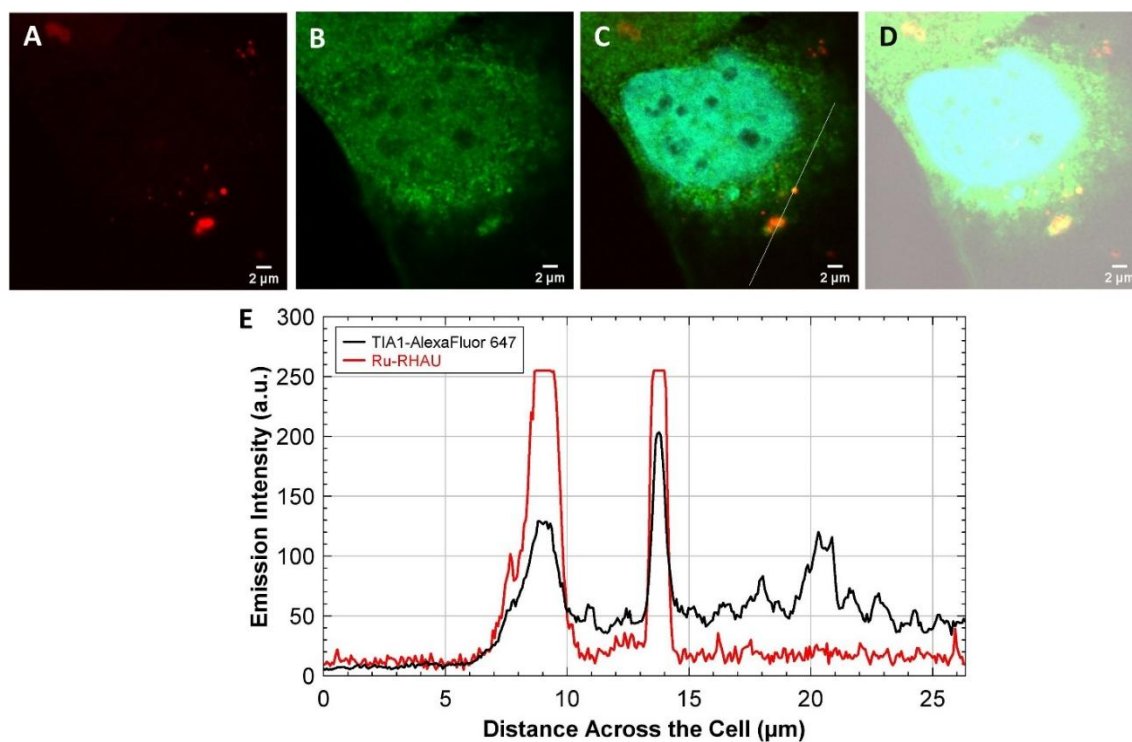


Figure S3.27 Confocal imaging of **MCF-7** cells treated with **(A)** Ru-RHAU (25 μM, 24 h) and immunostained with **(B)** TIA-1 where **(C)** is the overlay of the channels with Hoechst 33342 nuclear stain (2 μg/mL, 7 min) and **(D)** with the corresponding phase contrast image. The plot profile **(E)** was generated using the ROI indicated by the white line in **(C)**. 100x oil.

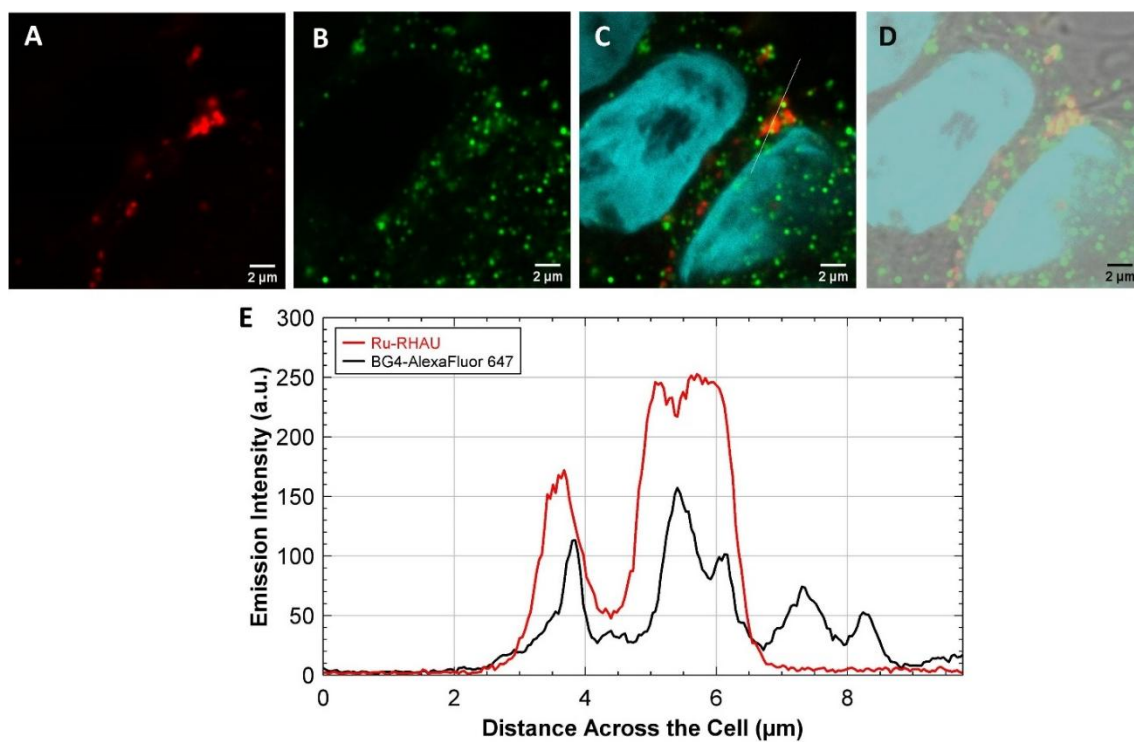


Figure S3.28 Confocal imaging of **MCF-7** cells treated with **(A)** Ru-RHAU (40 μM , 6 h) and immunostained with **(B)** BG4 where **(C)** is the overlay of the channels with Hoechst 33342 nuclear stain (2 $\mu\text{g/mL}$, 7 min) and **(D)** with the corresponding phase contrast image. The plot profile **(E)** was generated using the ROI indicated by the white line in **(C)**. 100x oil.

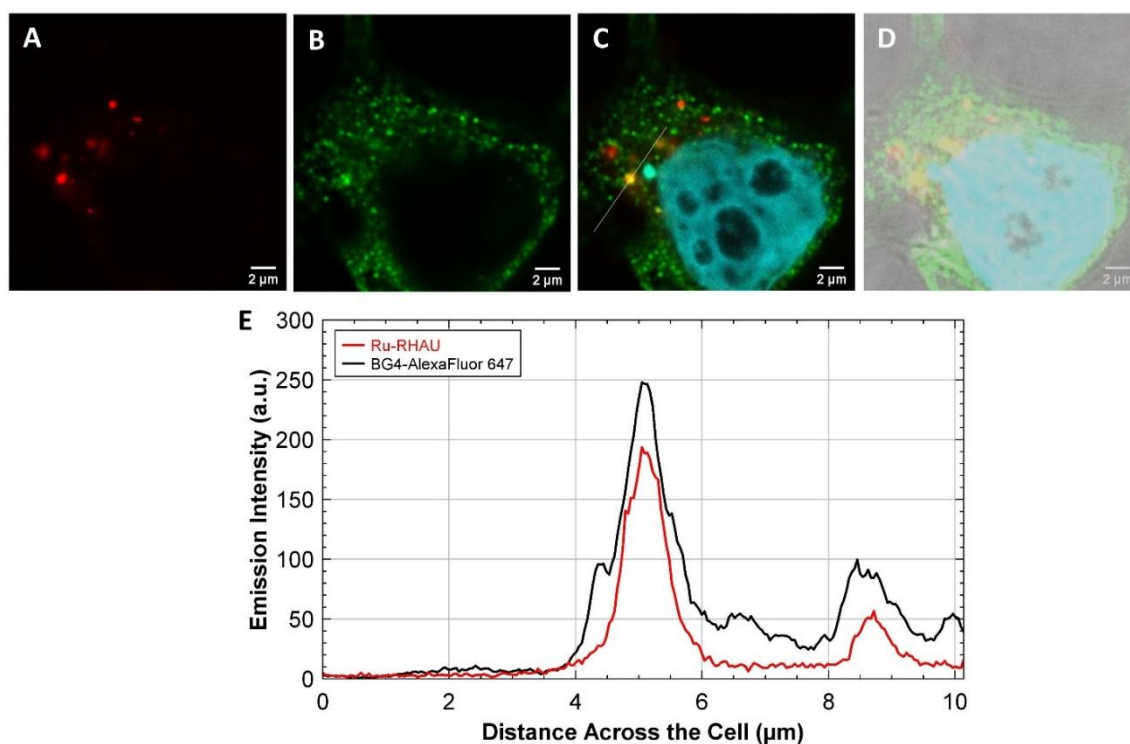


Figure S3.29 Confocal imaging of **MCF-7** cells treated with **(A)** Ru-RHAU (25 μ M, 24 h) and immunostained with **(B)** BG4 where **(C)** is the overlay of the channels with Hoechst 33342 nuclear stain (2 μ g/mL, 7 min) and **(D)** with the corresponding phase contrast image. The plot profile **(E)** was generated using the ROI indicated by the white line in **(C)**. 100x oil.

S3.2.5 SG Disassembly

HeLa cells were prepared as per routine imaging studies and incubated with either 40 μ M (6 h) or 25 μ M Ru-RHAU (24 h). Cells were imaged every 15 minutes for 2.5 h to monitor the disassembly of SGs over time. Hoechst 33342 was added to stain the nucleus prior to imaging. N=2.

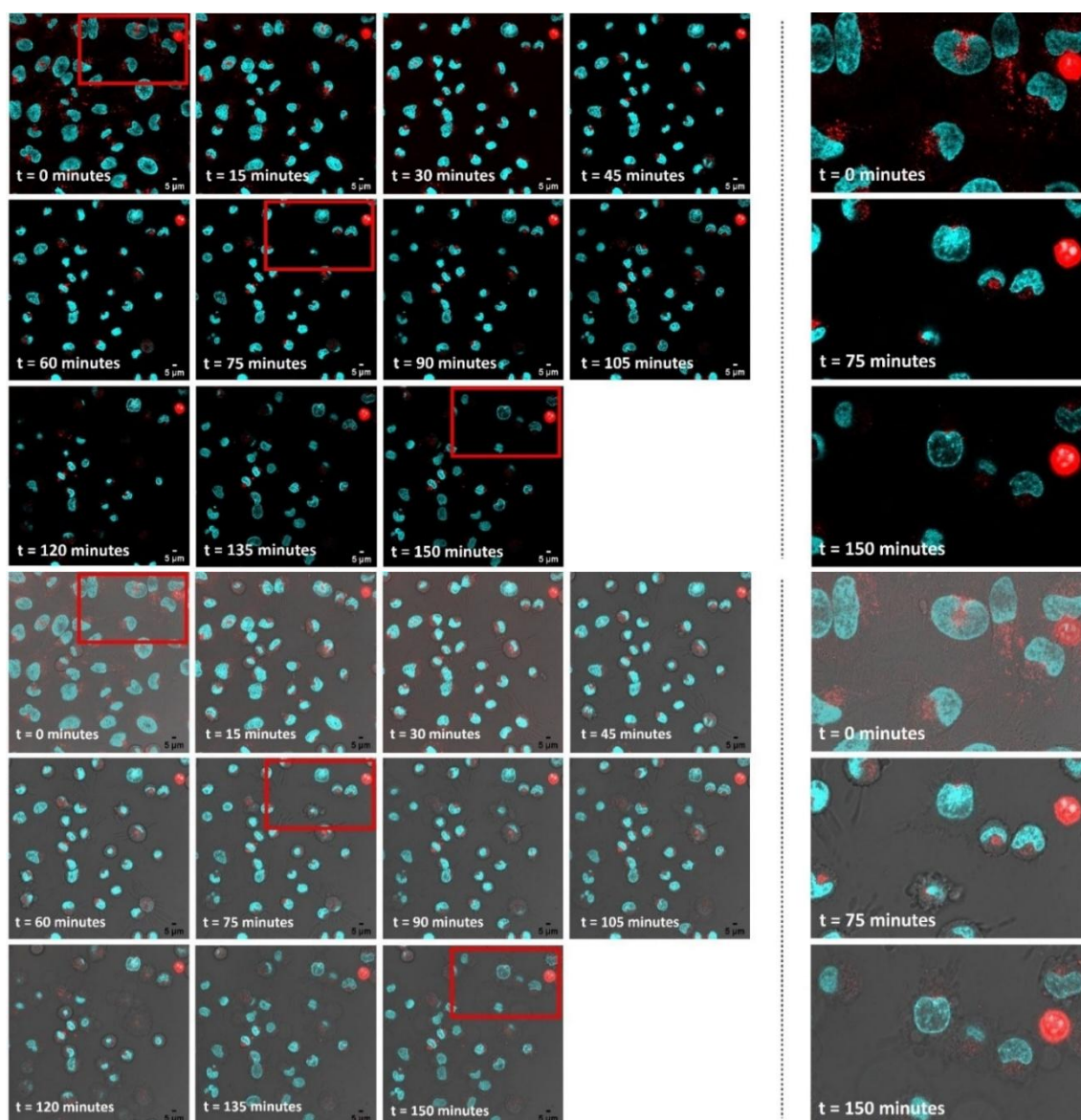


Figure S3.30 Disassembly over Ru-RHAU (40 μ M, 6 h) in live HeLa cells over time with corresponding phase contrast images in gray, indicating changes in cell morphology over time. Timings are indicated on each image where t = the time since Ru-RHAU has been removed. Zoomed images at t = 0, 75 and 150 minutes are indicated by a red box and shown in the columns on the right. Scale bars read 5 μ m.

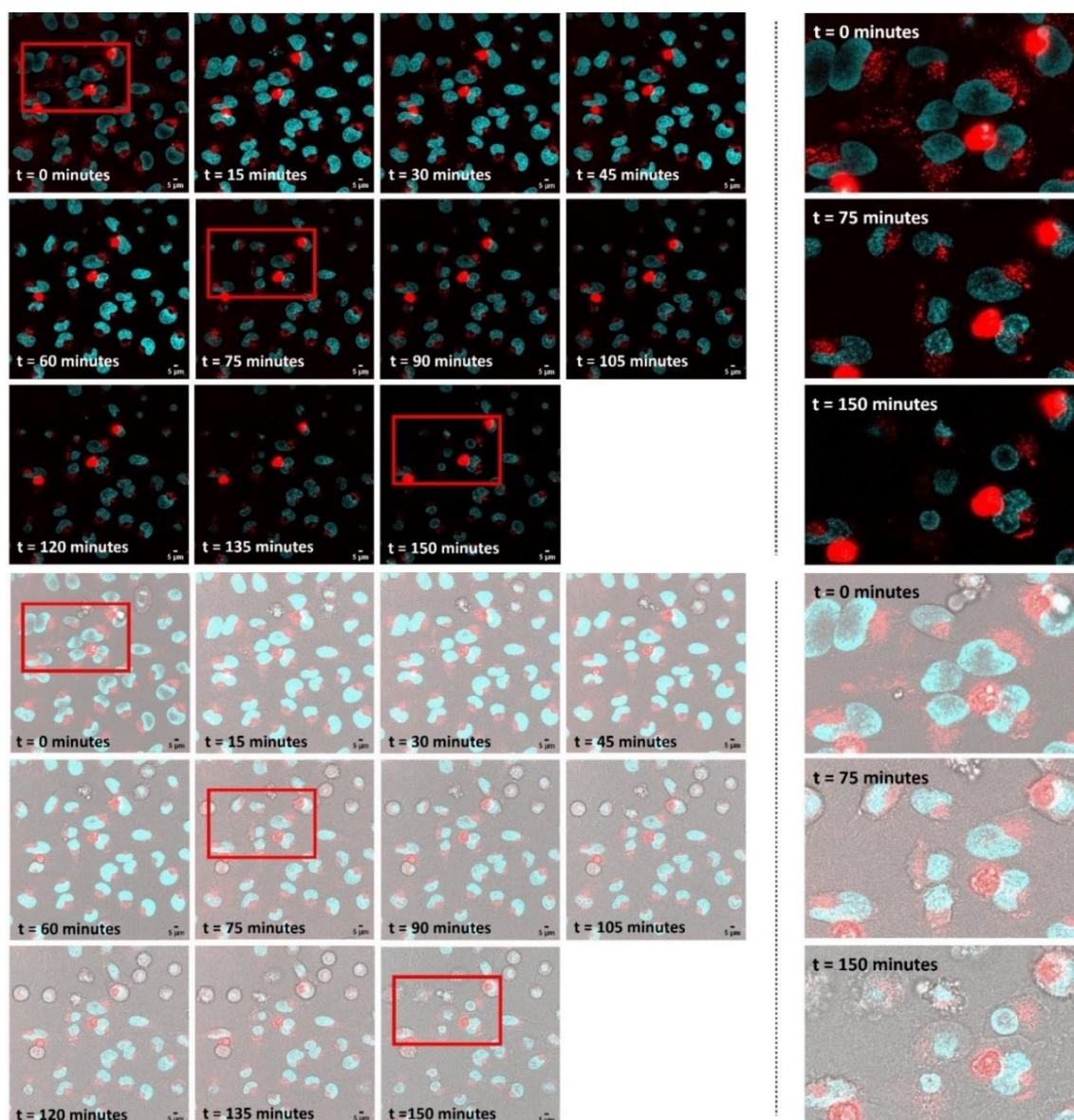


Figure S3.31 Disassembly of Ru-RHAU (25 μM , 24 h) in live HeLa cells over time with corresponding phase contrast images in gray, indicating changes in cell morphology over time. Timings are indicated on each image where t = the time since Ru-RHAU has been removed. Zoomed images at t = 0, 75 and 150 minutes are indicated by a red box and shown in the columns on the right. Scale bars read 5 μm .

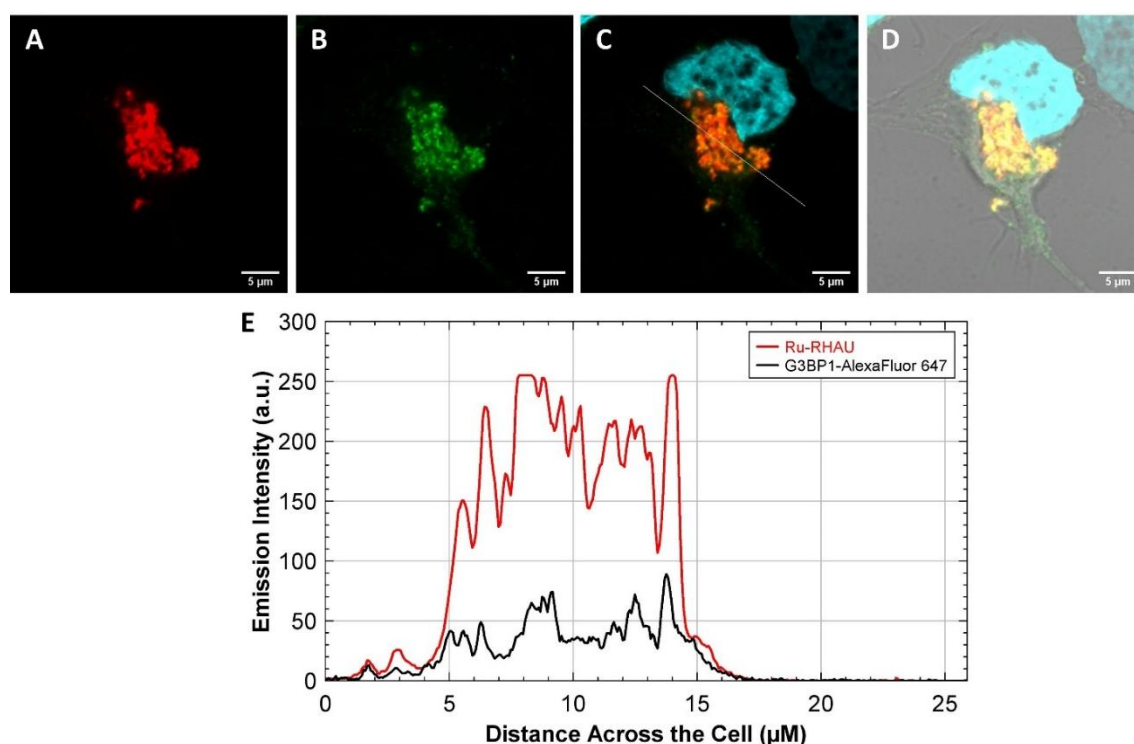


Figure S3.32 Large pathological SG in a fixed **HeLa** cell stained with **(A)** Ru-RHAU 25 μM , 24 h and **(B)** G3BP1 and the corresponding overlay with **(C)** Hoechst 33342 (2 $\mu\text{g/mL}$, 7 minutes) nuclear staining and **(D)** corresponding phase contrast channel. The ROI used to generate the co-localisation graph in **(E)** is indicated by the white line in **(C)**.

S3.2.6 Ru-RHAU with External Stress Stimuli

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% CO_2 . The cells were treated with heat shock (42°C) for 1 h in phenol red free media to induce stress granules. The cells were imaged immediately after the addition of Ru-RHAU (10 μM) at a heated stage set to 42°C . Ru-RHAU was excited at 488 nm and the emission range set to between 550 and 800 nm. DRAQ7 (3 μM) was added each well prior to imaging to confirm cell viability. MCF-7 cells were seeded at 1×10^5 cells/mL in 8 chamber slides and allowed to grow for 48 h at 37°C with 5% CO_2 and treated with heat shock and Ru-RHAU using the same protocol.

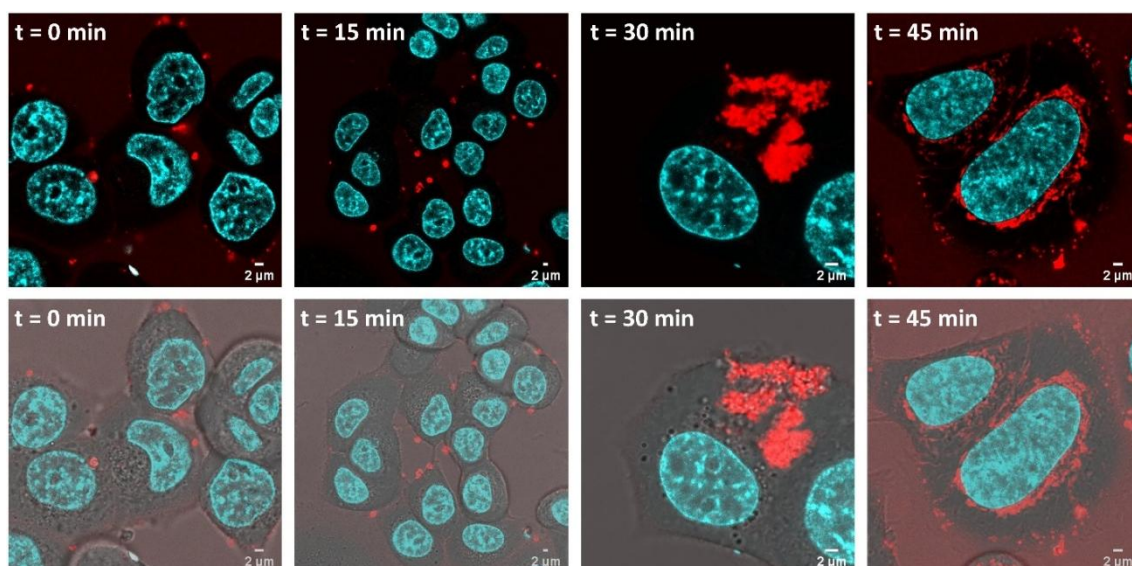


Figure S3.33 Confocal imaging of Ru-RHAU (10 μ M) in **MCF-7** cells that were pre-treated by heat shock (42 $^{\circ}$ C, 1 h) at t = 0, 15, 30 and 45 minutes after the addition of Ru-RHAU and their respective overlays with the phase contrast channel. DRAQ7 (3 μ M) was added to the cells and the lack of uptake confirmed good cell viability under imaging conditions.

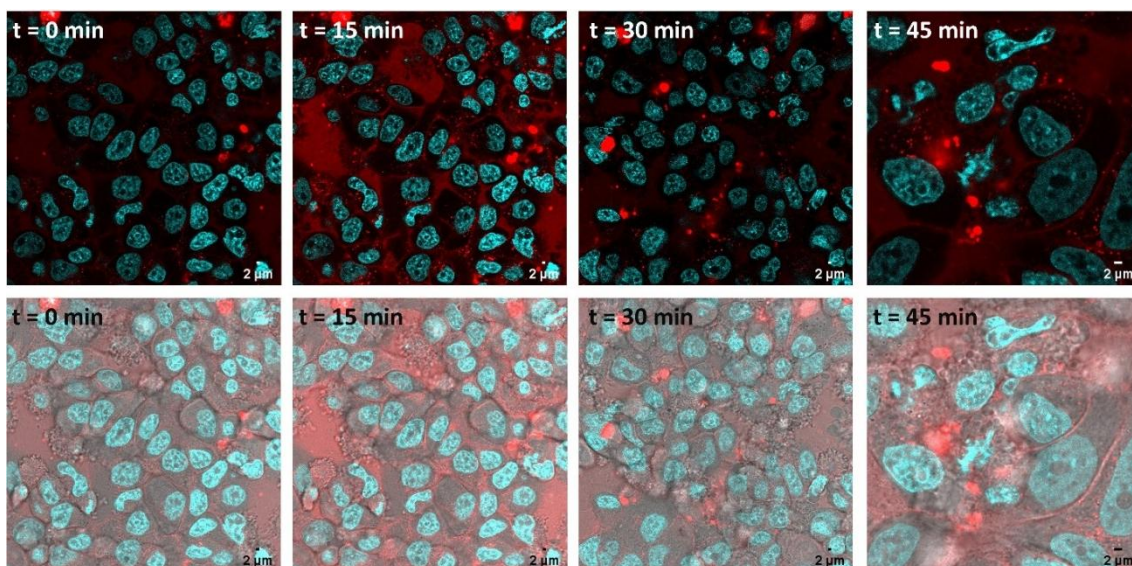


Figure S3.34 Confocal imaging of Ru-RHAU (10 μ M) in **HeLa** cells that were pre-treated by heat shock (42 $^{\circ}$ C, 1 h) at t = 0, 15, 30 and 45 minutes after the addition of Ru-RHAU and their respective overlays with the phase contrast channel. Images after t = 0 and t = 30 minutes uptake are also shown in Figure 3.6. DRAQ7 (3 μ M) was added to the cells and the lack of uptake confirmed good cell viability under imaging conditions.

S3.2.7 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 and 5 µM Ru-RHAU. Six replicate wells were treated for each concentration, and the plates incubated for 24 h or 6 h in the dark at 37 °C, additionally cells were incubated for 24 or 6 h and a 2 h incubation added to enable SG disassembly before assessing viability. 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 µL 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.

S3.2.8 Phototoxicity Studies

Phototoxicity plates were performed as described above. HeLa cells were treated with Ru-RHAU at 25, 22.5, 20, 17.5, 15, 10 and 5 µM for 24 h. The wells were then emptied and 100 µL phenol red free media added. Cells were irradiated at a total dose of 5 ± 0.29 J/cm² (0.5 h at 2.63 ± 0.16 mW/cm²) using a 470 nm LED (TeleOpto LEDA-X LED driver and array). The cells were incubated overnight after irradiation to facilitate cell recovery. The irradiation dose was established using a power meter (Edmund Optics, Coherent LaserCheck™). Cell viability was determined using the MTT assay.

S3.2.9 Autophagy Assay

For plate-based autophagy studies HeLa cells were seeded in black cell culture treated 96 well plates (Nunc) at 2×10^5 cells/mL and allowed to grow and attach for 24 h at 37 °C and 5 % CO₂. For cytotoxicity studies, the cells were then treated with 15, 25, 50 or 60 µM Ru-RHAU, and the plates incubated for 24 h in the dark at 37 °C. A solution of 500 nM Rapamycin and 10 µM Chloroquine (in phenol red free media supplemented with 5% FBS) was used as a positive control (cells treated for 17 h) and untreated cells in media were used as a negative control. Autophagy was assessed using an autophagy assay kit (abcam), which measures autophagic vacuoles. Cells were washed with 1X apoptosis buffer supplemented with 5% FBS before a 30-minute incubation with the dual detection reagent (1 µL/mL green detection reagent and 1 µL/mL Hoechst-33342). The cells were washed twice with 200 µL apoptosis buffer supplemented with 5% FBS, 100 µL of 1X apoptosis buffer added to each well and the plate read immediately. Fluorescence was measured using a BMG LABTECH CLARIOstar plate reader at Ex 480 nm / Em 530 nm for the green detection reagent and Ex 340 nm / Em 480 nm for Hoechst 33342. Autophagy is presented as a percentage (%) increase of green reagent intensity compared to untreated control cells, and cytotoxicity is presented as a % decrease of Hoechst-33342 compared to untreated control cells, where a decrease of $\geq 30\%$ indicates cytotoxicity. All conditions were performed in triplicate.

S3.2.10 FAM-FLICA Apoptosis Assay

The FAM-FLICA polycaspase assay (ImmunoChemistry Technologies) can detect a range of activated caspases, including caspase-1, -2, -3, -4, -5, -6, -7, -8 and -9. HeLa cells were seeded at 3×10^5 cells/mL in a black bottomed 96 well plate (Nunc) at a total volume of 100 µL per well and incubated overnight at 37 °C with 5% CO₂. A solution of Staurosporine (1 µM/3 h) in cell culture media was prepared as a positive control and untreated cells were used as a negative control. Experimental populations were exposed to the Ru-RHAU at 50 and 60 µM for 6 or 24 h in the dark. After the incubation with Ru-RHAU, both the test and control cell populations were incubated with the FLICA dye for 60 min at 37 °C and washed using the buffer provided as per manufacturer's protocol. All conditions were performed in triplicate and analyzed using a BMG Labtech ClarioSTAR plate reader at Ex 488 nm / Em 520 nm.

Appendix C: Supplementary Information – Chapter 4

S4.1 Materials and Methods

S4.1.1 Photophysical Characterisation

Absorbance measurements were made using by Dr. Lorcan Holden using a Jasco V670 spectrophotometer, data was analysed 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. Thiazole orange displacement studies were performed on Clariostar Plus plate reader. 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.

Chemicals required for synthesis were purchased from Sigma-Aldrich (Merck) or Fluorochem and used without further purification.

S4.1.2 DNA Binding Studies (Performed by Dr. Lorcan Holden)

Absorbance and luminescence titrations were performed in tandem using an adapted literature protocol.^[1] DNA stock concentrations were maintained at 1 mM in KPi and titrated into a 10 µM 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.^[2] 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.

S4.1.3 Circular Dichroism (Performed by Dr. Lorcan Holden)

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.

S4.1.4 Computational Studies (Performed by Professor Conor Long)

Quantum calculations were performed using the Gaussian 16 suite as implemented on Kay at the Irish Centre for High-End Computing (ICHEC).^[3] All Density Functional calculations used the B3LYP hybrid functional coupled with a double zeta quality basis set (LanL2DZ) and modelled in water using the Polarizable Continuum Method.^[4–7] All structures were optimised to tight convergence. Excited state energies and electron density maps were extracted from Time-Dependent Density Functional Calculations. Electron Density Difference maps were obtained by subtracting the density map of the ground-state from the map of each excited state, volumes colored blue represent regions where the electron density is less in the excited state compared to the ground-state and those colored red are regions in the excited-state of increased electron density compared to the ground state. GaussView (revision 6.0.16) was used to visualise the results of the quantum calculations.^[8]

S4.1.5 Synthetic Procedures (Performed by Dr. Lorcan Holden)

Phendione-DC3, 5,6-Diamino-1,10-phenanthroline, tetraazaphenanthrene and [Ru(tap)₂Cl₂] were synthesised as previously reported.^[1,9,10]

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): 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

[Ru(tap)₂Cl₂] (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 NH₄PF₆ 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 NH₄PF₆ to precipitate the product as a PF₆ 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 (MeCN/H₂O/20%KNO₂), yielding 60 mgs (48%). ¹H NMR (600 MHz, CD₃CN) 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

Table S4.1 DNA oligonucleotide sequences studied

Name	Sequence (5'-3')	Reference
22AG	AGGGTTAGGGTTAGGGTTAGGG	[11]
HRCC	GGGGGTTGGGTATGGGGAGGGGGG	[12]
PMPS	GGGACGCGGGCGGGGGATATAGGG	[12]
KSS	GGGGAGGGGTGTTTAAGGGGTTGGCTAGGG	[12]
dsDNA	CAATCGGATCGAATTCGATCCGATTG	[13]

S4.2 NMR Spectra

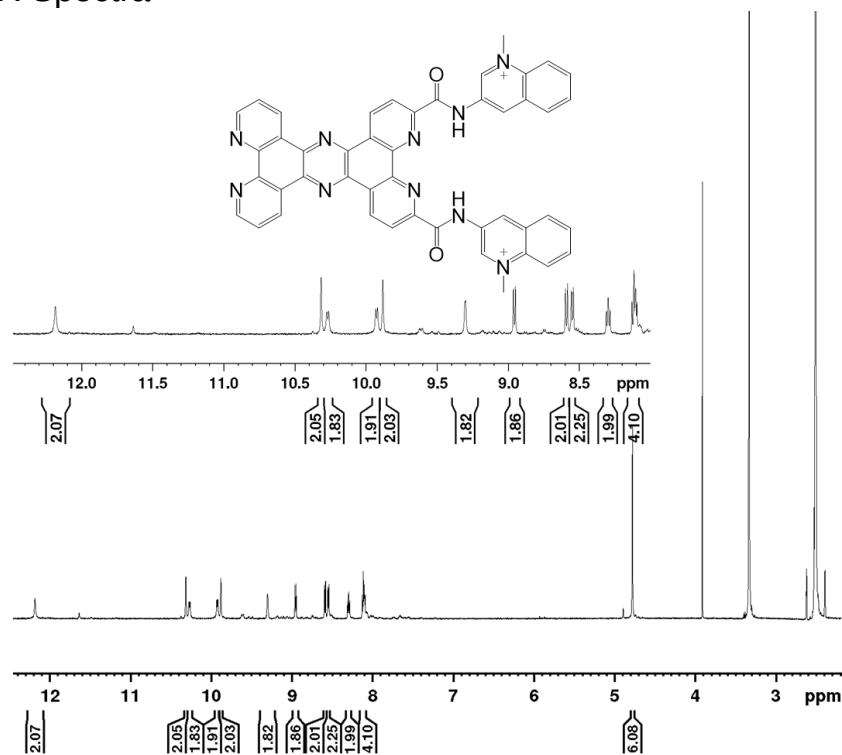


Figure S4.1 ^1H NMR (600 MHz) spectra of TPPHz-PDC3 in DMSO- d_6 . Inset shows aromatic region.

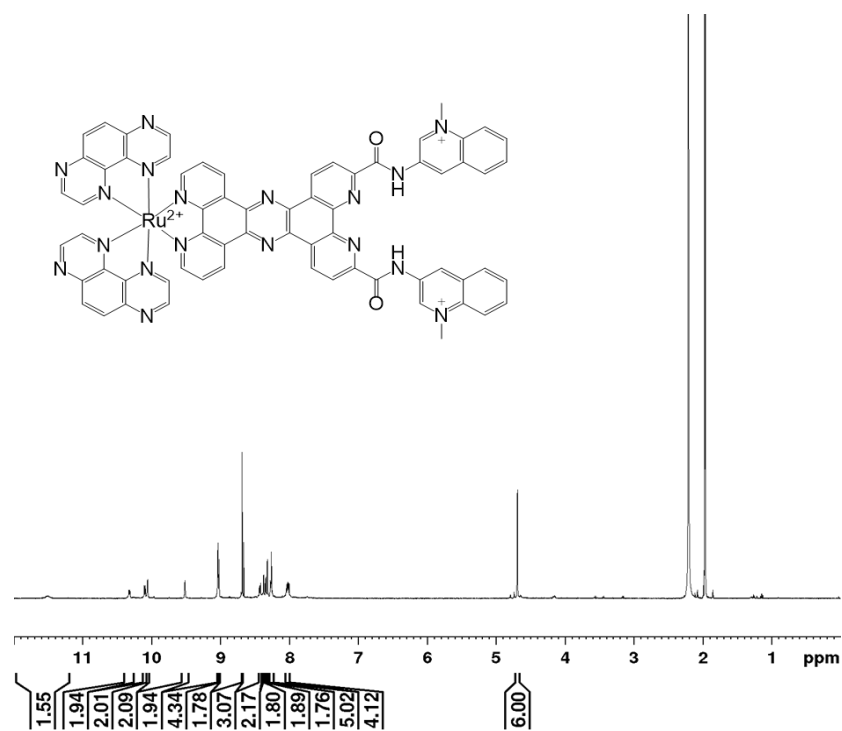


Figure S4.2 ^1H NMR (600 MHz) of Ru-TAP-PDC3 in CD_3CN .

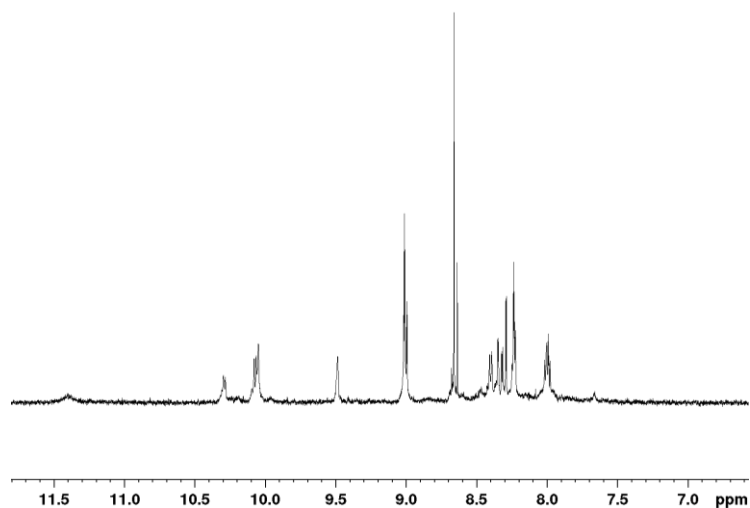


Figure S4.3 ^1H NMR (600 MHz) Expanded to show aromatic region Ru-TAP-PDC3 in CD_3CN .

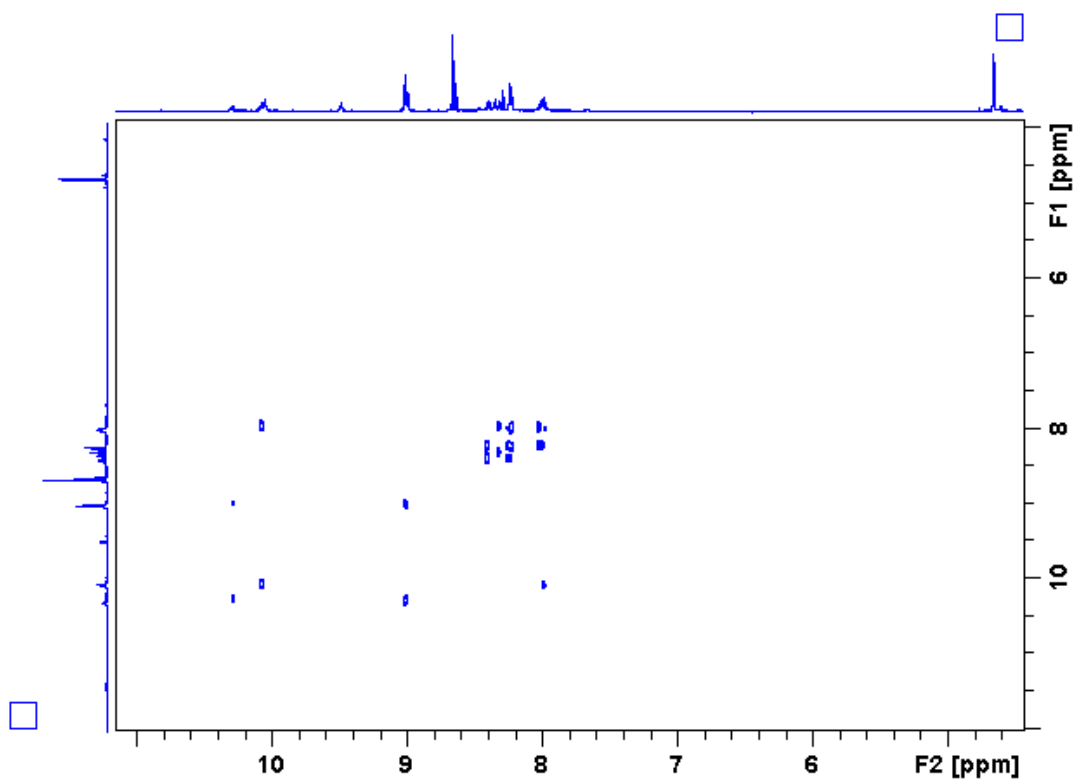


Figure S4.4 ^1H NMR (600 MHz) COSY spectra of Ru-TAP-PDC3 in CD_3CN .

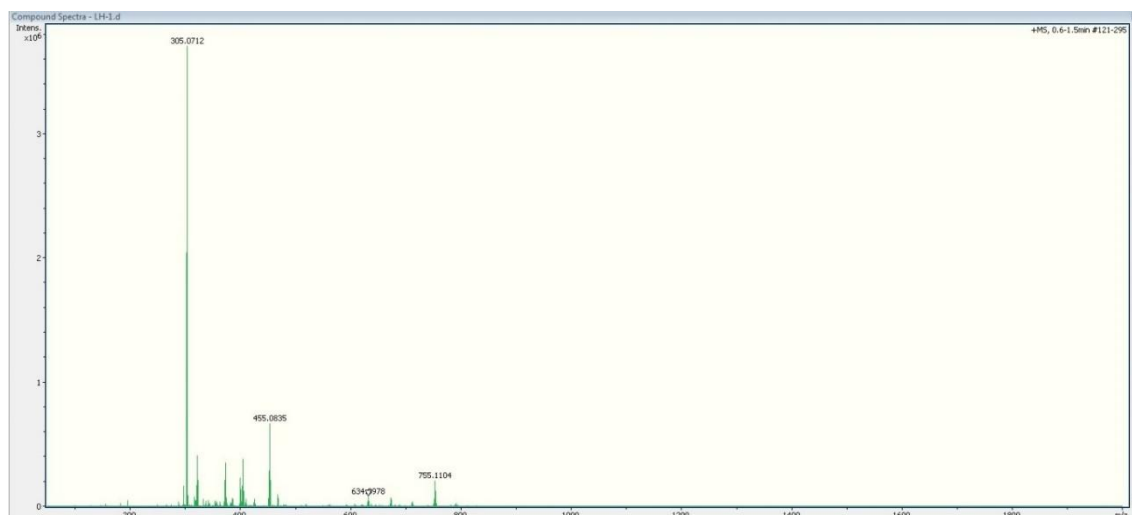


Figure S4.5 Full ESI Mass spec of Ru-TAP-PDC3.

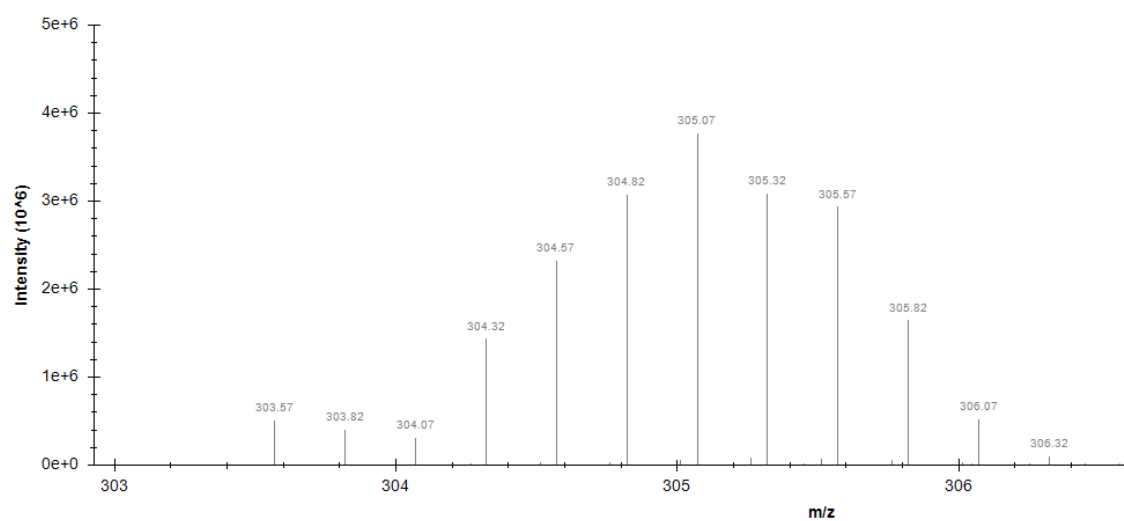


Figure S4.6 ESI mass Spec of Ru-TAP-PDC3 expanding the 4+ m/z region.

S4.3 HPLC, Cyclic Voltammetry and 77K Data

Ru tap HPLC

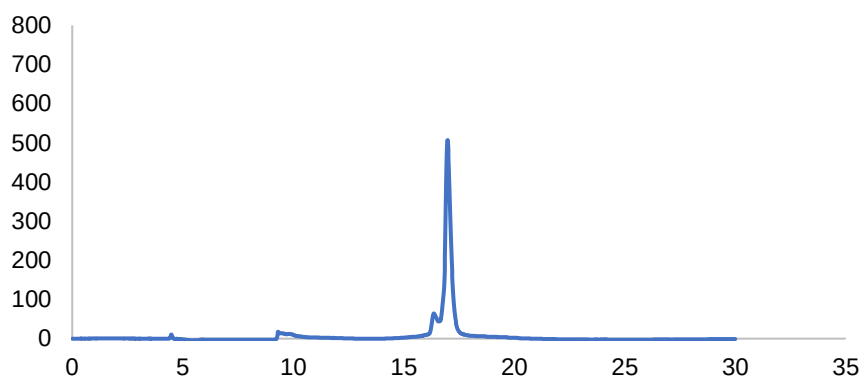


Figure S4.7 HPLC chromatogram of Ru-TAP-PDC3, indicating purity of >95% based on absorbance at 280 nm.

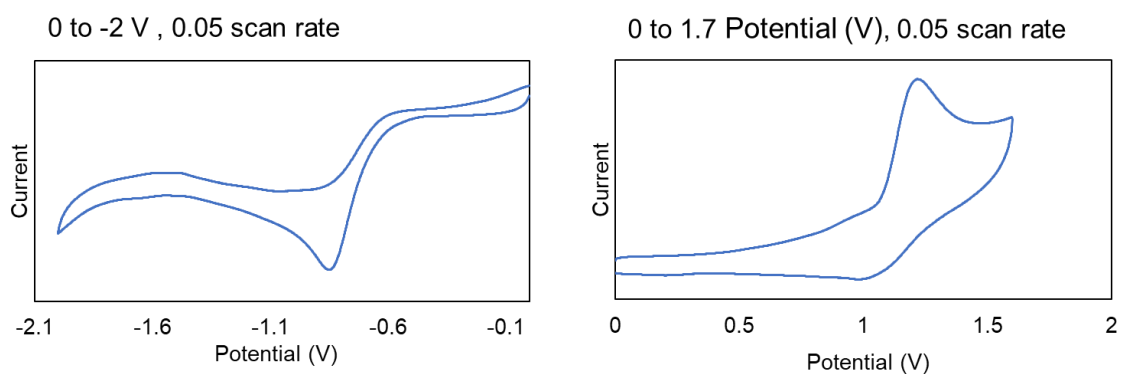


Figure S4.8 Cyclic voltammetry of Ru-TAP-PDC3 in MeCN (1 mM), at a scan rate of 0.05 V.S Voltammetry was carried out at a glassy carbon working electrode, using a Pt coil auxiliary electrode and Ag/AgCl reference electrode.

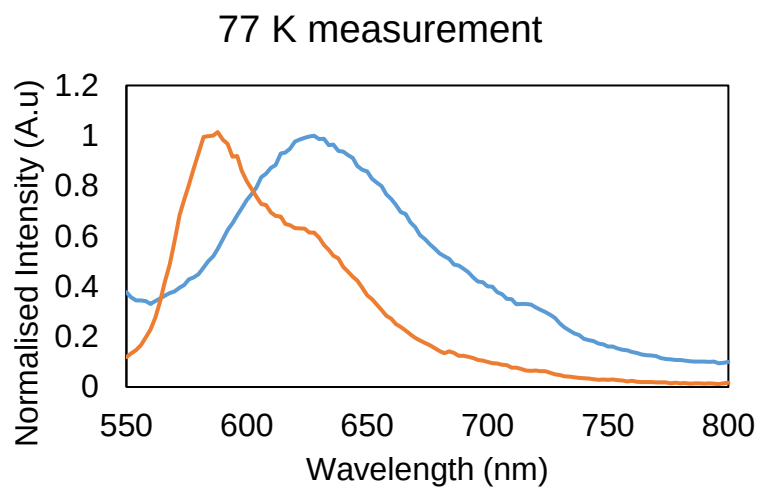


Figure S4.9 Emission Spectrum of Ru-TAP-PDC3 in ethanol:methanol (4:1) taken at 77 K (Blue) and 293 K (Orange), excitation wavelength 470 nm.

S4.4 DNA Studies

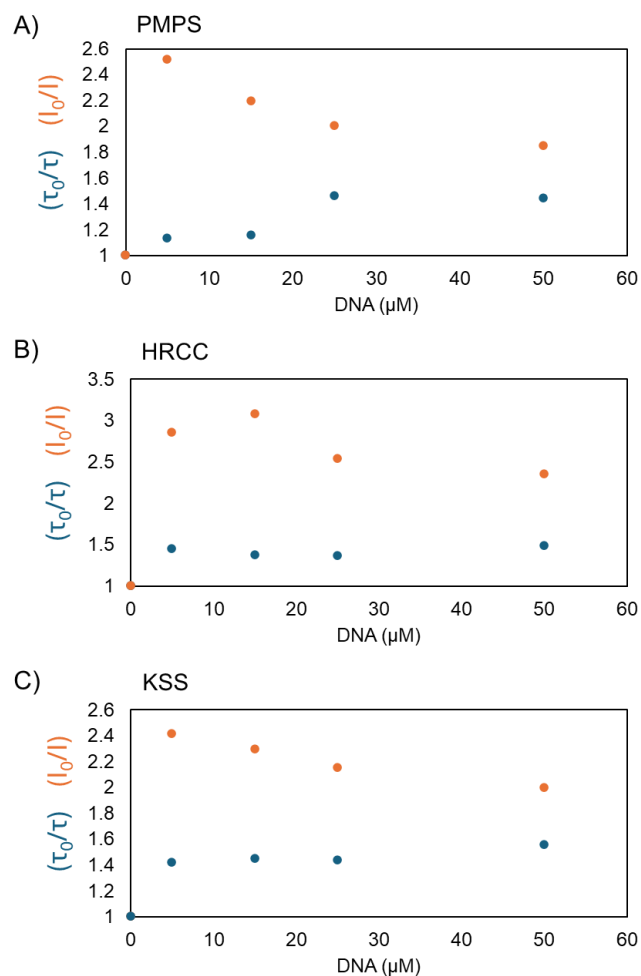


Figure S4.10 Stern-Volmer plots of steady state and emission lifetime quenching data for Ru-TAP-PDC3 (5 μM) with increasing concentrations of the mitoG4 **(A)** PMPS, **(B)** HRCC and **(C)** KSS from 0 to 50 μM. Luminescence intensity-based data is shown in orange. Emission lifetime data is presented in blue. As emission lifetime is essentially unchanged beyond the first data point (1:1 concentration), this indicates, as expected, that the quenching with G4 is primarily static, consistent with association of the complex with the G4 structure.

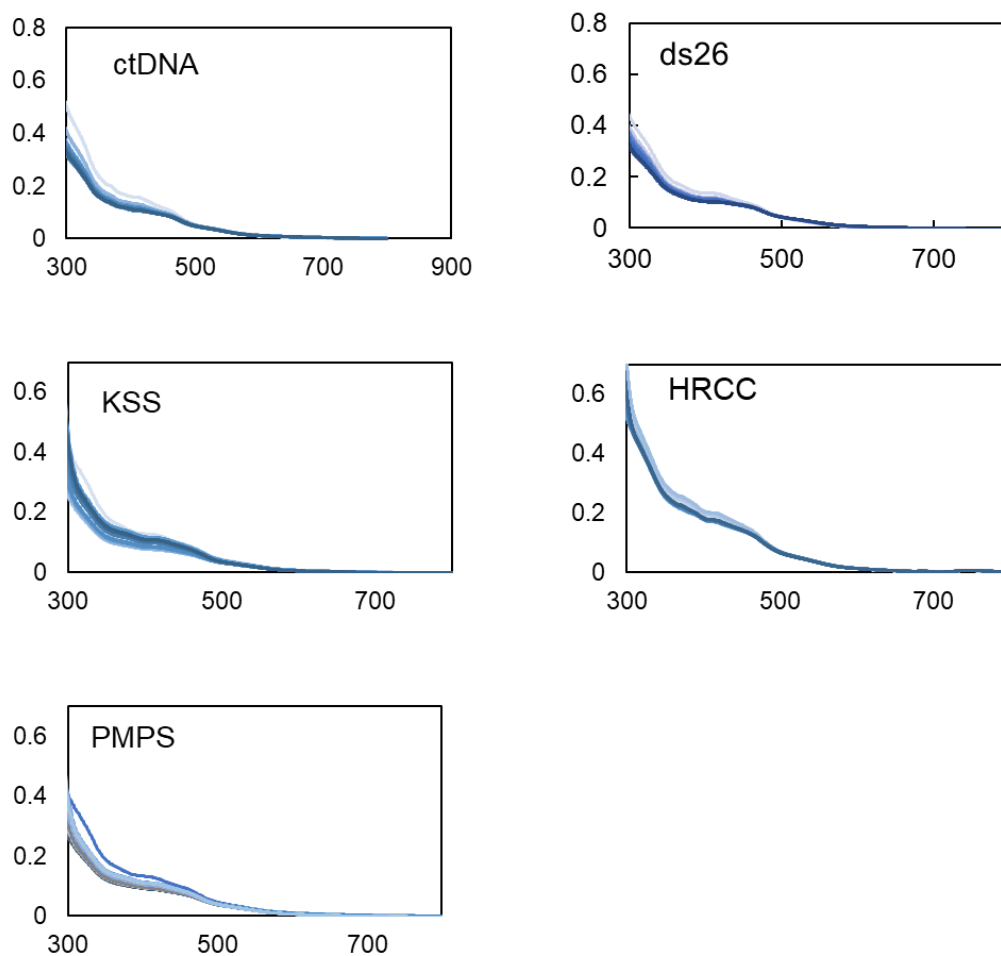


Figure S4.11 Absorption titrations of Ru-TAP-PDC3 (10 μM) against ctDNA, ds26, KSS, HRCC and PMPS (0-50 μM for ctDNA and dsDNA) and (0-30 μM for KSS, HRCC and PMPS) in KPi buffer.

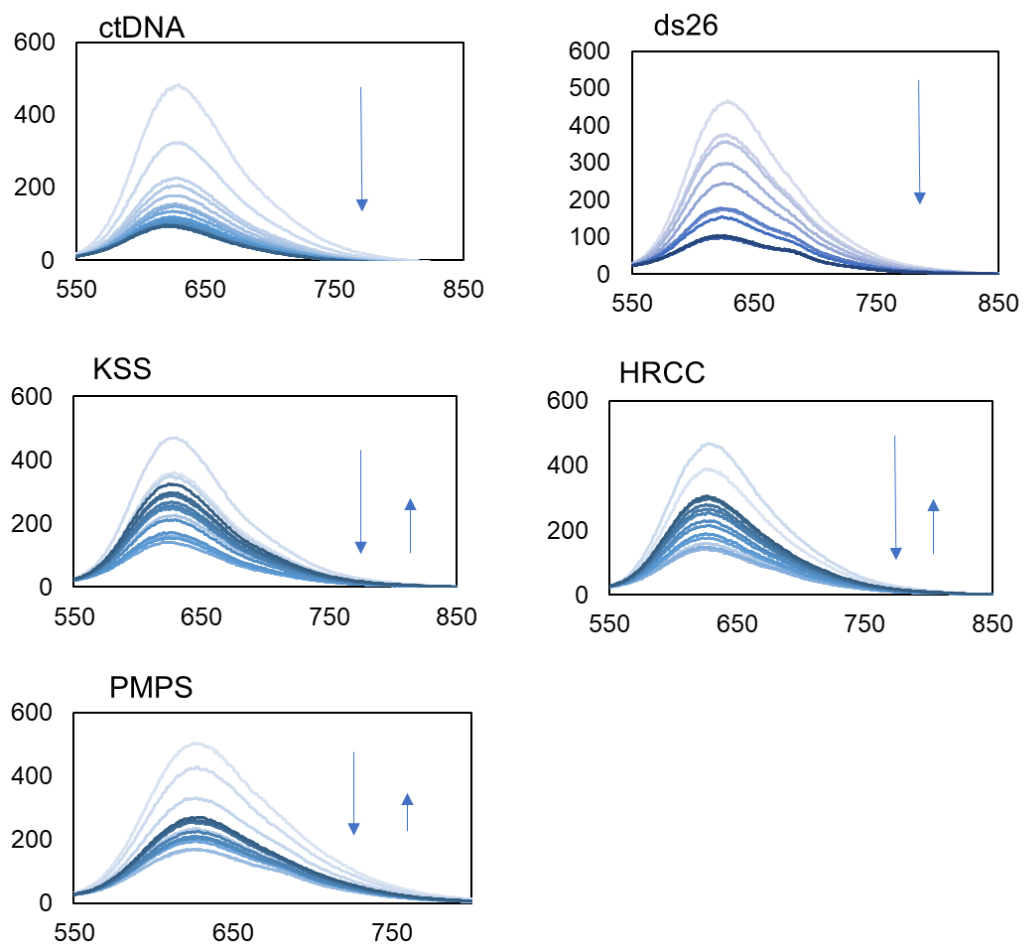


Figure S4.12 Luminescence titrations of Ru-TAP-PDC3 (10 μM) against ctDNA, ds26, KSS, HRCC and PMPS (0-50 μM for ctDNA and dsDNA) and (0-30 μM for KSS, HRCC and PMPS) in KPi buffer. ($\lambda_{\text{excitation}} = 470 \text{ nm}$).

S4.5 Circular Dichroism

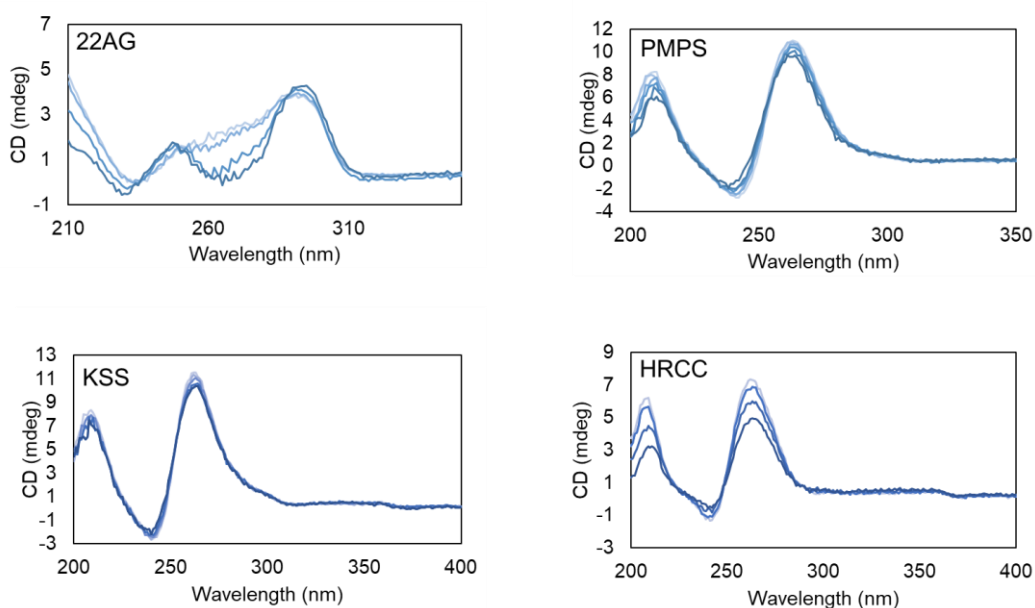


Figure S4.13 Circular dichroism response of mitoG4s (5 μ M) to increasing concentrations of Ru-TAP-PDC3 (2.5 – 25 μ M).

S4.6 Cellular Studies

S4.6.1 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 (Gibco) supplemented with 10% foetal 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.

S4.6.2 Confocal Imaging

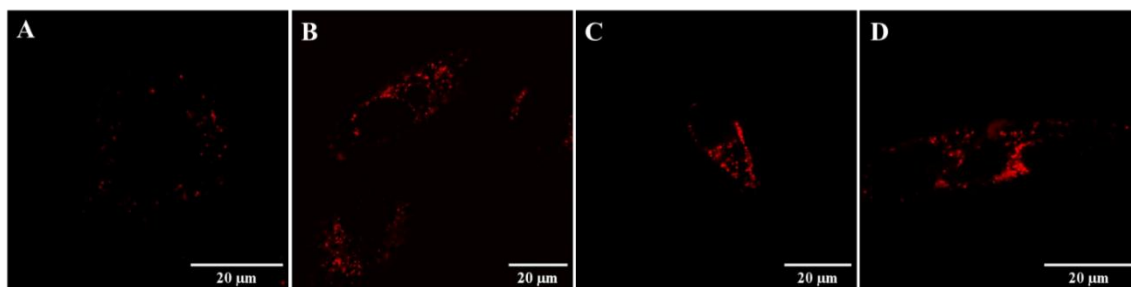


Figure S4.14 Confocal imaging of **(A)** 30 **(B)** 50 **(C)** 100 and **(D)** 200 μM Ru-TAP-PDC3 in live HeLa cells at 24 h incubation. A 405 nm laser was used to excite the cells and emission collected between 580 – 750 nm.

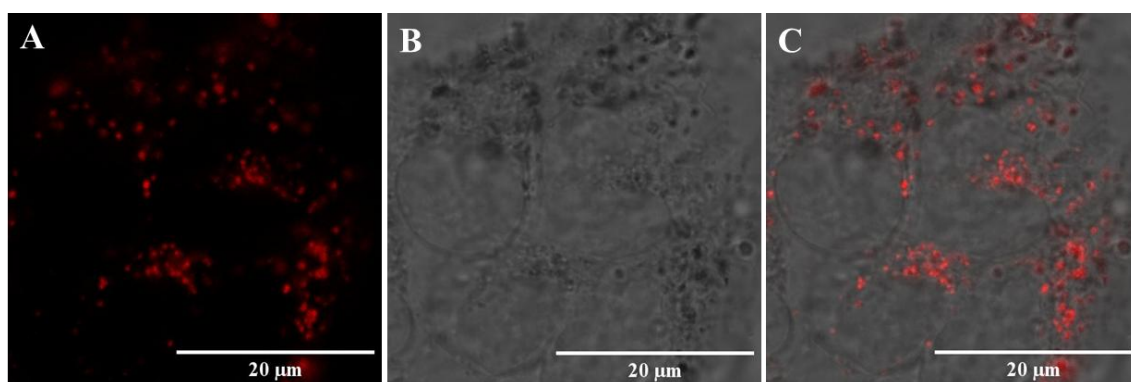


Figure S4.15 Confocal images of **(A)** 50 μM Ru-TAP-PDC3 in HEK-293 cells **(B)** brightfield and **(C)** overlay.

S4.6.3 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 26. Additional co-localisation studies were completed in HEK-293 cells with Late Endosomes-GFP (CellLight) and Golgi-RFP (CellLight) and in HeLa cells with Hoechst 33342. Cells were treated with Ru-TAP-PDC3 at 50 μM 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 580-750 nm.

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 μL/mL, 24 h) Ex 488 nm, Em 495-540 nm, Golgi-RFP (10 μL/mL, 24 h) Ex 555 nm, Em 580-590 nm and Hoechst 33342 (1 μg/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.

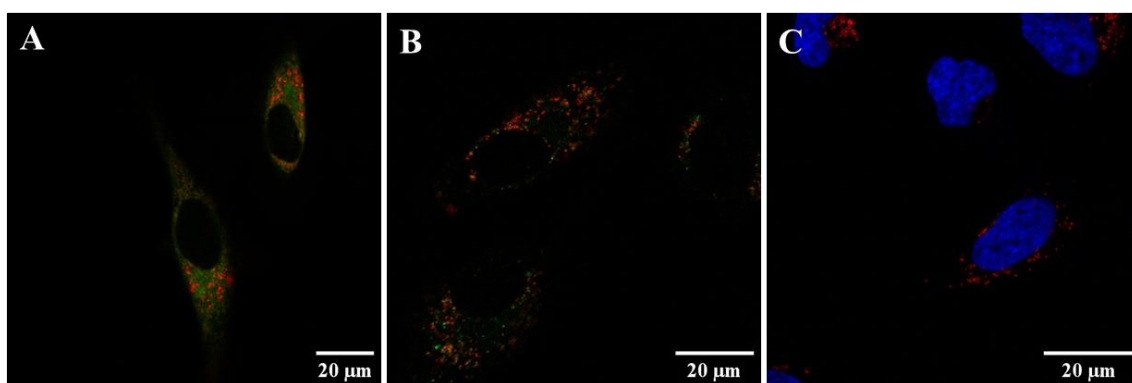


Figure S4.16 Confocal images of Ru-TAP-PDC3 (50 μM, 24 h) overlay with **(A)** BioTracker 488 ($r = 0.6 \pm 0.11$), **(B)** LysoTracker Green DND-26 ($r = 0.46 \pm 0.08$) and **(C)** Hoechst 33342 ($r = 0.08 \pm 0.01$).

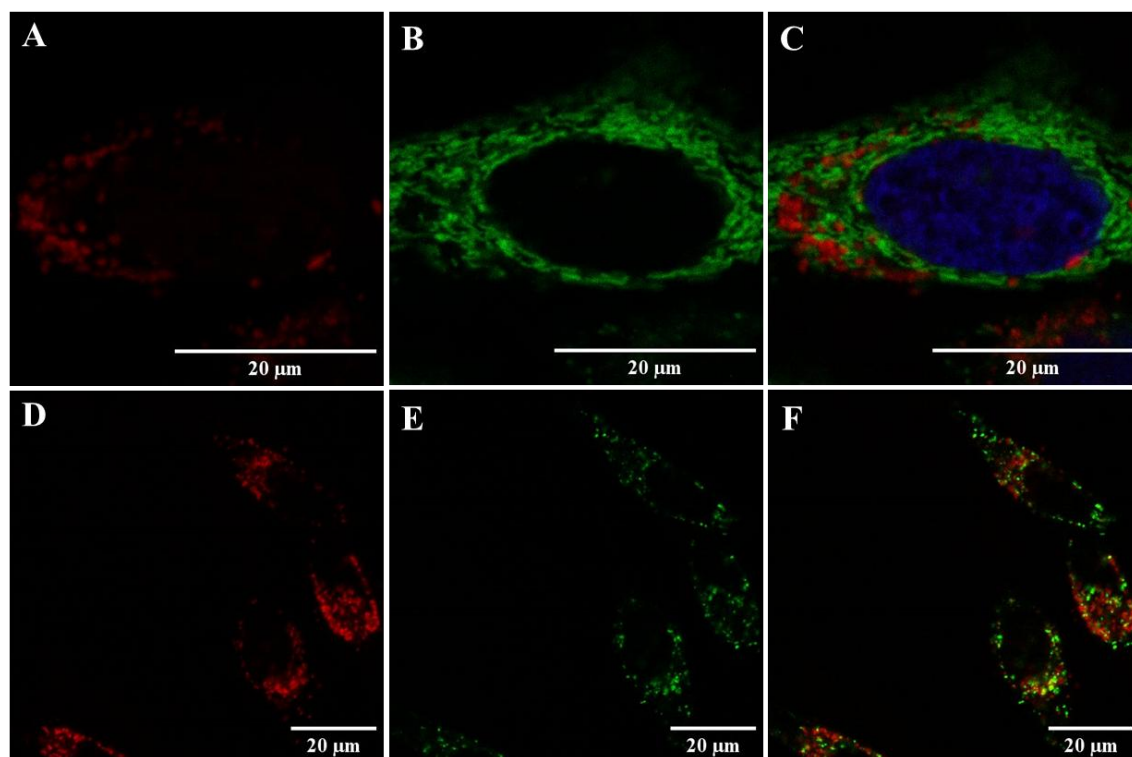


Figure S4.17 Ru-TAP-PDC3, 50 μM at 48 h incubation with **(A-C)** BioTracker 488 ($r = 0.33 \pm 0.04$) with Hoechst 33342 nuclear staining for contrast and **(D-F)** LysoTracker Green DND-26 ($r = 0.39 \pm 0.06$).

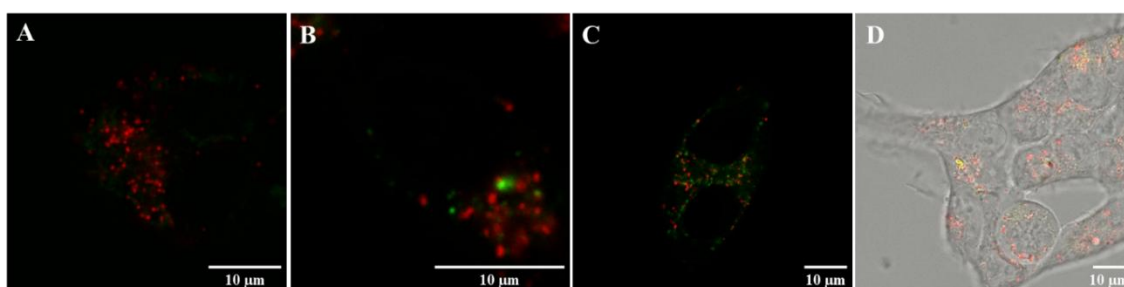


Figure S4.18 Confocal images of Ru-TAP-PDC3 (red) after 24 h incubation at 50 μ M, with **(A)** BioTracker 488 ($r = 0.30 \pm 0.04$), **(B)** LysoTracker Green DND-26 ($r = 0.39 \pm 0.01$), **(C)** Late Endosomes-GFP ($r = 0.49 \pm 0.14$) and **(D)** Golgi-RFP ($r = 0.28 \pm 0.09$) with brightfield overlay. The fusion of late endosomes with lysosomes is an established part of the endocytic pathway, so localisation in both cellular compartments is not surprising.^[14] Late endosomes serve as hubs for the local translation of mitochondrial RNAs, essential for mitochondrial maintenance.^[15] Therefore, while Ru-TAP-PDC3 is mainly localising in the late endosomes of HEK-293, the complex remains linked to the mitochondria.

Table S4.2 Summary of the co-localisation results of Ru-TAP-PDC3 (50 μ M, 24 h) in live HEK-293 and HeLa cell monolayers

Cell Line	Commercial Dye	Pearson's Coefficient $r =$	Incubation Time (hours)
HeLa	BioTracker 488 mitochondrial dye	0.60 ± 0.11	24
		0.33 ± 0.04	48
HEK-293		0.30 ± 0.04	24
HeLa	LysoTracker Green DND-26	0.46 ± 0.08	24
		0.39 ± 0.06	48
HEK-293		0.39 ± 0.01	24
HeLa	Hoechst 33342	0.08 ± 0.01	24
HEK-293	Golgi-RFP	0.28 ± 0.09	24
	Rab7a-GFP	0.49 ± 0.14	

S4.6.4 BG4 Immunostaining

HeLa cells were seeded on poly-D-lysine coated coverslips at 1×10^5 cells/mL in a 12-well tissue culture plate (Corning) and incubated for 24 h at 37 °C. Cells were treated with 50 μ M Ru-TAP-PDC3 for 24 h in the dark at 37 °C before fixation.

Cells were fixed with 4% paraformaldehyde (BioLegend) for 15 minutes at room temperature (RT), and permeabilised with 0.01% Triton X-100 (Invitrogen) for 5 minutes at RT. Cells were washed three times with 1X PBS. 5% goat serum was used to as a blocking buffer (1 h, RT). Cells were incubated with anti-G4 antibody BG4 (Merck, 1:50) overnight at 4 °C, washed twice with PBS and incubated for 2 h at RT with Flag-Tag (Cell Signaling Technology, 1:500, anti-rabbit mAb). The cells were then incubated for 3 h with goat anti-rabbit AlexaFluor 647 (Invitrogen, 1:500) at RT, and Hoechst 33342 (2 μ g/mL) for 7 minutes at RT. Cells were washed twice with cold PBS between each incubation. Coverslips were mounted on glass slides using Prolong Gold Antifade (Invitrogen), incubated for 24 h at RT in the dark, sealed and stored at 4 °C prior to confocal imaging.

Samples shown in Figure S4.19 were prepared using this protocol to determine the effect of irradiation on the mitoG4s, where the cells were treated with Ru-TAP-PDC3 at 3 μ M (24 h) prior to irradiation at 470 nm with a total irradiation dose of 5 J/cm² (30 minutes at 2.8 mW/cm²) while control cells were incubated in the dark during the irradiation period.

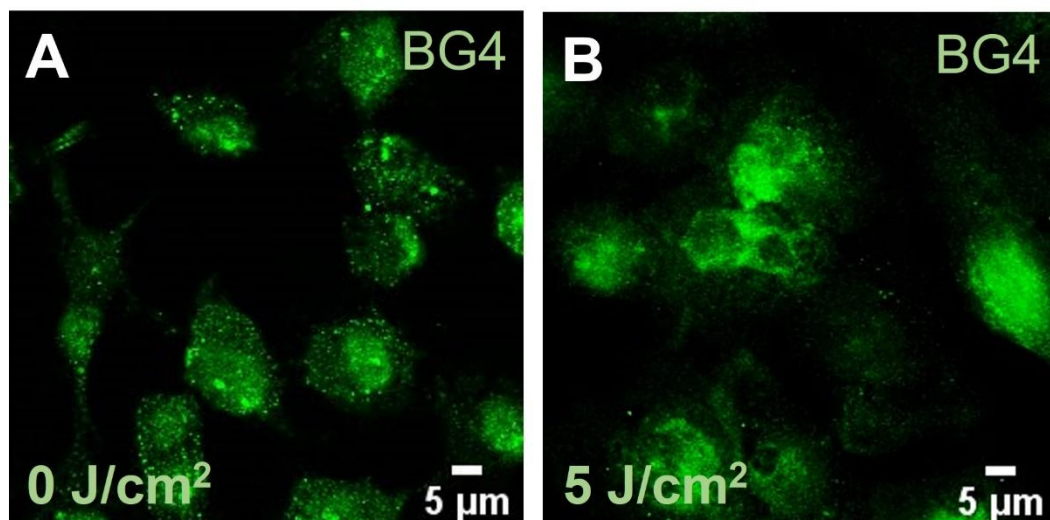


Figure S4.19 BG4 staining in cells pre-treated with Ru-TAP-PDC3 (3 μ M, 24 h) **(A)** without irradiation and **(B)** irradiated at a total dose of 5 J/cm² using a 470 nm LED.

S4.6.5 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.

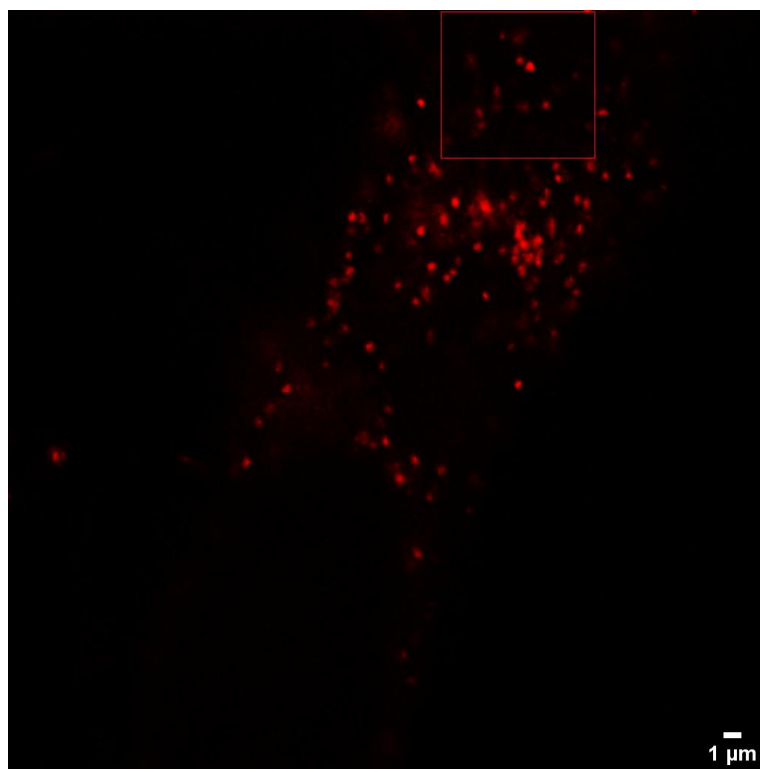


Figure S4.20 Deconvolved STED image of Ru-TAP-PDC3 (50 μ M, 24 h) in a live HeLa cell, deconvolved using Huygens Professional deconvolution software. The region of interest (ROI) marked by the red box corresponds to the images in Figure 4.3C comparing the confocal, gated STED and deconvolved STED resolution of the Ru-TAP-PDC3 foci.

S4.6.6 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 3 μ M, washed twice with 1X PBS and counterstained with PicoGreen (Invitrogen) 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, 5, 10 or 15 minutes of continuous irradiation using a 405 nm laser (total irradiation dose 1.2 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 and to confirm that there was negligible photobleaching of PicoGreen under excitation at 405 nm (Figure S4.21). Control studies with DRAQ7 confirmed good cell viability under these conditions (Figure S4.22).

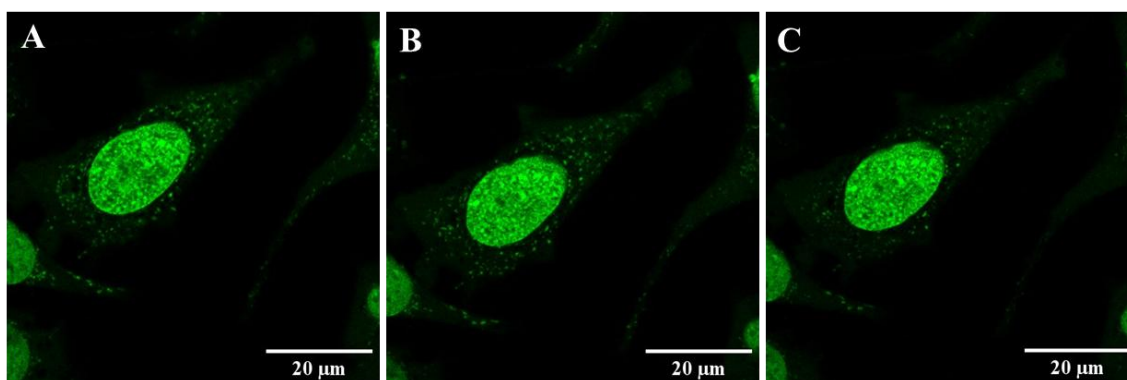


Figure S4.21 Confocal fluorescence imaging of PicoGreen (2 μ L/mL) in HeLa cells at **(A)** 0, **(B)** 2.5 and **(C)** 5 minutes continuous irradiation using a 405 nm laser (total irradiation dose of 0.5 J/cm²). PicoGreen is susceptible to photobleaching thus a control study of PicoGreen in the absence of Ru-TAP-PDC3 was necessary to confirm depletion was not the result of photobleaching.

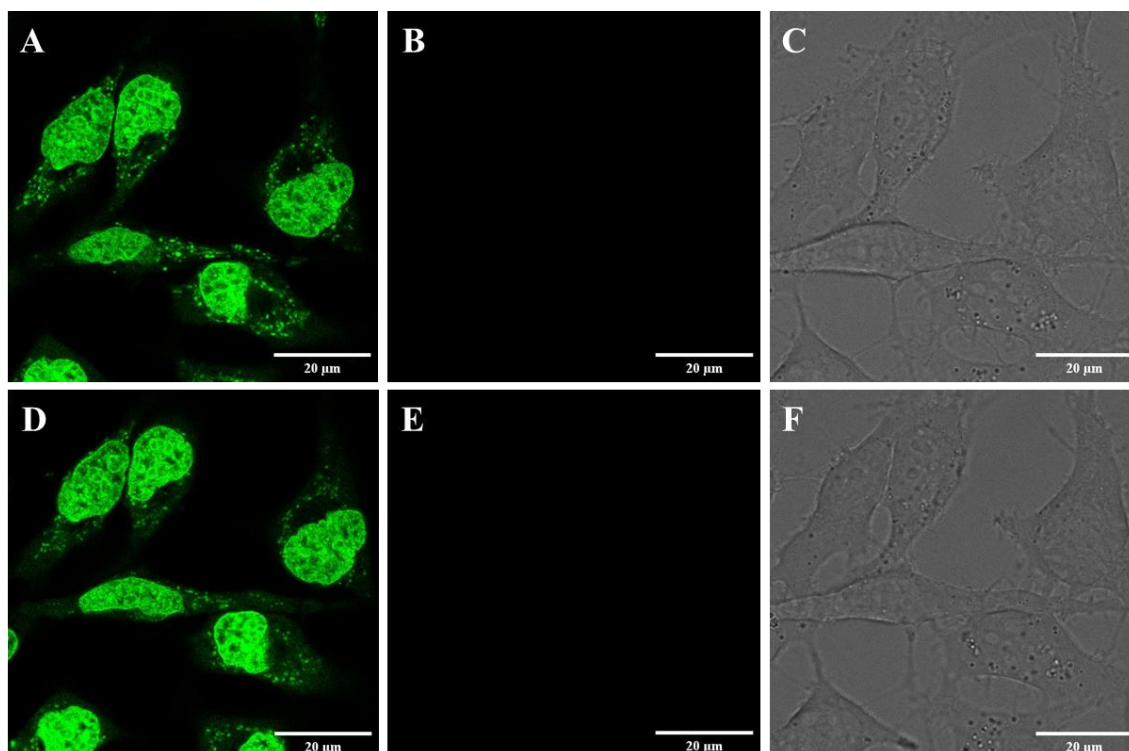


Figure S4.22 Confocal imaging of PicoGreen (2 $\mu\text{L/mL}$) in HeLa cells at **(A)** 0 and **(B)** 5 minutes continuous irradiation using a 405 nm laser (total irradiation dose of 0.5 J/cm^2). A lack of DRAQ7 (3 μM) uptake after **(C)** 0 and **(E)** 5 minutes irradiation confirms PicoGreen is not phototoxic towards HeLa cells under the experimental conditions. Cell morphology can be seen at **(D)** 0 and **(F)** 5 minutes irradiation. Scale bar reads 20 μm .

S4.6.7 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 μL respectively and left to grow and attach for 24 h at 37 $^\circ\text{C}$ and 5% CO_2 . For 24 h dark toxicity studies, HeLa cells were treated with 200, 175, 150, 100, 75, 50, 25 and 10 μM Ru-TAP-PDC3 and HEK-293 cells treated with 200, 100, 50, 25 μM . The cells were treated in triplicate and the plates incubated for 24 h in the dark at 37 $^\circ\text{C}$. 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 $^\circ\text{C}$ 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.

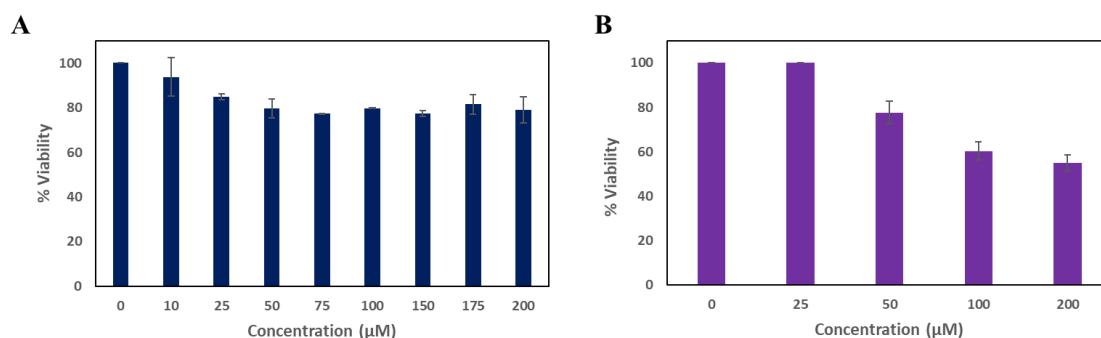


Figure S4.23 Dark toxicity results of Ru-TAP-PDC3 in **(A)** HeLa and **(B)** HEK-293 cell monolayers at 24 h incubation. A PI value of 52 was determined for HEK-293 cells at 24 h incubation, with a phototoxic IC_{50} of $3.88 \pm 0.14 \mu M$. Error bars show standard deviation of $n=3$ measurements.

S4.6.8 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 $5 \pm 0.54 J/cm^2$ (0.5 h at $2.81 \pm 0.30 mW/cm^2$) or $15 \pm 1.6 J/cm^2$ (1.5 h at $2.81 \pm 0.30 mW/cm^2$) 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.

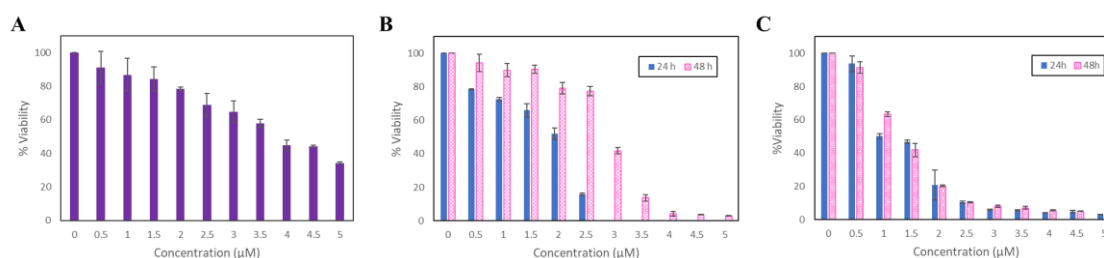


Figure S4.24 Phototoxicity results of Ru-TAP-PDC3 in **(A)** HEK-293 (24 h incubation, $5 \pm 0.54 J/cm^2$) and HeLa cells at 24 h or 48 h incubation prior to irradiation with a 470 nm LED at a total irradiation dose of **(B)** $5 \pm 0.54 J/cm^2$ or **(C)** $15 \pm 1.6 J/cm^2$. Error bars show standard deviation of $n=3$ measurements.

S4.6.9 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.^[16] 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.^[17,18] HIF-2 α and the lesser studied HIF-3 α are also activated at low oxygen concentrations.^[19] 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.^[20,21]

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.

S4.6.10 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 μM CoCl_2 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.^[22,23]

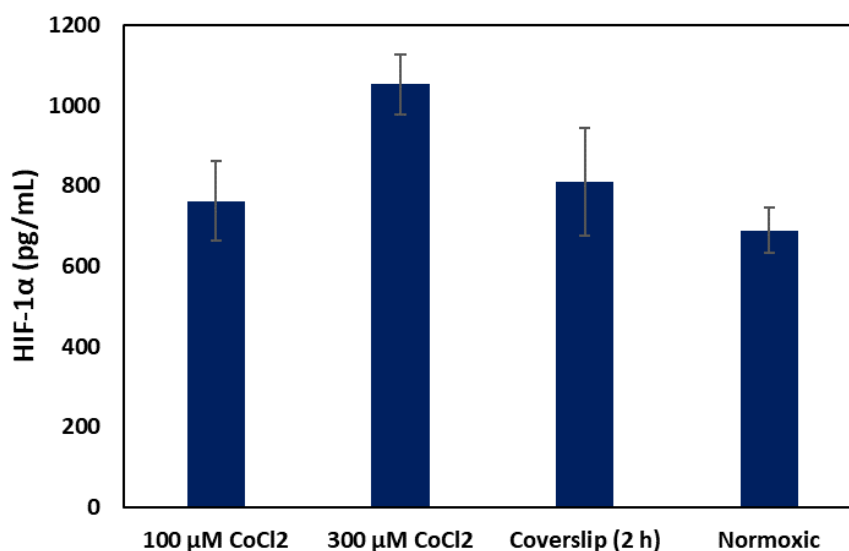


Figure S4.25 Results of HIF-1 α ELISA, the values were calculated using a standard curve of known HIF-1 α concentrations. Error bars show standard deviation of $n = 3$ measurements.

S4.6.11 FAM-FLICA Polycaspase Activity: Fluorescence Assay

The polycaspase assay detects multiple activated caspases, including caspase-1, -2, -3, -4, -5, -6, -7, -8 and -9. 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.^[24]

HeLa cells were seeded at 3×10^5 cells/mL in a 96 well plate (Nunc) at a total volume of 100 μL per well and incubated overnight at 37 °C with 5% CO_2 . The following control populations were prepared for each cell line: negative (untreated) control and positive control cells treated with Staurosporine (1 μM /3 h). Experimental populations were exposed to the Ru-TAP-PDC3 at 1 and 3 μM (above and below the phototoxic IC_{50}) for 24 h and either placed in the dark or irradiated for 30 minutes using a 470 nm LED ($5 \pm 0.54 \text{ J/cm}^2$). 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 ($3 \times 100 \mu\text{L}$) in a black bottomed 96-well plate using BMG Labtech ClarioSTAR plate reader set at 488 nm excitation and 520 nm emission.

S4.6.12 JC-1 – Mitochondrial Membrane Potential: Fluorescence Assay

$\Delta\Psi\text{m}$ 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 $100 \mu\text{L}$ per well and incubated overnight at 37°C with 5% CO_2 . The following control populations were prepared: negative (untreated) control and positive control cells treated with carbonyl cyanide m-chlorophenyl hydrazone (CCCP, $20 \mu\text{M}/2 \text{ h}$). Experimental populations were exposed to Ru-TAP-PDC3 at 1 and $3 \mu\text{M}$ (above and below the normoxic light IC_{50}) for 24 h and either placed in the dark or irradiated for 30 minutes (total irradiation dose of $5 \pm 0.54 \text{ J}/\text{cm}^2$) or 1.5 hours (total irradiation dose of $15 \pm 1.6 \text{ J}/\text{cm}^2$) 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 ($3 \times 100 \mu\text{L}$) 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 $\Delta\Psi\text{m}$. 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 $\Delta\Psi\text{m}$.

S4.6.13 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 $100 \mu\text{M}$ 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.

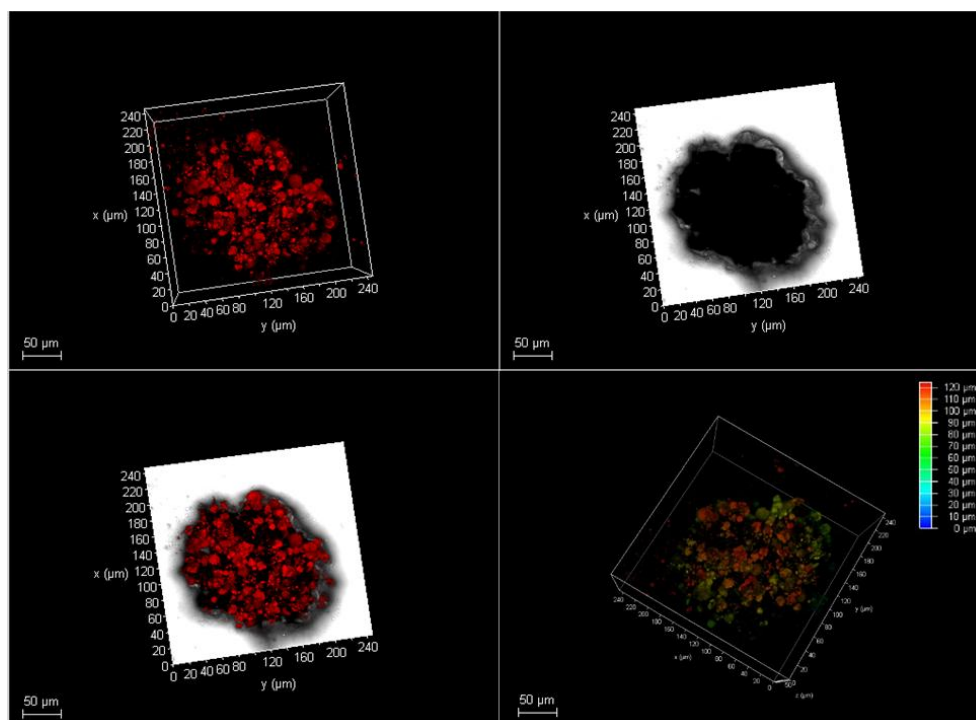


Figure S4.26 3D reconstruction of Ru-TAP-PDC3 (100 μ M, 24 h) in HeLa spheroids, with depth coding image shown. Ru-TAP-PDC3 was excited using a 405 nm laser and emission collected between 580 – 750 nm. Scale bars read 50 μ m.

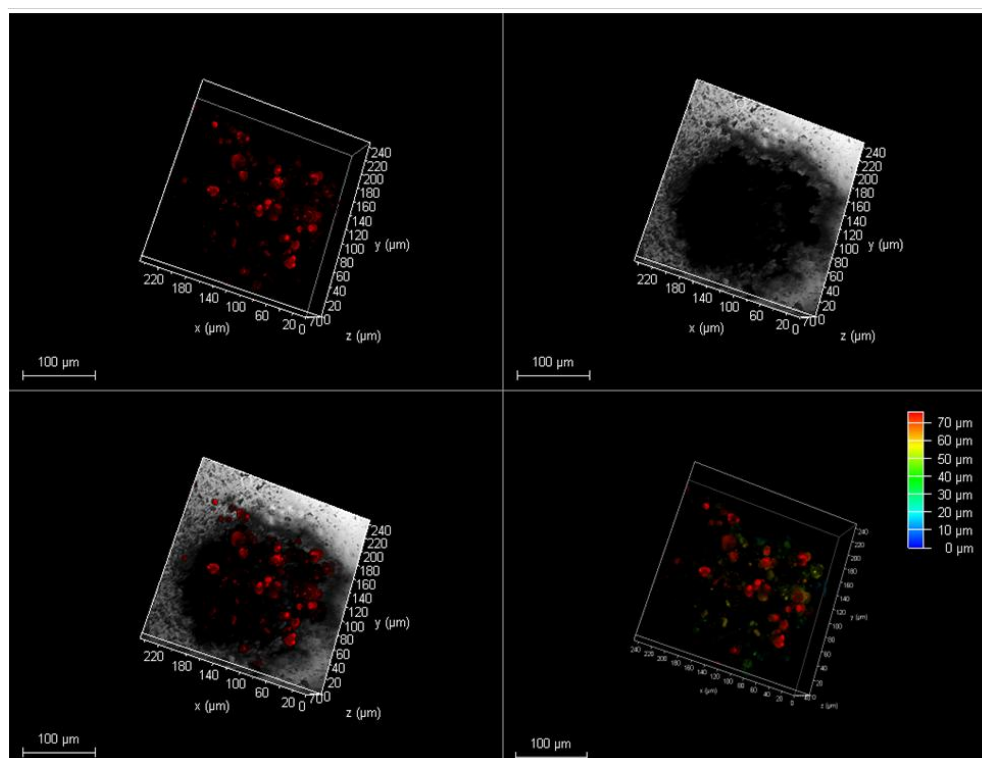


Figure S4.27 3D reconstruction of Ru-TAP-PDC3 (100 μ M, 24 h) in HEK-293 spheroids, with depth coding image shown. Scale bars read 100 μ m.

S4.6.14 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.

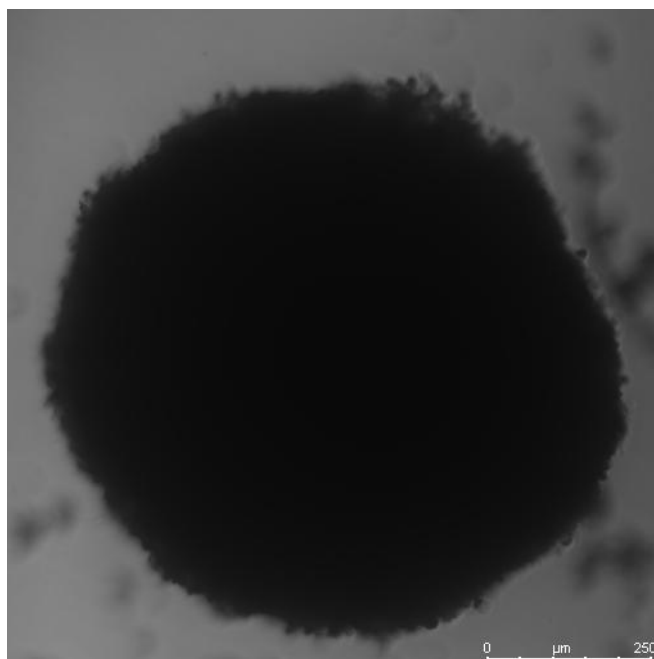


Figure S4.28 Brightfield image of a HeLa spheroid grown in a round bottom 96 well plate for 96 hours taken using a 10x air objective. This image is indicative of the size of the spheroids prepared for the toxicity studies.



Figure S4.29 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.

S4.6.15 DNA Cleavage Studies using pUC19 plasmid DNA

Samples were prepared with 400 ng of pUC19 plasmid DNA (NEB, N3041S) and exposed to Ru-PDC3 to a final ratio of 1:1, 1:2, 1:5, 1:10 (pUC19:Ru-PDC3) plasmid:Ru-complex, in presence of 25 mM NaCl in a total volume of 10 μ L using 80 mM HEPES buffer. Samples were irradiated in a 96 well plate using a 470 nm LED array plate (TeleOpto LEDA-X) connected to a LED array driver (Teleopto, LAD-1 driver) with exposure times from 30 seconds up to 30 minutes at a maximum total dose of 7.52 ± 0.49 J/cm². For dark conditions, samples were covered with aluminium foil and kept in the dark. The reaction was quenched adding loading buffer, 6X, (NEB, B7021S) and samples were loaded onto an agarose gel (0.75%) stained with SYBR[™] Safe DNA gel stain, 1X (Invitrogen, S33102). The gel was run in TAE buffer at 70 V for 70 min using a Bio-Rad wide-cell mini sub system with a Bio-Rad basic Power Pac[™] and imaged using a an Accuris[™] SmartDoc[™] 2.0 Imaging Enclosure paired with an Android camera device.

S4.6.16 DNA Cleavage and ROS scavenger studies

Samples were prepared exposing 400 ng of pUC19 plasmid DNA (NEB, N3041S) to Ru-PDC3 to a final ration of 1:1 plasmid: Ru-complex, with 25 mM NaCl, with and without 5% sodium azide (NaN_3) in a total volume of 10 μL using 80 mM HEPES buffer. Next, samples were irradiated in a 96well plate using a 470 nm LED (TeleOpto LEDA-X LED driver and array) with exposure times from 30 seconds up to 30 minutes at a maximum total dose of $7.52 \pm 0.49 \text{ J/cm}^2$. For dark conditions, samples were covered with aluminium foil and kept in the dark. The reaction was quenched by adding loading buffer, 6X, (NEB, B7021S) and samples were loaded onto an agarose gel (1.2%) stained with SYBR[™] Safe DNA gel stain, 1X (Invitrogen, S33102). The gel was run in TAE buffer at 65 V for 90 min using a Bio-Rad wide-cell mini sub system with a Bio-Rad basic Power Pac[™] and imaged using an Accuris[™] SmartDoc[™] 2.0 Imaging Enclosure paired with an Android camera device.

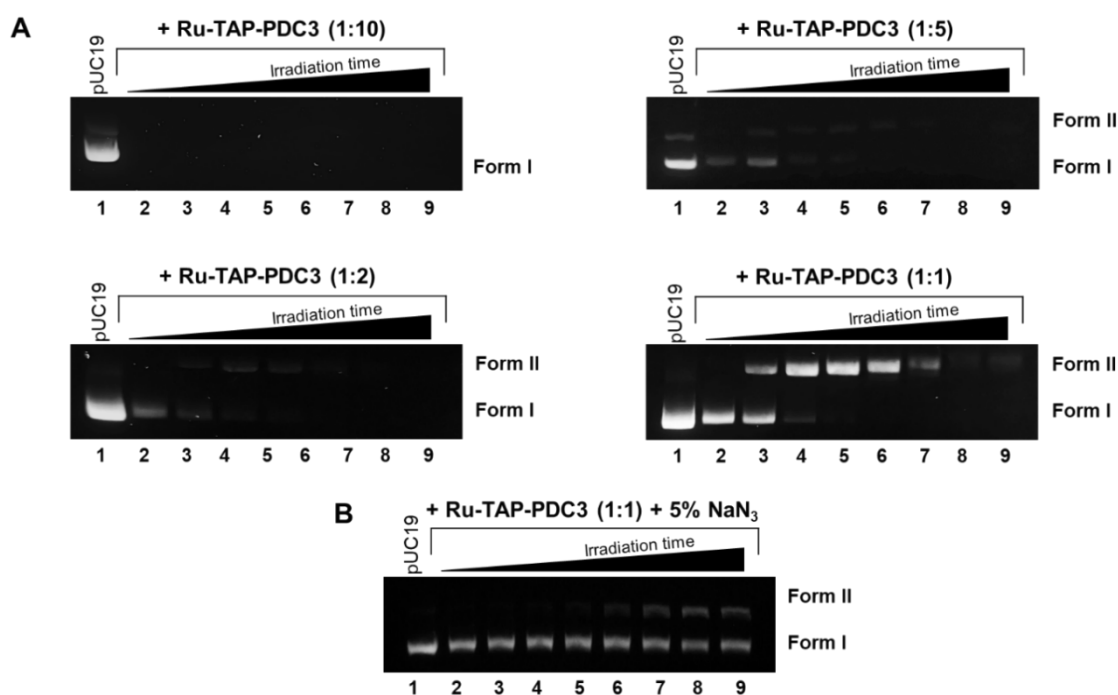


Figure S4.30 DNA cleavage reactions and ROS scavenger study performed by Dr. Giuseppe Avella. Gel electrophoresis of 400 ng of supercoiled pUC19 plasmid exposed to Ru-TAP-PDC3 in a buffer solution of 25 mM NaCl and 80 mM HEPES. **(A)** DNA cleavage reactions with 1:10, 1:5, 1:2 and 1:1 of DNA to Ru-TAP test ratios. In presence of excess Ru-TAP extensive cleavage to the plasmid is evident even under very low irradiation doses, consistent with cell studies. **(B)** DNA-PDC3 cleavage reactions in the presence of singlet oxygen scavenger sodium azide (5% NaN₃), with DNA:Ru-TAP-PDC3 at a 1:1 ratio confirm the cleavage is reduced/slower but still persists in absence of singlet oxygen. 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).

S4.7 References

- [1] L. Holden, K. S. Gkika, C. S. Burke, C. Long, T. E. Keyes, *Inorg. Chem.* **2023**, 62, 2213–2227.
- [2] E. Largy, F. Hamon, M.-P. Teulade-Fichou, *Anal Bioanal Chem* **2011**, 400, 3419–3427.
- [3] M. ea Frisch, G. Trucks, H. B. Schlegel, G. Scuseria, Ma. Robb, J. Cheeseman, G. Scalmani, V. Barone, G. Petersson, H. Nakatsuji, **2016**.
- [4] A. Becke, *Chem. Phys* **n.d.**, 98, 5648.
- [5] C. Lee, W. Yang, R. G. Parr, *Physical review B* **1988**, 37, 785.
- [6] T. H. Dunning Jr, P. J. Hay, **1976**.
- [7] J. Tomasi, B. Mennucci, R. Cammi, *Chem. Rev.* **2005**, 105, 2999–3094.
- [8] R. Dennington II, T. Keith, J. Millam, *GaussView*, **2016**.
- [9] J. Bolger, A. Gourdon, E. Ishow, J.-P. Launay, *Inorg. Chem.* **1996**, 35, 2937–2944.
- [10] C. S. Burke, A. Byrne, Tia. E. Keyes, *J. Am. Chem. Soc.* **2018**, 140, 6945–6955.
- [11] A. T. Phan, J.-L. Mergny, *Nucleic Acids Res* **2002**, 30, 4618–4625.
- [12] S. K. Bharti, J. A. Sommers, J. Zhou, D. L. Kaplan, J. N. Spelbrink, J.-L. Mergny, R. M. Brosh, *J Biol Chem* **2014**, 289, 29975–29993.
- [13] H. Bertrand, A. Granzhan, D. Monchaud, N. Saettel, R. Guillot, S. Clifford, A. Guédin, J.-L. Mergny, M.-P. Teulade-Fichou, *Chemistry – A European Journal* **2011**, 17, 4529–4539.
- [14] J. L. Jeger, *Mol Biol Rep* **2020**, 47, 9801–9810.
- [15] J.-M. Cioni, J. Q. Lin, A. V. Holtermann, M. Koppers, M. A. H. Jakobs, A. Azizi, B. Turner-Bridger, T. Shigeoka, K. Franze, W. A. Harris, C. E. Holt, *Cell* **2019**, 176, 56-72.e15.
- [16] Y. Du, J. Han, F. Jin, Y. Du, *Pharmaceutics* **2022**, 14, 1763.
- [17] K. J. Steinberger, T. D. Eubank, *Oxygen (Basel)* **2023**, 3, 45–76.
- [18] E. Khakshour, M. T. Bahreyni-Toossi, K. Anvari, M. A. Shahram, F. Vaziri-Nezamdoust, H. Azimian, *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* **2024**, 828, 111848.
- [19] M. Y. Koh, G. Powis, *Trends Biochem Sci* **2012**, 37, 364–372.
- [20] M. Di Mattia, A. Mauro, S. Delle Monache, F. Pulcini, V. Russo, P. Berardinelli, M. R. Citeroni, M. Turriani, A. Peserico, B. Barboni, *Cells* **2022**, 11, 461.
- [21] Y. Yuan, G. Hilliard, T. Ferguson, D. E. Millhorn, *Journal of Biological Chemistry* **2003**, 278, 15911–15916.
- [22] J. Liu, A. W. Prentice, G. J. Clarkson, J. M. Woolley, V. G. Stavros, M. J. Paterson, P. J. Sadler, *Advanced Materials* **2023**, 35, 2210363.

- [23]K. R. Pitts, C. F. Toombs, *American Journal of Physiology-Heart and Circulatory Physiology* **2004**, 287, H1801–H1812.
- [24]Z. Darzynkiewicz, P. Pozarowski, B. W. Lee, G. L. Johnson, *Methods Mol Biol* **2011**, 682, 103–114.

Appendix D: Supplementary Information – Chapter 5

S5.1 Photophysical Characterisation Data (Courtesy of Dr. Ruben Arturo Arellano-Reyes)

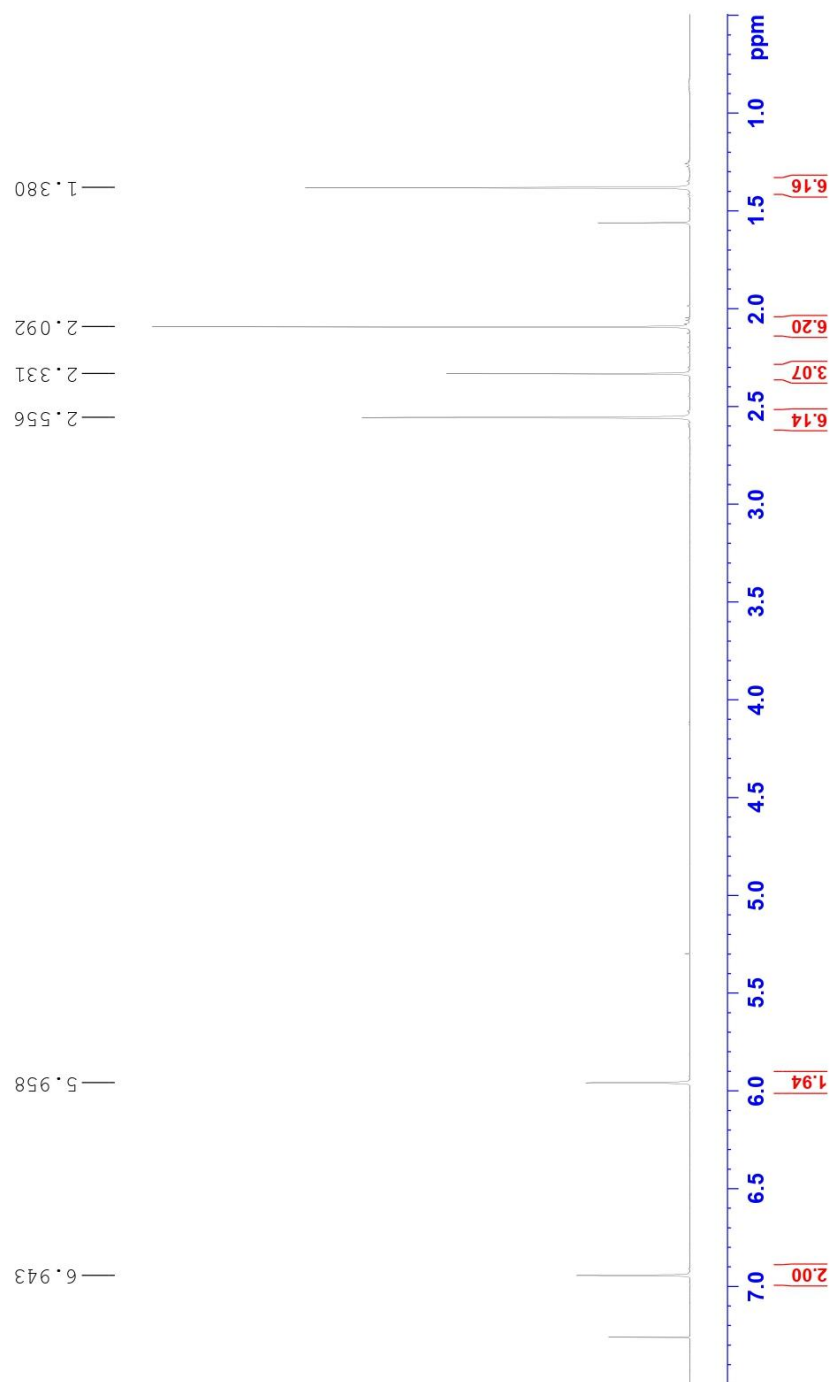


Figure 5.1 ^1H -NMR spectra of Mesityl BODIPY in CDCl_3 at 600 MHz.

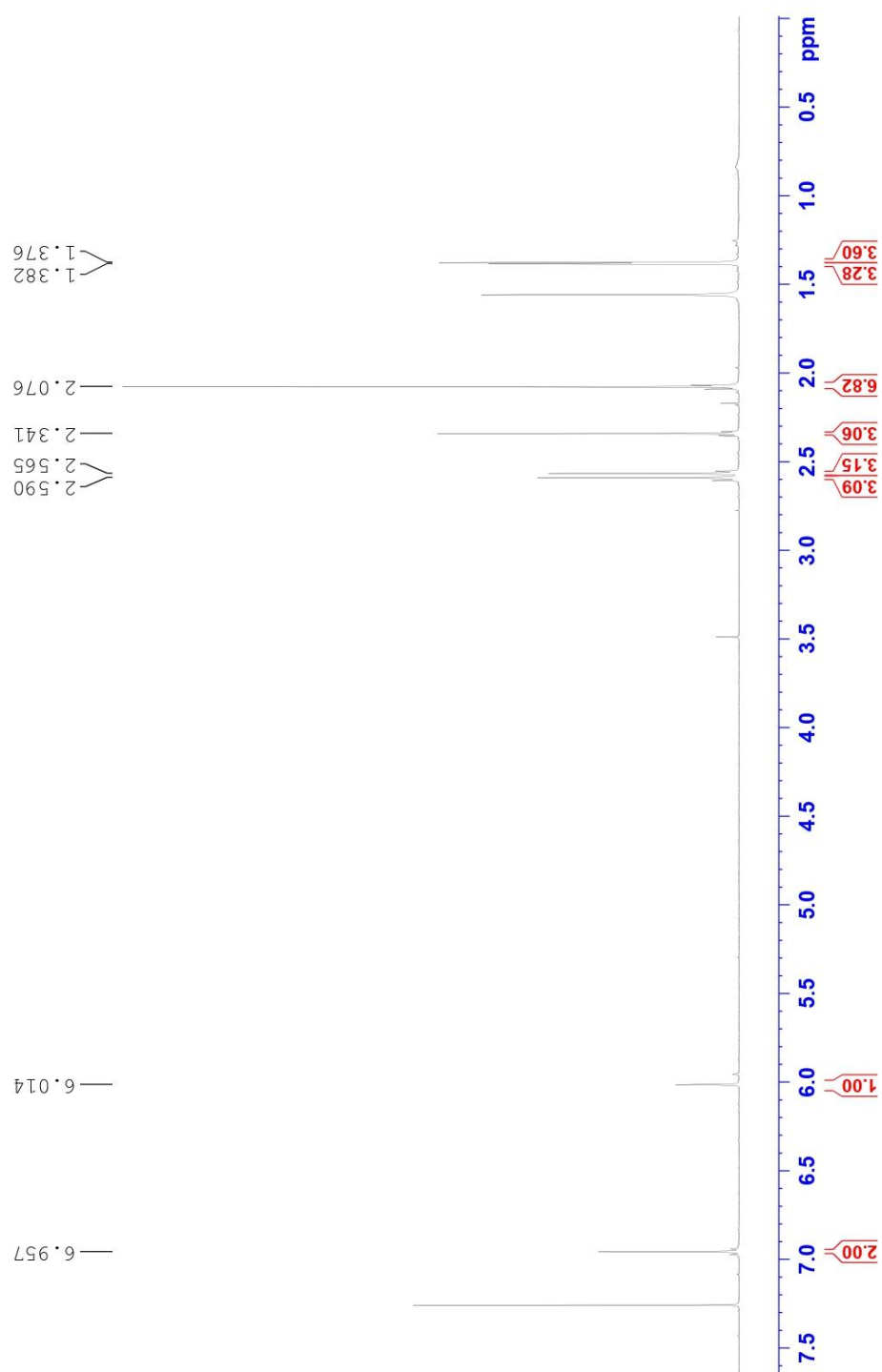


Figure S5.2 ¹H-NMR spectra of 2-BrMesityl BODIPY in CDCl₃ at 600 MHz.

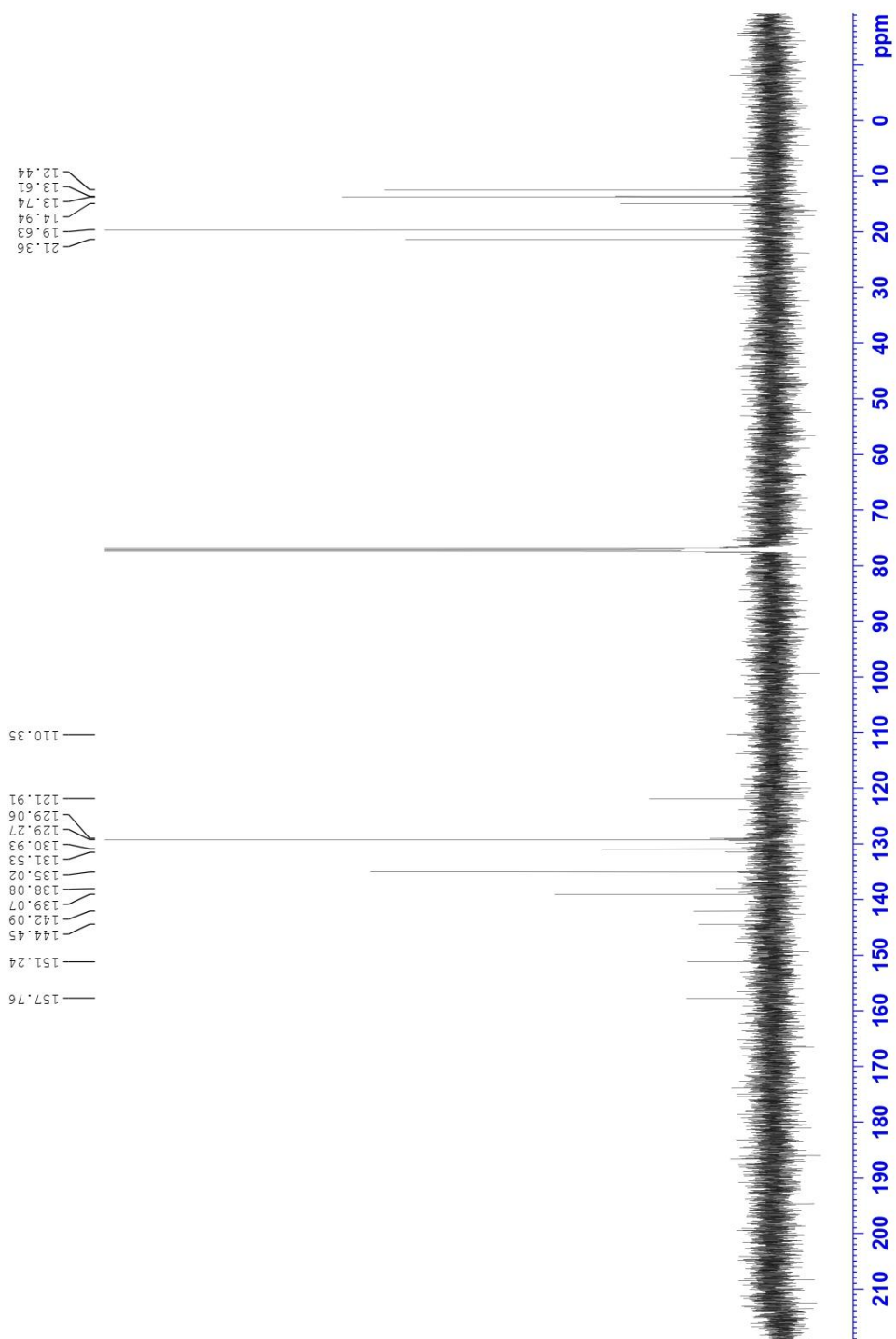


Figure S5.3 ^{13}C -NMR spectra of 2-BrMesityl BODIPY in CDCl_3 at 150 MHz.

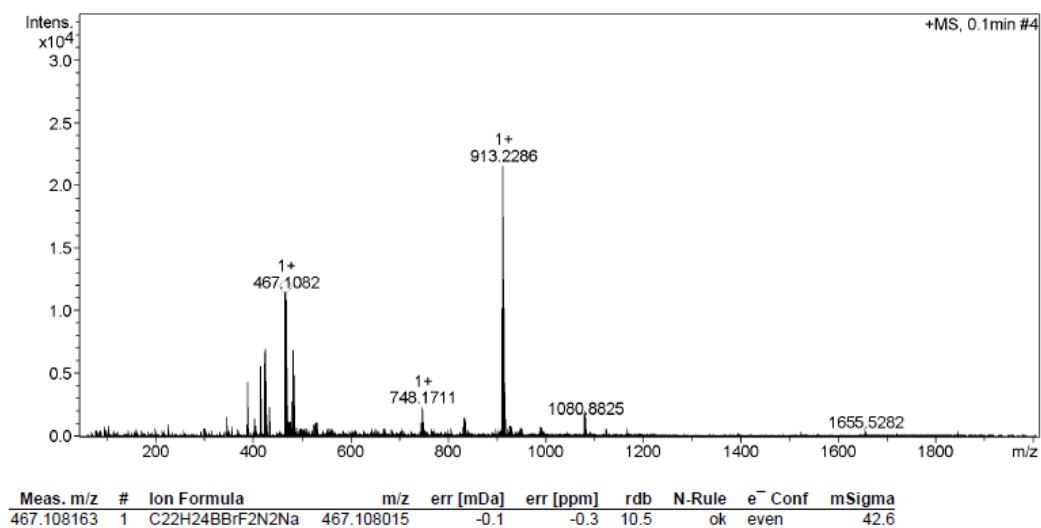


Figure S5.4 HR-MS (ESI-TOF) positive scan of 2-BrMesityl BODIPY.

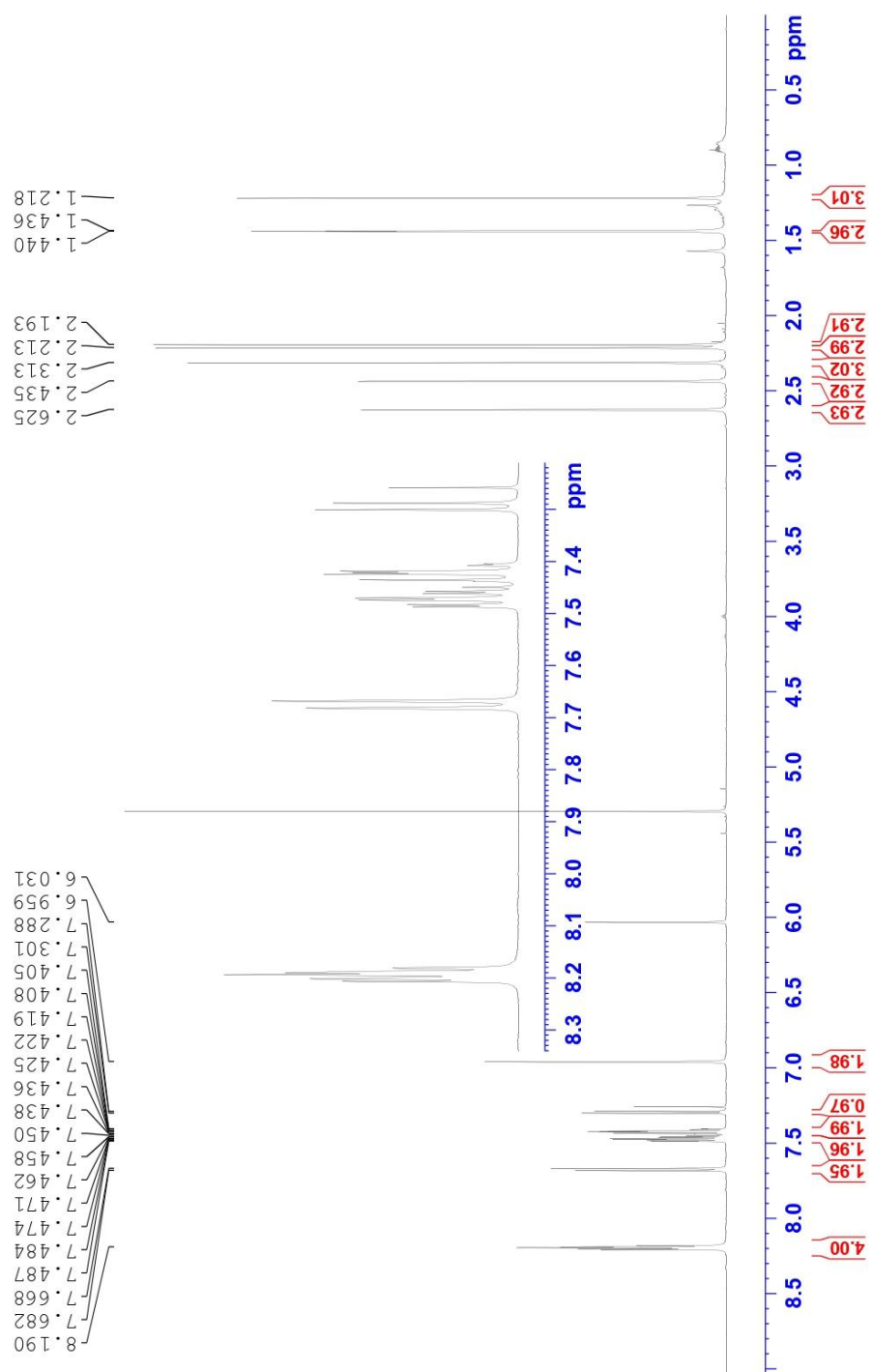


Figure S5.5 ^1H -NMR spectra of MB2P in CDCl_3 at 600 MHz.

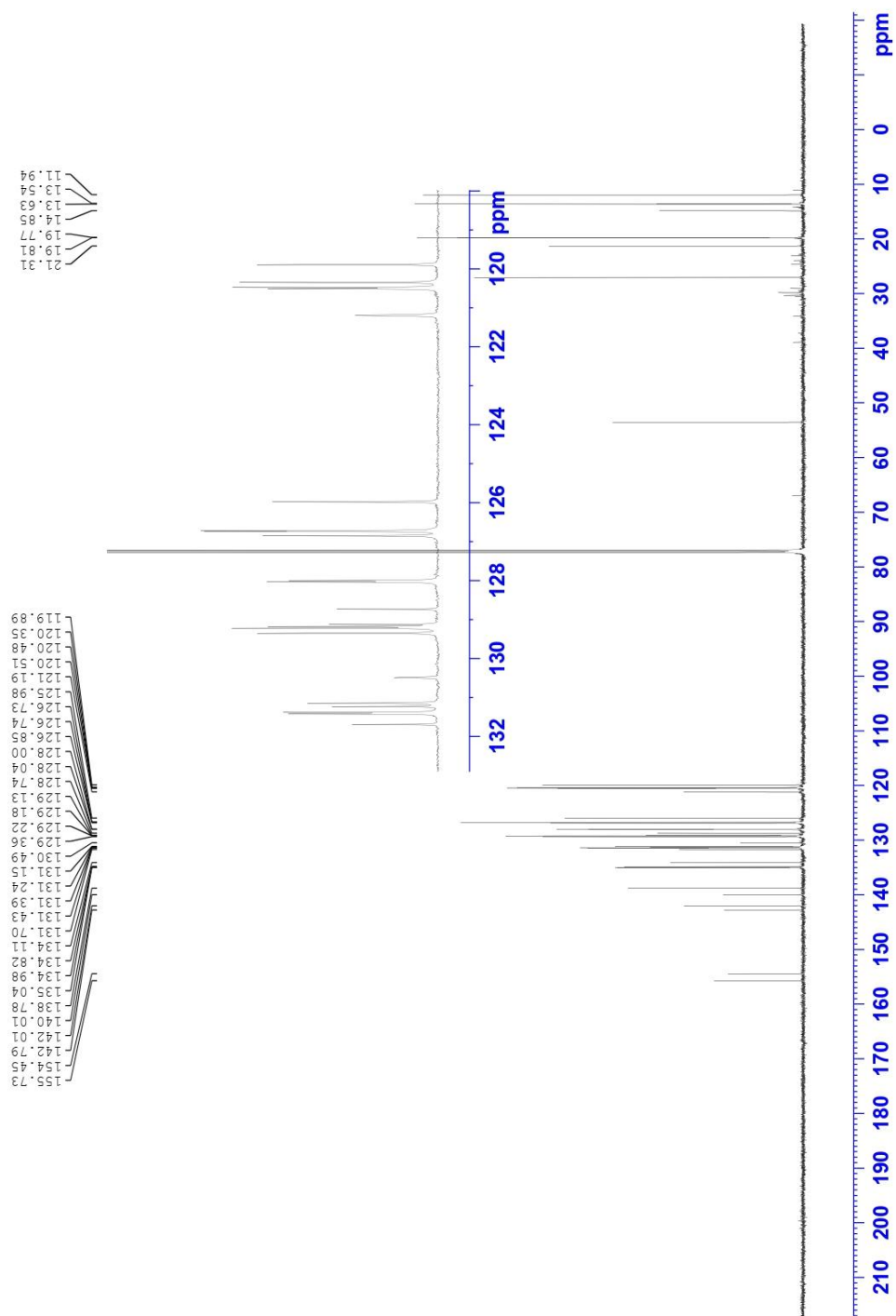


Figure S5.6 ^{13}C -NMR spectra of MB2P in CDCl_3 at 150 MHz.

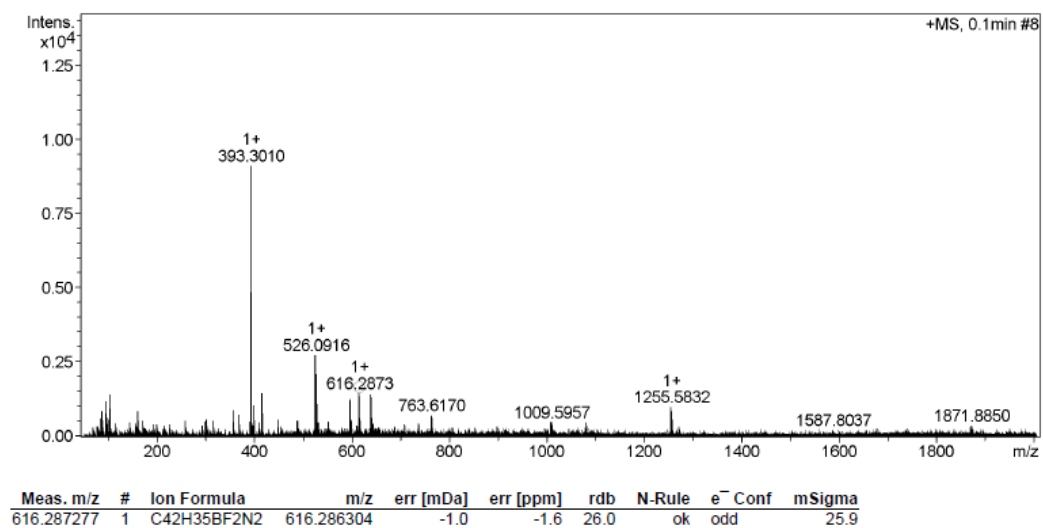


Figure S5.7 HR-MS (ESI-TOF) positive scan of MB2P.

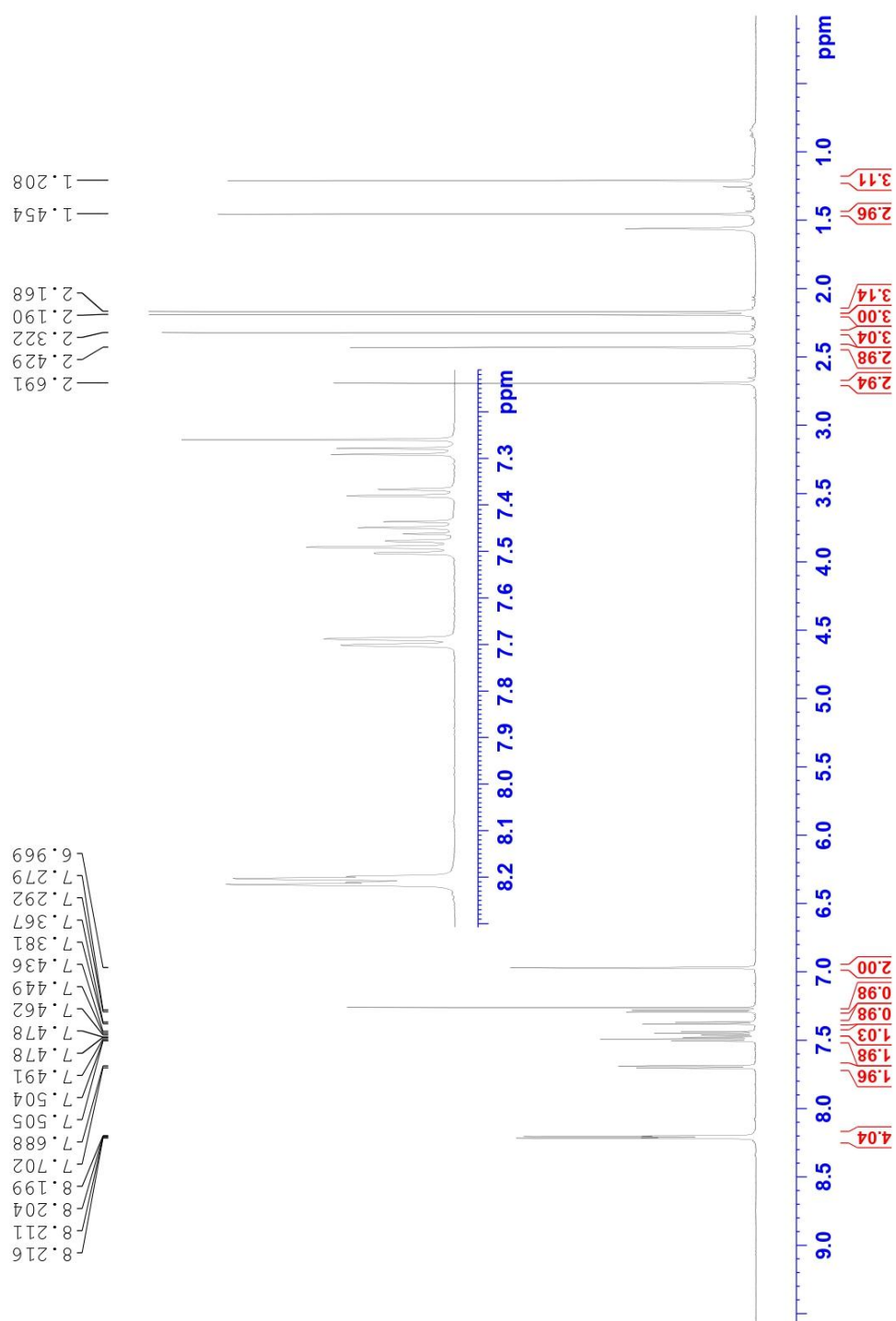


Figure S5.8 ^1H -NMR spectra of MB2PI in CDCl_3 at 600 MHz.

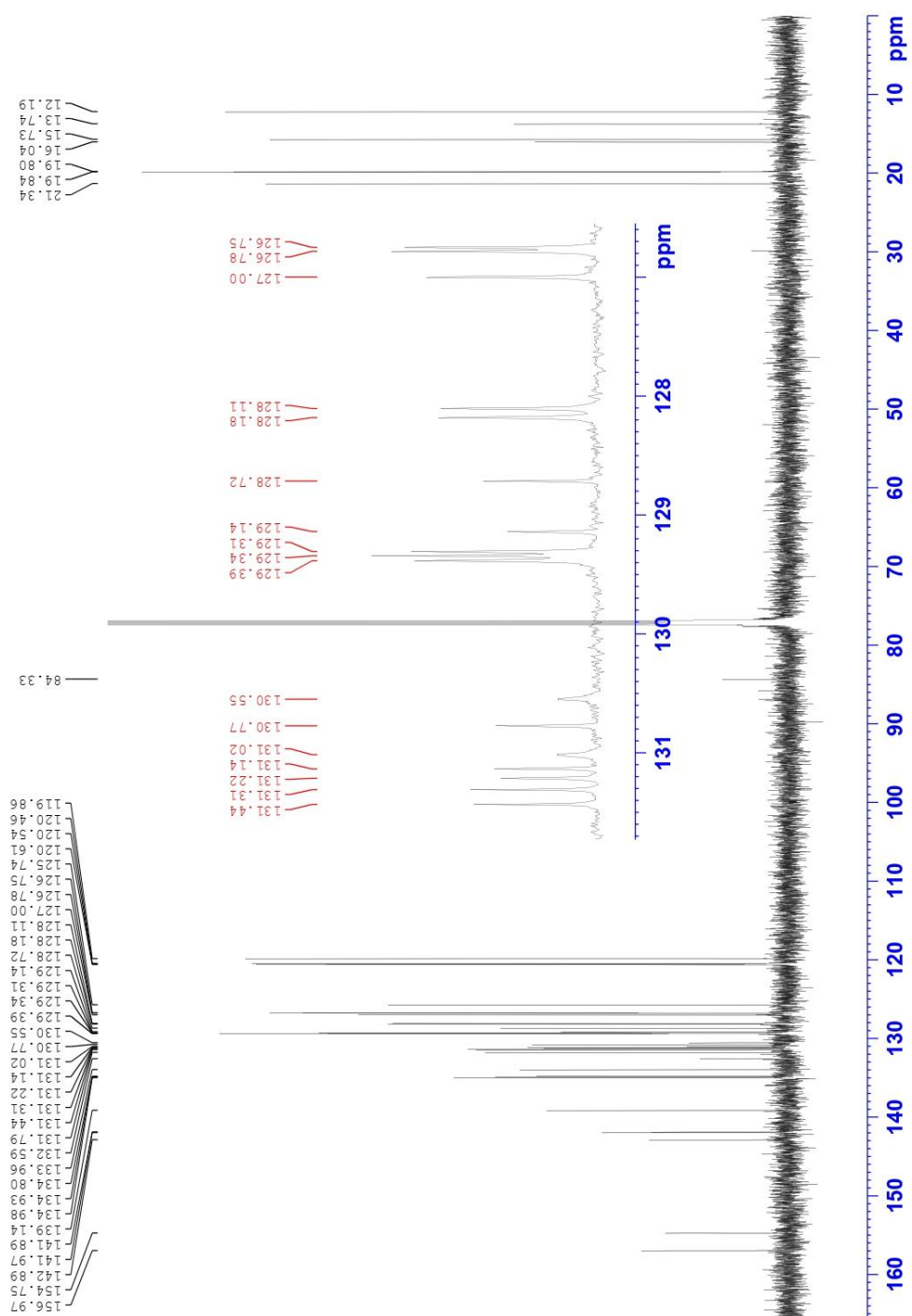


Figure S5.9 ¹³C-NMR spectra of MB2PI in CDCl₃ at 150 MHz.

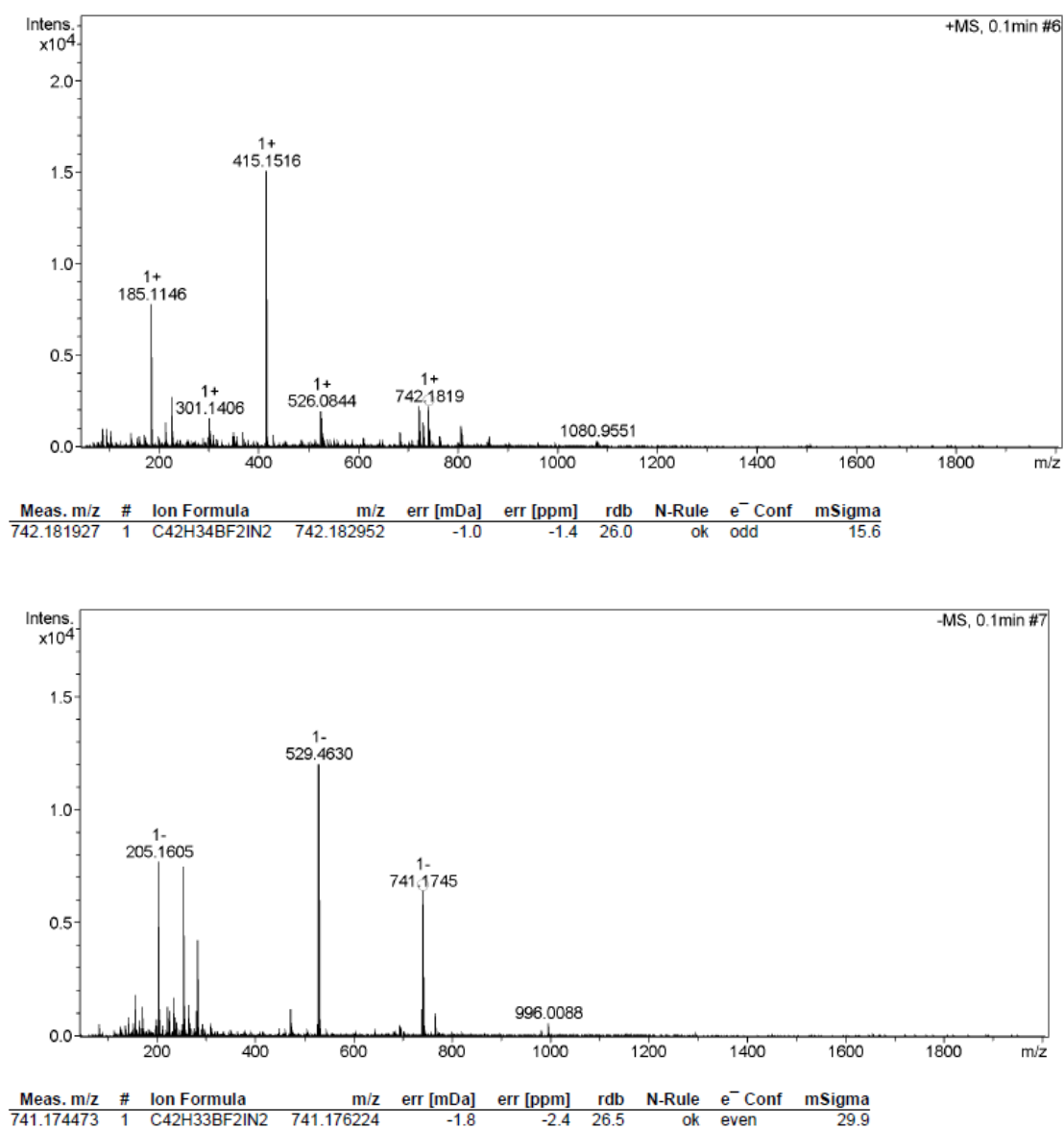


Figure S5.10 HR-MS (ESI-TOF) positive and negative scans of MB2PI.

S5.2 Crystallography (Courtesy of Dr. James N. McPherson and Professor Vickie McKee)

Table S5.1 Crystal data and structure refinement for MB2P·0.475(CHCl₃)

Empirical formula	C _{42.48} H _{35.48} BCl _{1.43} F ₂ N ₂
Formula weight	673.23
Temperature/K	100.01(11)
Crystal system	monoclinic
Space group	P2 ₁ /c
a/Å	22.5919(8)
b/Å	12.5812(5)
c/Å	25.2321(9)
α/°	90
β/°	96.352(3)
γ/°	90
Volume/Å ³	7127.8(5)
Z	8
ρ _{calc} g/cm ³	1.255
μ/mm ⁻¹	1.584
F(000)	2812.0
Crystal size/mm ³	0.609 × 0.043 × 0.032
Radiation	Cu Kα (λ = 1.54184)
2θ range for data collection/°	7.05 to 133.202
Index ranges	-26 ≤ h ≤ 26, -14 ≤ k ≤ 10, -28 ≤ l ≤ 30
Reflections collected	52467
Independent reflections	12557 [R _{int} = 0.0886, R _{sigma} = 0.0612]
Data/restraints/parameters	12557/3043/1179
Goodness-of-fit on F ²	1.059
Final R indexes [I >= 2σ (I)]	R1 = 0.1039, wR2 = 0.2897
Final R indexes [all data]	R1 = 0.1305, wR2 = 0.3148
Largest diff. peak/hole / e Å ⁻³	0.81/-0.40
CCDC deposition number	2157191

The data were collected at 100(1)K on a Synergy, Dualflex, AtlasS2 diffractometer using CuKα radiation (λ = 1.54184 Å) and the *CrysAlis PRO* 1.171.40.67a suite¹. Using SHELXLE² and Olex2³ the structure was solved by dual space methods (SHELXT⁴) and refined on F² using all the reflections (SHELXL-2018/3⁵). All the non-hydrogen atoms were refined using anisotropic atomic displacement parameters, except for the carbon atoms of the overlapping, partial-occupancy chloroform solvate molecules and the 5% occupancy chlorine atoms.

SAME restraints were applied to all the perylene groups, to the disordered section of the BODIPY in mol 1, and to the chloroform molecules. Hydrogen atoms were inserted at

calculated positions using a riding model. The crystals diffracted poorly and, despite a long collection time (ca. 80 hr), the data set is very weak and the structure shows significant disorder. Consequently, the precision of the structure determination is reduced but the main features are clear. Crystal parameters, data collection and structure refinement details are summarised in Table S1. CCDC 2157191 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

1. Rigaku Oxford Diffraction, (2019), CrysAlisPro Software system, version 1.171.40.67a, Rigaku Corporation, Oxford, UK.
2. C.B. Hübschle, G.M. Sheldrick and B. Dittrich. *J. Appl. Cryst.*, 2011, **44**, 1281-1284.
3. O.V. Dolomanov, L.J. Bourhis, R.J. Gildea, J.A.K. Howard & H. Puschmann. *J. Appl. Cryst.*, 2009, **42**, 339-341
4. G.M. Sheldrick, *Acta Cryst.*, 2015, **A71**, 3-8.
5. G.M. Sheldrick, *Acta Cryst.*, 2015, **C71**, 3-8.

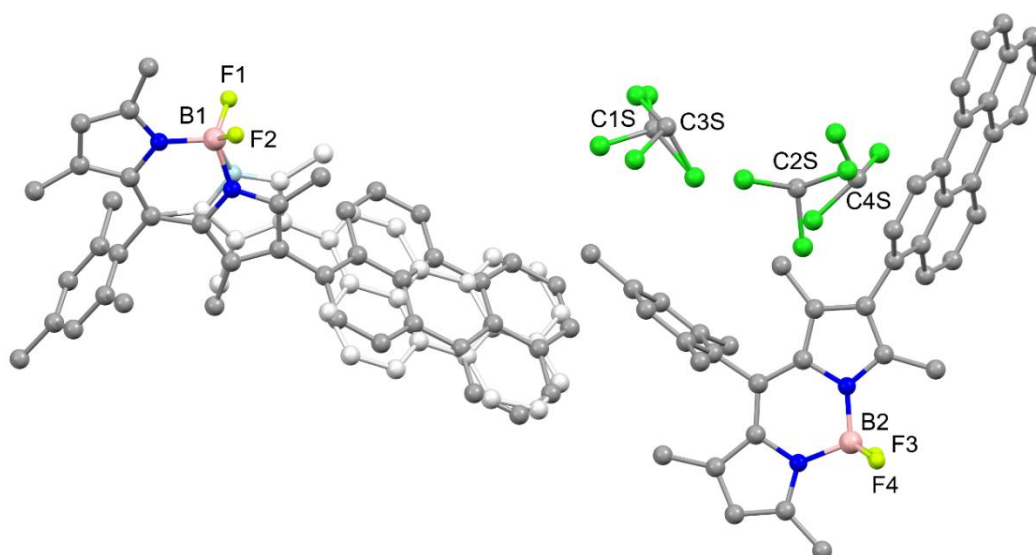


Figure S5.11 Asymmetric unit showing disorder in the molecule containing B1 (minor component shown in paler colours).

The asymmetric unit (above) contains two independent $C_{42}H_{35}BF_2N_2$ molecules and some chloroform solvate. In the molecule containing B1 (mol1), the perylene group is disordered by rotation of 180° about the C–C bond linking it to the BODIPY section and the site occupancies refined to 0.74 and 0.26 for the major and minor components, respectively. A slight displacement of the linked 5-membered ring allows a close spatial overlap of the two perylene components (Figures S5.12 & S5.13).

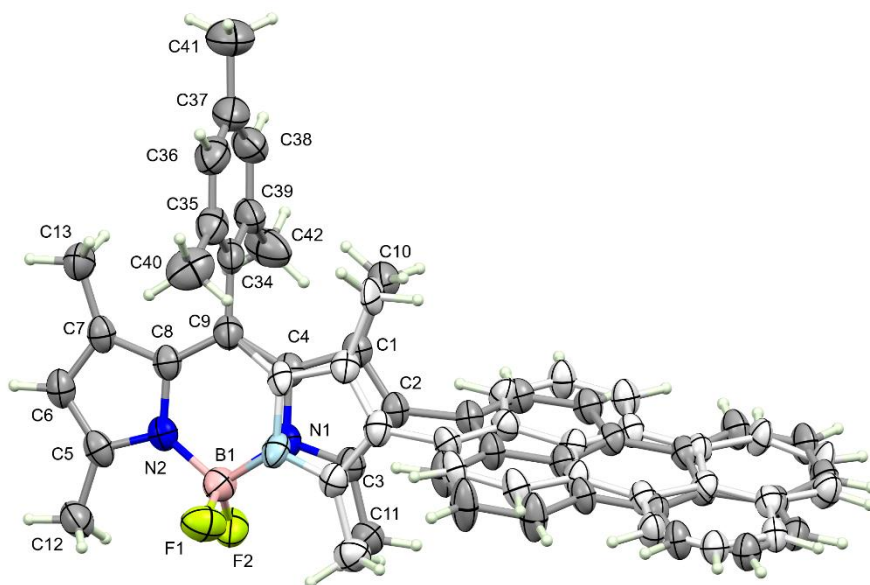


Figure S5.12 Disordered molecule 1 (50% probability ellipsoids) showing numbering scheme for the BODIPY section. Minor component shown in paler colours.

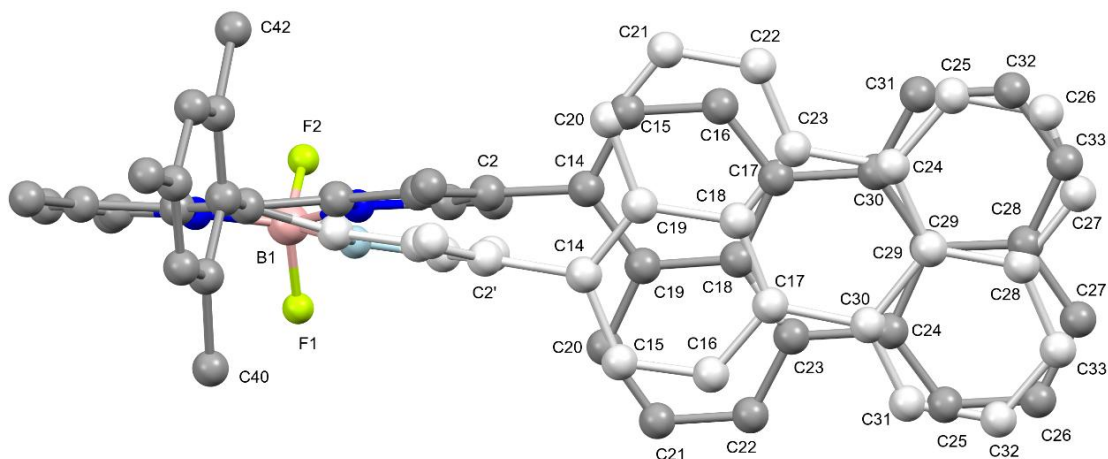


Figure S5.13 Disordered mol1 showing numbering scheme for the perylene sections. Minor component shown in paler colours.

The ADPs of the second molecule (mol2, containing B2) suggest a small amount of disorder in the perylene group (Figure S5.14) but this has not been modelled, given the low data quality and the more significant disorders elsewhere. The conformations of the two molecules are similar and the small differences (Figure S5.15) can probably be ascribed to intermolecular interactions in the solid state. In each case the mean plane of the perylene group is rotated with respect to the mean plane of the BODIPY core, the interplanar angles are $69.8(1)^\circ$ and $73.5(3)^\circ$ for the major and minor components of mol1 and $73.23(6)^\circ$ for mol2.

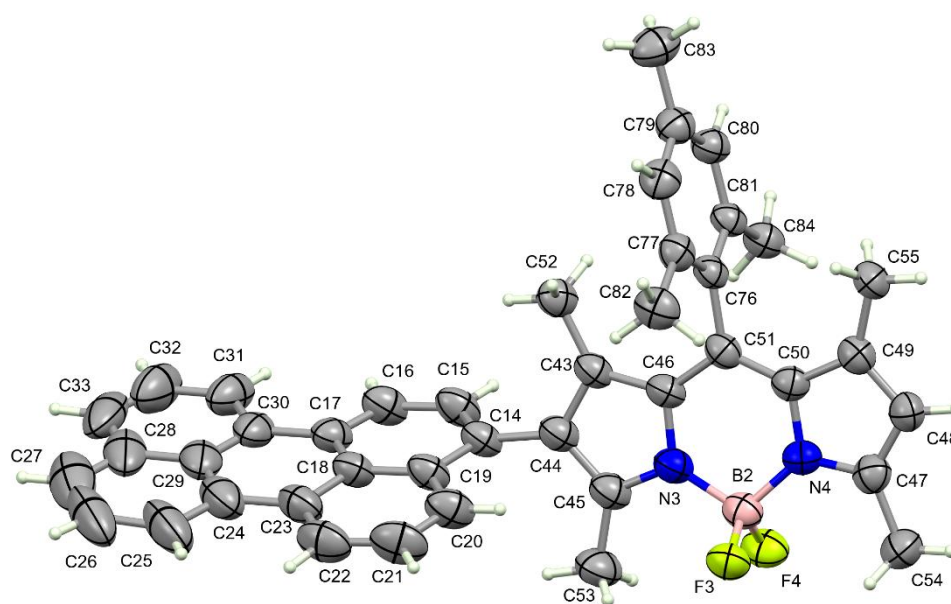


Figure S5.14 Mol 2 (50% probability ellipsoids) showing numbering scheme.

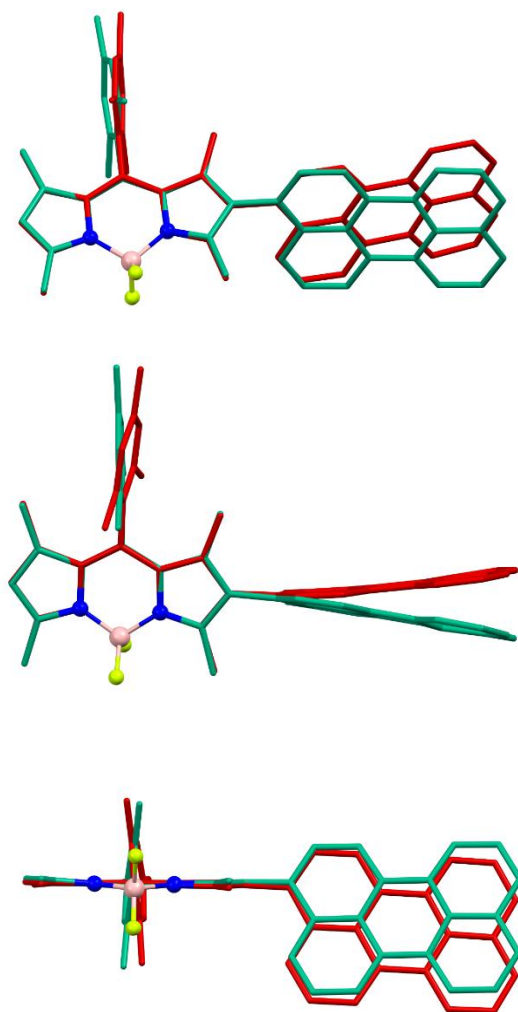


Figure S5.15 Overlays of mol1 (green) and mol2 (red), based on fitting the N and F atoms only. Only the major component of the disorder is shown for mol1 and it is inverted relative to the asymmetric unit coordinates (permitted in this centrosymmetric space group).

Mol1 is paired with a symmetry equivalent by inversion (symmetry operation 1-x, 1-y, 1-z) to generate π -stacked dimeric units; this stacking does not extend further through the structure (Figure S5.16). There are no equivalent π -interactions involving mol2 but there is a set of reasonably convincing (sp^2)C–H $\cdots$ F hydrogen bonds linking the mol2 units into chains running parallel to the *b* axis (Figure S5.17).

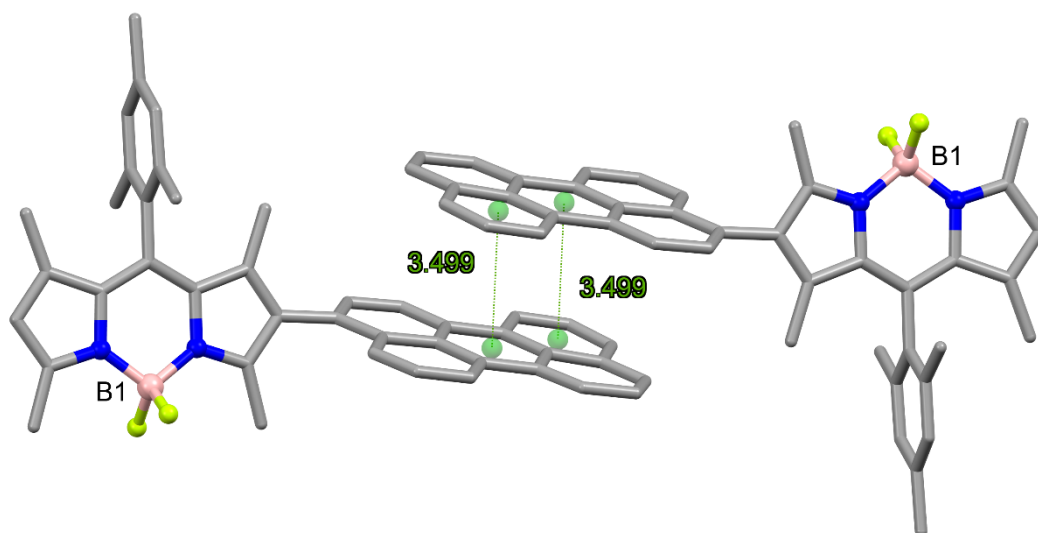


Figure S5.16 π -stacking showing centroid-centroid distances (Å) between mol1 and its symmetry equivalent under 1-x, 1-y, 1-z.

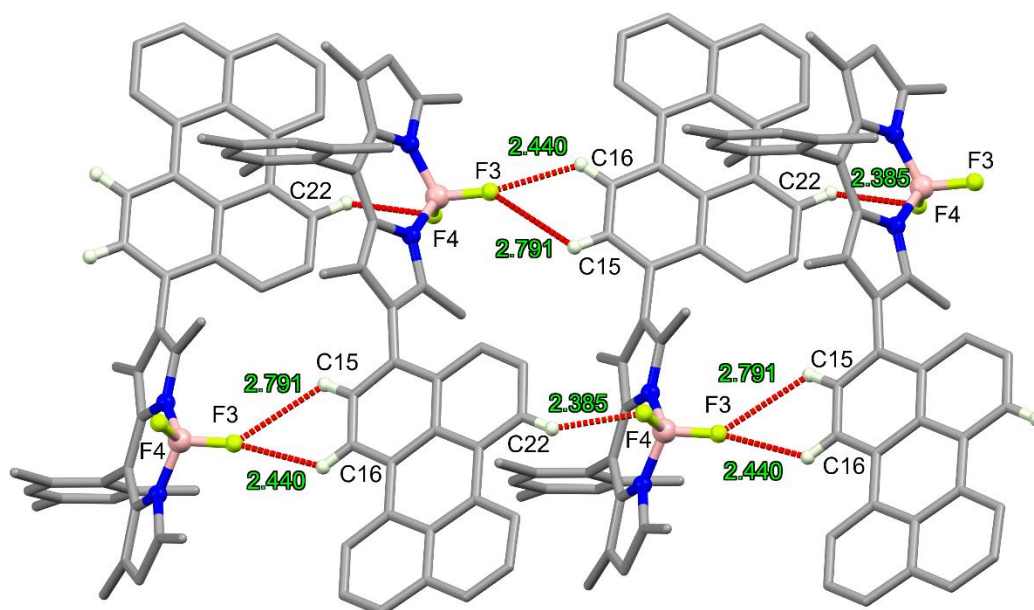


Figure S5.17 Dashed red lines indicate (sp²)C–H...F interactions linking the mol2 units into chains running parallel to the b axis.

Examination of the unit cell packing (Figures S5.18 & S5.19) shows the solvate chloroform molecules lying in columns parallel to the b axis, the solvate was modelled over four overlapping sites with occupancies of 0.35, 0.40, 0.15 and 0.05 for the molecules centered on C1S, C2S, C3S and C4S, respectively. The site occupancies were adjusted by hand to minimise the residual electron density in this region. Part-occupancy carbon atoms C1S, C2S and the 0.05 occupancy chlorine atoms were refined isotropically, while a fixed isotropic parameter was used for C3S and C4S.

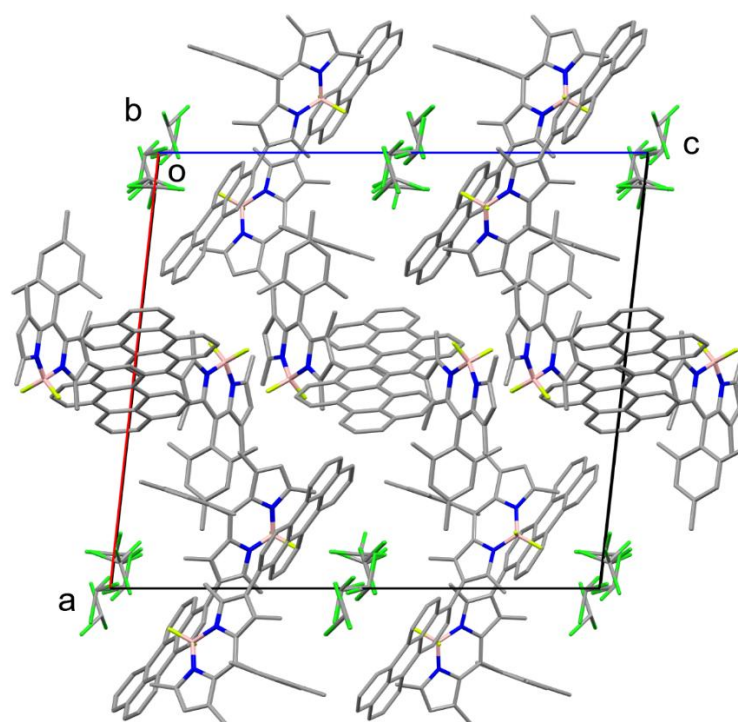


Figure S5.18 Unit cell packing viewed down the b axis. Perylene disorder and hydrogen atoms removed for clarity.

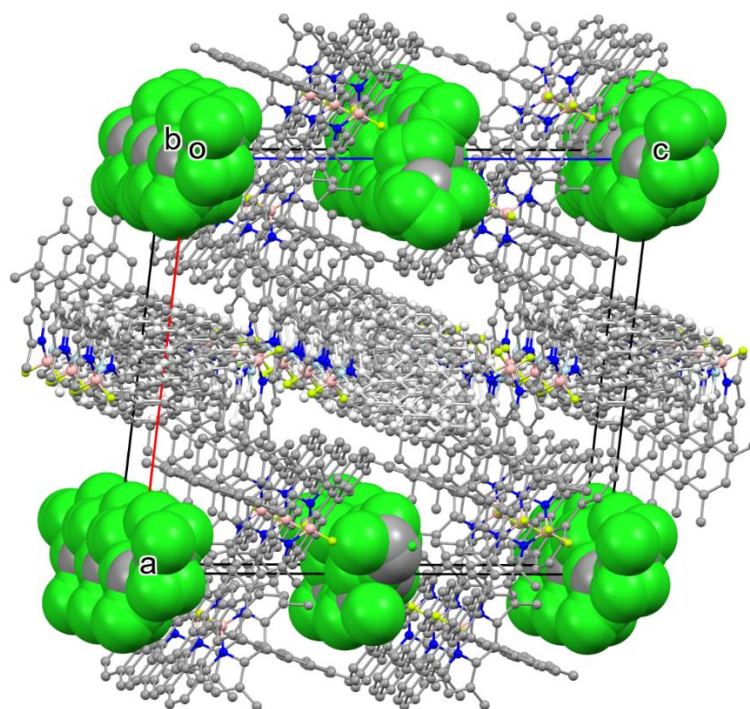


Figure S5.19 Unit cell packing showing partial-occupancy chloroform molecules (spacefill) in channels parallel to the b axis. Hydrogen atoms removed for clarity.

S5.3 Photophysical Data

S5.3.1 BODIPY-erylene in PBS and Cell Culture Media

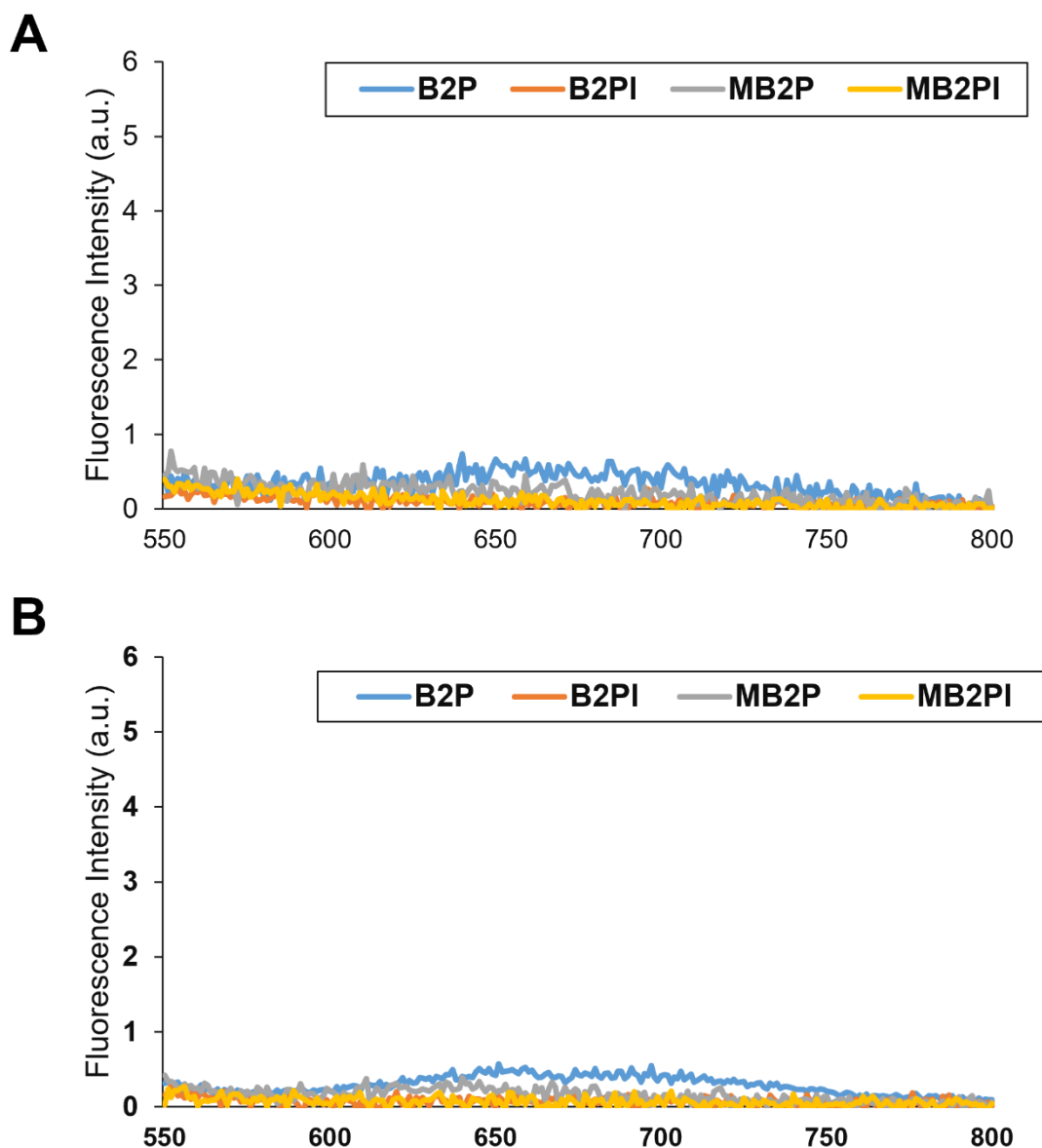


Figure S5.20 Emission of B2P, B2PI, MB2P and MB2PI (10 μ M) in **(A)** phenol red free DMEM cell culture media and **(B)** PBS with 10% DMSO. B2P/MB2P were excited at 515 nm and B2PI/MB2PI excited at 532 nm and data was recorded with a slid width of 2.5-2.5 nm to match the photophysical studies reported in Figures 5.1, 5.2 and Table 5.1.

S5.3.2 Response to Viscosity in Solution

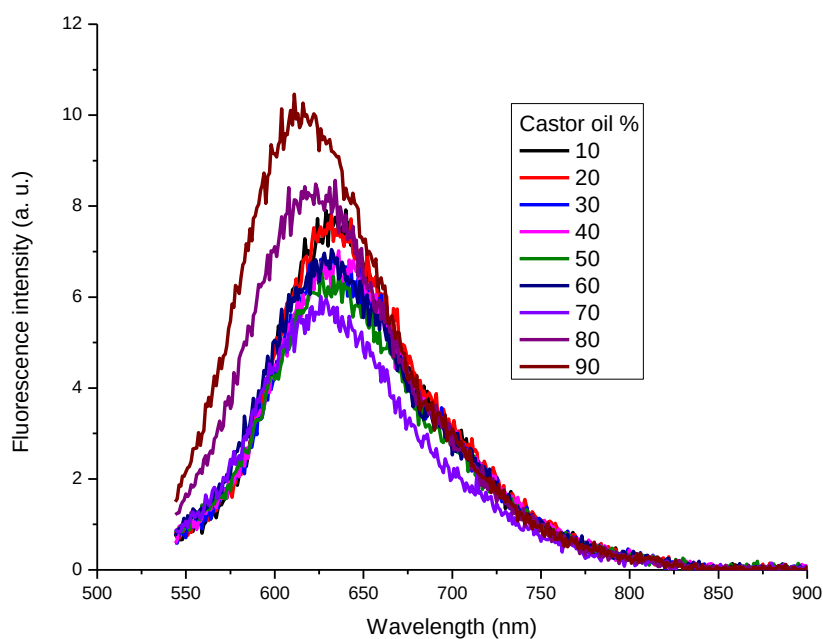


Figure S5.21 Emission of MB2PI (10 μ M) in toluene with varying concentrations of castor oil. Figure courtesy of Dr. Ruben Arturo Arellano-Reyes.

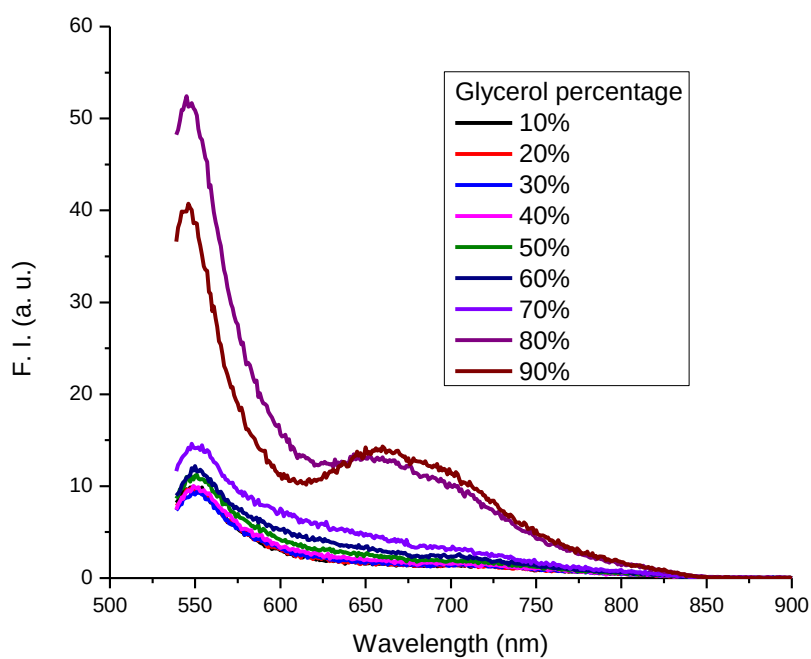


Figure S5.22 Emission of MB2PI (10 μ M) in methanol with varying concentrations of glycerol where F.I. indicates fluorescence intensity. Figure courtesy of Dr. Ruben Arturo Arellano-Reyes.

S5.4 Cellular Studies

S5.4.1 Uptake in Live Cell Monolayers

CHO cells were incubated in 75cm² cell culture flasks (Corning) with DMEM/F-12 Ham medium (Sigma-Aldrich) with L-glutamine and sodium bicarbonate supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin-streptomycin (Sigma-Aldrich). MCF-7 cells were incubated in 25cm² cell culture flasks with RPMI-1640 medium (Sigma-Aldrich) with L-glutamine and sodium bicarbonate supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin. Cells were incubated at 37 °C with 5% CO₂ and detached from the flask at 90% confluency using a 1X Trypsin-EDTA solution (Sigma-Aldrich). Cells were counted using the EVE automated cell counter (NanoEnTek) and seeded at 1 x 10⁵ cells per mL in a 4 or 8 chamber μ -slide (ibidi) for cell imaging and left for 24 h at 37 °C. Cells were rinsed with 1X PBS (Gibco) and stained with B2P or B2PI at 10 μ M and MB2P or MB2PI at 30 μ M for 17 h. Cells were washed twice with PBS and covered with phenol red free RPMI medium (Gibco) for imaging. Live cells were imaged using a Leica SP8 confocal microscope with 63x oil immersion lens and heated stage at 37 °C.

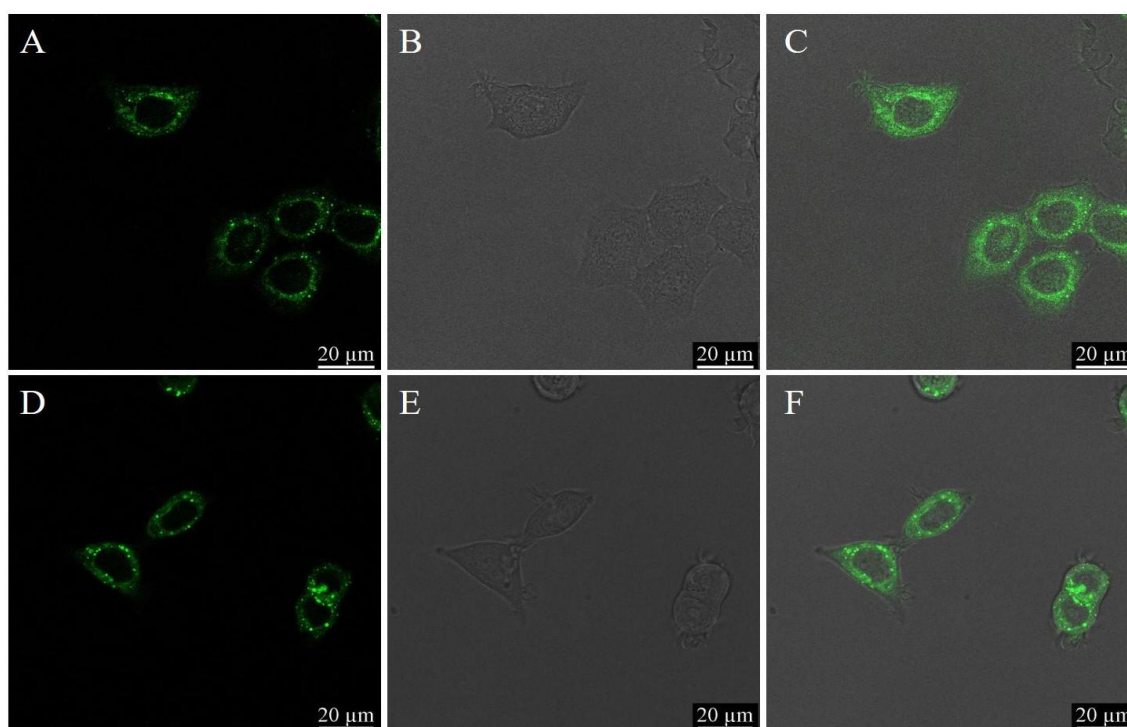


Figure S5.23 Confocal imaging of 10 μ M B2P in MCF-7 cells after **(A-C)** 4 and **(D-F)** 17 h incubation.

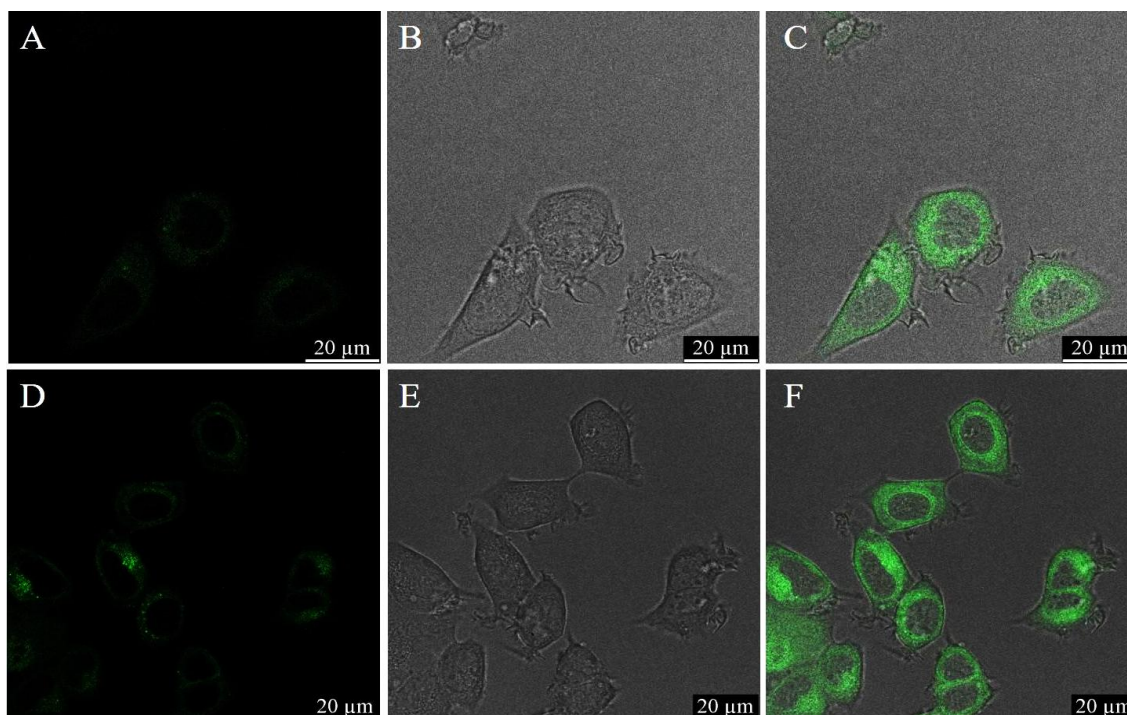


Figure S5.24 Confocal imaging of 10 μM B2PI in MCF-7 cells after **(A-C)** 4 and **(D-F)** 17 h incubation.

S5.4.2 Localisation in Live Cell Monolayers

For co-localisation studies, LysoTracker Deep Red, ER Tracker Blue-White, PyLa-C17Cer or BODIPY 493/503 were added to CHO and MCF-7 cells after the 17 h incubation period with the BODIPY probes. Cells were washed twice with PBS and imaged in phenol red free media. The BODIPY-perylene compounds were excited at their respective λ_{abs} and emission for co-localisation studies was collected from 580-640 nm, where the emission collection window was shortened depending on the λ_{em} of the BODIPY derivative being tested to ensure minimal overlap with the commercial dyes. Moreover, images were obtained by sequential sequencing with gating applied. The conditions for co-localisation studies are as follows: LysoTracker Deep Red (100 nM, 1 h) Ex 647, Em 665-685 nm; ER Tracker Blue/White (1 μM , 45 minutes) Ex 405 nm, Em 435-560 nm; PyLa-C17Cer (2 μM , 2 h) Ex 405 nm, Em 430-550 nm and BODIPY 493/503 (2 μM , 30 min) Ex 493 nm, Em 500-515 nm. Co-localisation analysis was performed using ImageJ.

Table S5.2 Pearson's coefficient values of the BODIPY-erylene compounds in CHO and MCF-7 cells with LysoTracker Deep Red (100 nM, 1 h), ER Tracker Blue/White (1 μ M, 45 min), PyLa-C17Cer (2 μ M, 2 hours) and BODIPY 493/503 (2 μ M, 30 min).

BODIPY	Cell Line	Pearson Coefficient (r)			
		LysoTracker Deep Red	ER Tracker Blue/White	PyLa-C17Cer	BODIPY 493/503
B2P	CHO	0.34 $\pm$ 0.04	0.17 $\pm$ 0.06	0.69 $\pm$ 0.03	0.78 $\pm$ 0.09
	MCF-7	0.75 $\pm$ 0.11	0.14 $\pm$ 0.07	0.55 $\pm$ 0.07	0.75 $\pm$ 0.07
B2PI	CHO	0.65 $\pm$ 0.05	0.46 $\pm$ 0.04	0.65 $\pm$ 0.04	
	MCF-7	0.71 $\pm$ 0.03	0.19 $\pm$ 0.05	0.54 $\pm$ 0.05	
MB2P	CHO	0.59 $\pm$ 0.08	0.56 $\pm$ 0.02	0.67 $\pm$ 0.09	0.70 $\pm$ 0.03
	MCF-7	0.57 $\pm$ 0.06	0.54 $\pm$ 0.04	0.67 $\pm$ 0.03	0.66 $\pm$ 0.11
MB2PI	CHO	0.44 $\pm$ 0.07	0.46 $\pm$ 0.01	0.33 $\pm$ 0.06	0.23 $\pm$ 0.04
	MCF-7	0.59 $\pm$ 0.09	0.53 $\pm$ 0.04	0.49 $\pm$ 0.04	0.45 $\pm$ 0.06

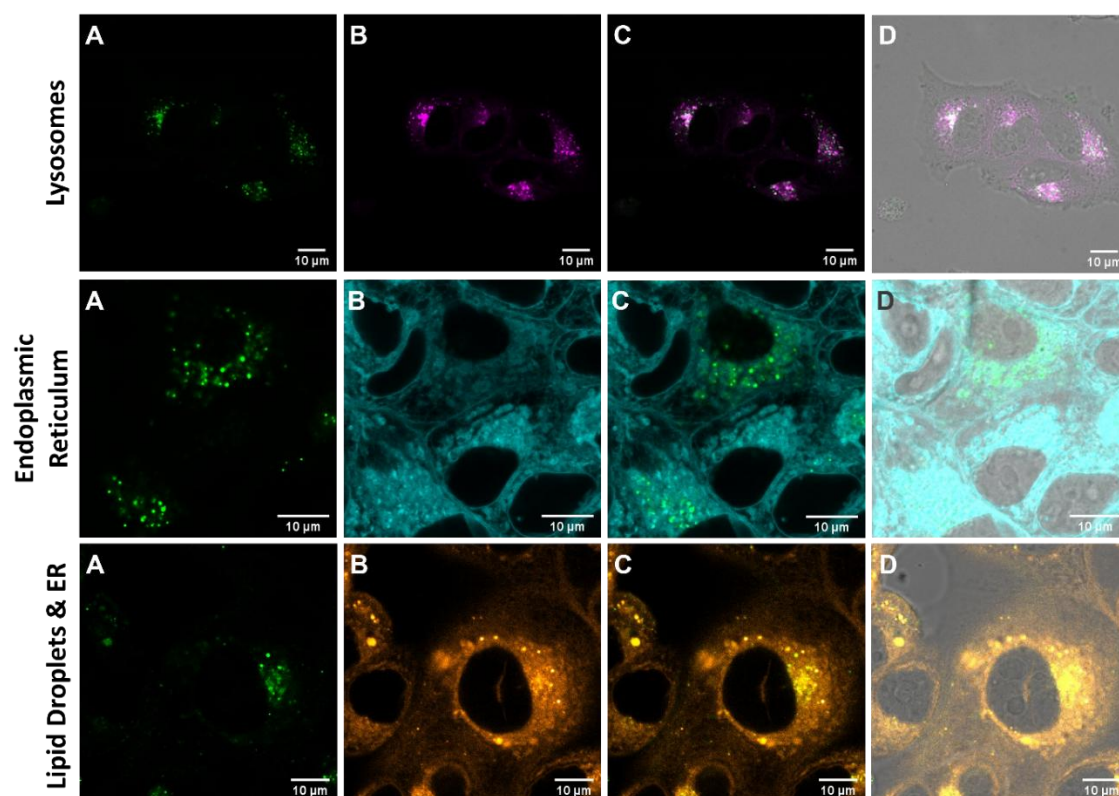


Figure S5.25 Co-localisation imaging of B2PI (10 μ M, 17 h) with LysoTracker Deep Red, ER Tracker Blue/White and PyLa-C17Cer. Images are labelled with the corresponding dyes in MCF-7 cells, where **(A)** is the B2PI channel, **(B)** indicates the co-localisation dyes where LysoTracker is indicated by magenta, ER Tracker Blue/White is cyan and PyLa-C17Cer is orange. The overlay is shown with and without the phase contrast channel in **(C)** and **(D)**.

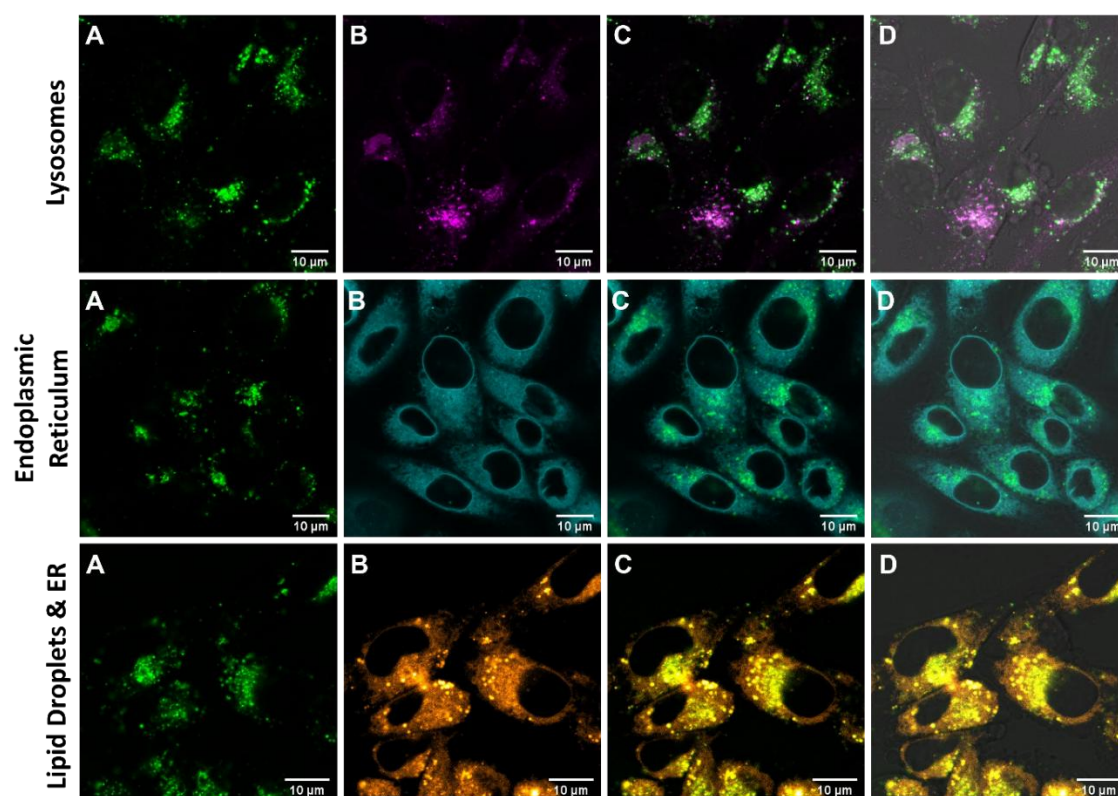


Figure S5.26 Co-localisation imaging of B2PI (10 μ M, 17 h) with LysoTracker Deep Red, ER Tracker Blue/White and PyLa-C17Cer. Images are labelled with the corresponding dyes in CHO cells, where **(A)** is the B2PI channel, **(B)** indicates the co-localisation dyes where LysoTracker is indicated by magenta, ER Tracker Blue/White is cyan and PyLa-C17Cer is orange. The overlay is shown with and without the phase contrast channel in **(C)** and **(D)**.

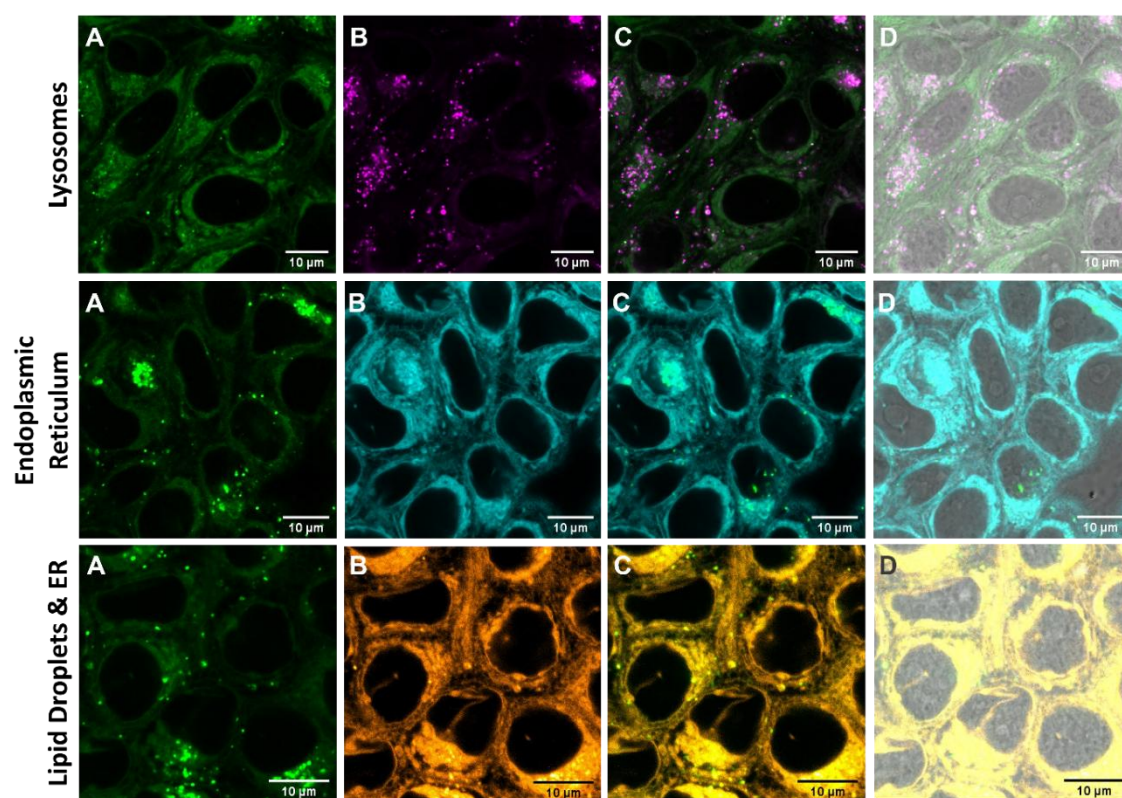


Figure S5.27 Co-localisation imaging of M2P (30 μ M, 17 h) with LysoTracker Deep Red, ER Tracker Blue/White and PyLa-C17Cer. Images are labelled with the corresponding dyes in MCF-7 cells, where **(A)** is the B2PI channel, **(B)** indicates the co-localisation dyes where LysoTracker is indicated by magenta, ER Tracker Blue/White is cyan and PyLa-C17Cer is orange. The overlay is shown with and without the phase contrast channel in **(C)** and **(D)**.

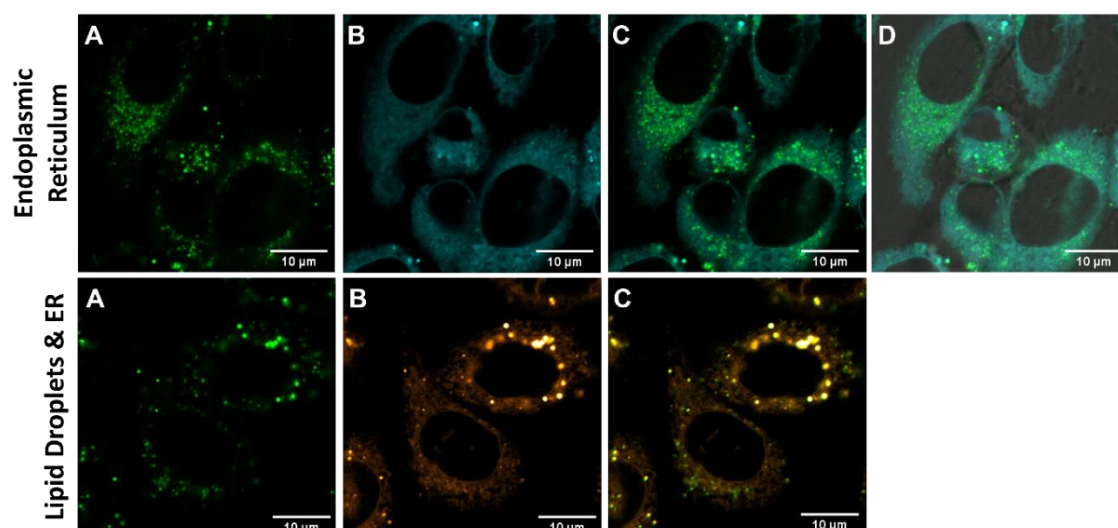


Figure S5.28 Co-localisation imaging of M2P (30 μ M, 17 h) with ER Tracker Blue/White and PyLa-C17Cer. Images are labelled with the corresponding dyes in CHO cells, where **(A)** is the B2PI channel, **(B)** indicates the co-localisation dyes where LysoTracker is indicated by magenta, ER Tracker Blue/White is cyan and PyLa-C17Cer is orange. The overlay is shown with and without the phase contrast channel in **(C)** and **(D)**.

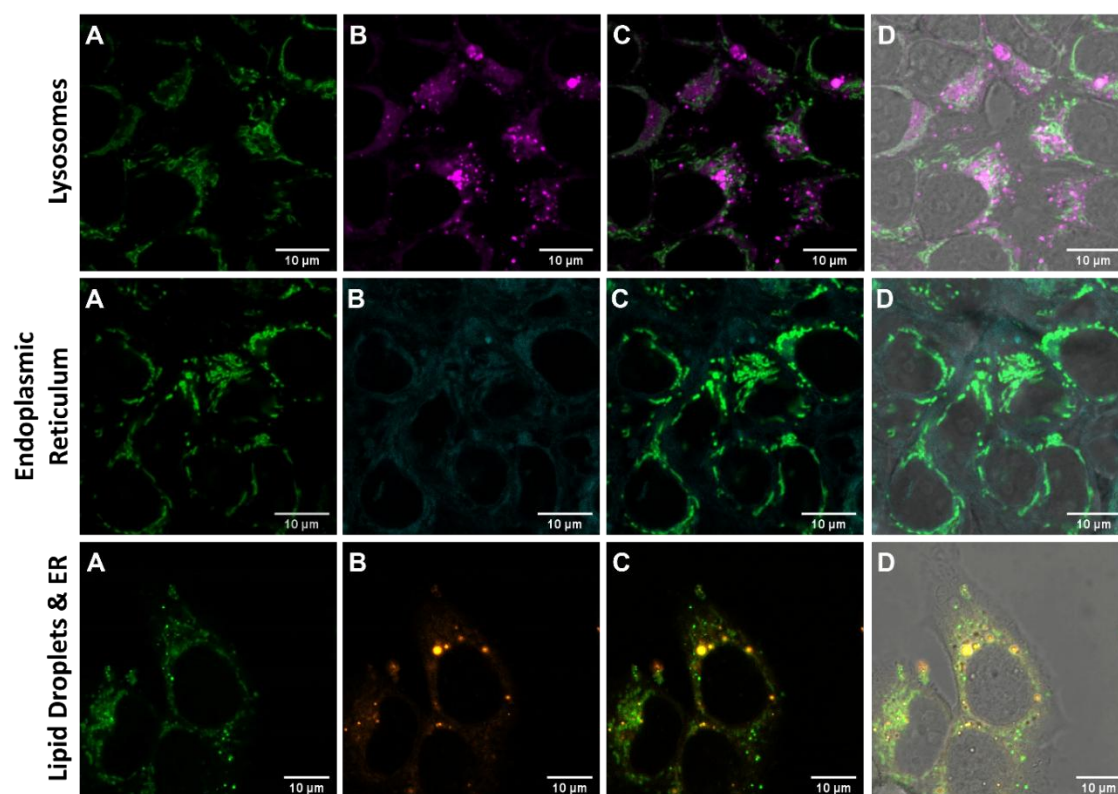


Figure S5.29 Co-localisation imaging of MB2PI (30 μ M, 17 h) with LysoTracker Deep Red, ER Tracker Blue/White and PyLa-C17Cer. Images are labelled with the corresponding dyes in MCF-7 cells, where **(A)** is the B2PI channel, **(B)** indicates the co-localisation dyes where LysoTracker is indicated by magenta, ER Tracker Blue/White is cyan and PyLa-C17Cer is orange. The overlay is shown with and without the phase contrast channel in **(C)** and **(D)**.

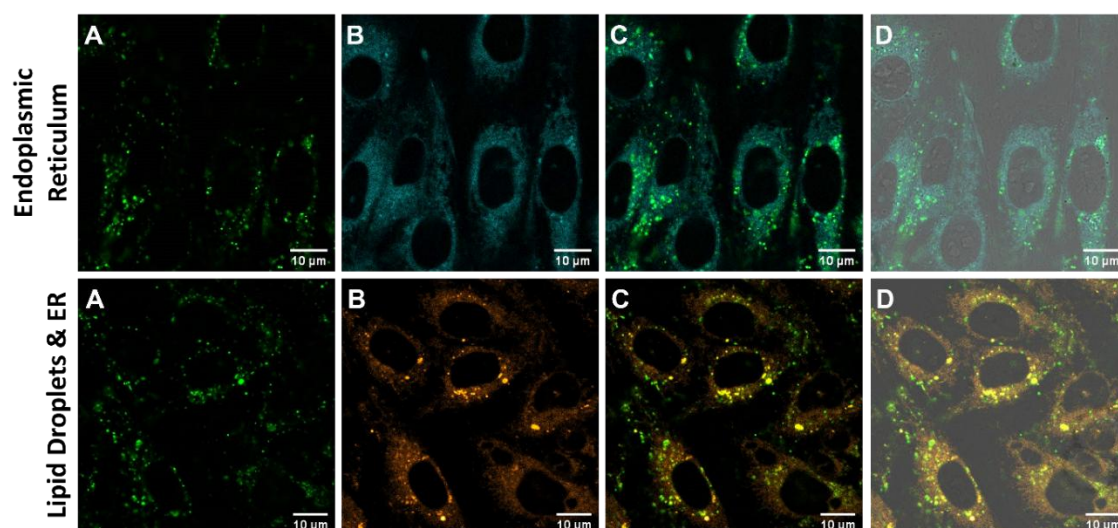


Figure S5.30 Co-localisation imaging of MB2PI (30 μ M, 17 h) with ER Tracker Blue/White and PyLa-C17Cer. Images are labelled with the corresponding dyes in CHO cells, where **(A)** is the B2PI channel, **(B)** indicates the co-localisation dyes where ER Tracker Blue/White is cyan and PyLa-C17Cer is orange. The overlay is shown with and without the phase contrast channel in **(C)** and **(D)**.

S5.4.3 Cytotoxicity Studies

Chinese Hamster Ovary (CHO) non-cancer and MCF-7 breast cancer cells were seeded in clear cell culture treated 96 well plates (Nunc) at 1×10^4 cells per 100 μ L and left for to grow and attach for 24 h at 37°C and 5 % CO₂. For 24 h dark toxicity studies, 50, 10, 5 and 1 μ M stock concentrations of B2P, B2PI, MB2P and MB2PI were prepared in media to a final DMSO concentration of $\leq 1\%$. 100 μ L of each concentration was added to the wells 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% Resazurin-media solution (alamarBlue, Invitrogen) was added to the wells and incubated 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 triplicate.

S5.4.4 Phototoxicity Studies

Phototoxicity and dark control plates were performed by incubating CHO or MCF-7 cells with B2P, B2PI, MB2P and MB2PI for 17 h to allow uptake, emptying the wells, replacing with phenol red free media, and irradiating at a total dose of $17 \pm 1.64 \text{ J/cm}^2$ (2 h at $2.37 \pm 0.23 \text{ mW/cm}^2$) using a 470 nm LED (TeleOpto LEDA-X LED driver and array). Control plates were incubated in the dark during the irradiation period (2 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 before any experiments were performed and each phototoxicity plate included untreated test wells for cell viability comparison. Viability was determined using the alamar blue assay as per the cytotoxicity protocol.

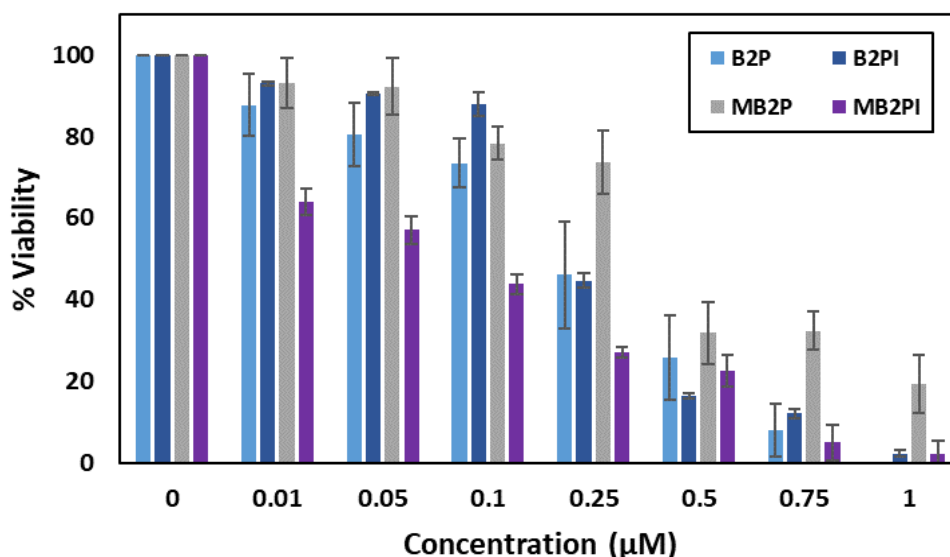


Figure S5.31 Phototoxicity results of MCF-7 cells. Cells were irradiated using a 470 nm LED and cell viability determined using the alamar blue assay. % Viability was determined relative to untreated control cells.

S5.4.5 DPBF Singlet O₂ study

To assess singlet oxygen (¹O₂) production and confirm the phototoxicity of our Ru(II) complexes was caused by the generation of reactive oxygen species (ROS), a ¹O₂ scavenger study was completed using 1,3-diphenylisobenzofuran (DPBF). A 20 mM solution of DPBF in methanol was prepared, and stocks of the BODIPY-perylene compounds in DMSO were prepared under absorbance matched concentrations, starting at a 30 μM concentration of each. Test solutions of 1:1, DPBF: BODIPY in methanol (final concentration of 10 mM DPBF) were added to a clear 96 well plate (Nunc) and absorbance was measured using a BMG LABTECH CLARIOstar plate reader. Each test condition was performed in triplicate.

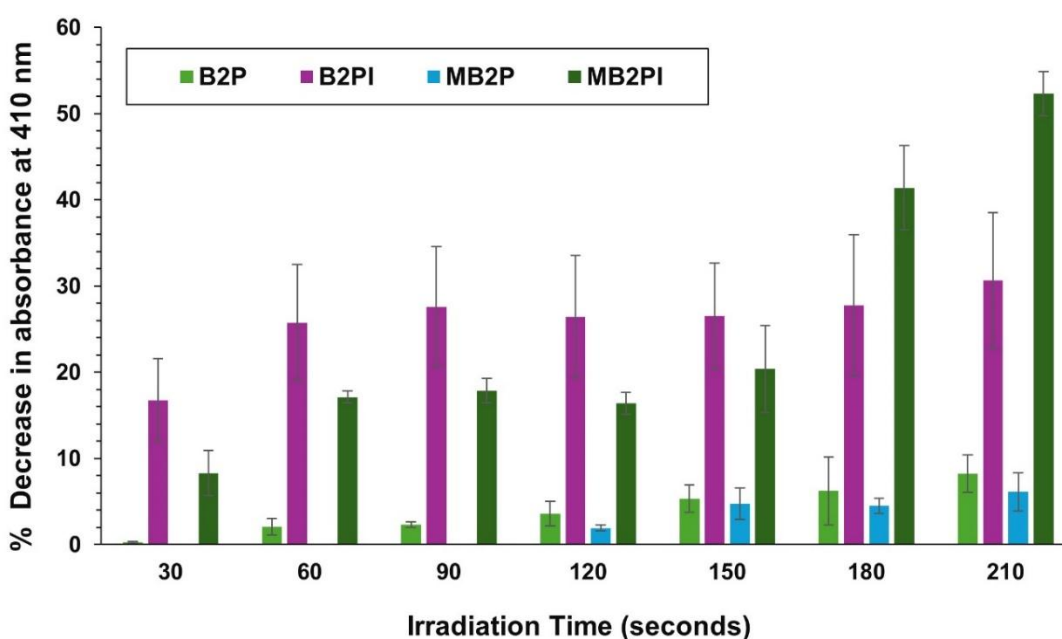


Figure S5.32 DPBF (10 mM) ¹O₂ scavenger assay with B2P, MB2P, B2PI or MB2PI in methanol from 0-210 seconds irradiation, where absorbance at 410 nm was recorded every 30 seconds.

S5.4.6 ROS Imaging Studies

CHO and MCF-7 cells were treated with MB2PI (30 μM, 17 h) for the ROS imaging studies. A stock of 5 mM H₂DCFDA was prepared in DMSO and diluted to 5 μM in PBS. The cells were treated with the 5 μM H₂DCFDA-PBS solution for 30 minutes at 37 °C and 5% CO₂ prior to imaging. The cells were then washed twice with PBS and imaged in phenol red free media (F12-Ham/DMEM for CHO cells and RPMI for MCF-7) supplemented with 10% FBS.

A 63x oil immersion objective was used to acquire images. The irradiance was determined using a power meter from Edmund Optics.

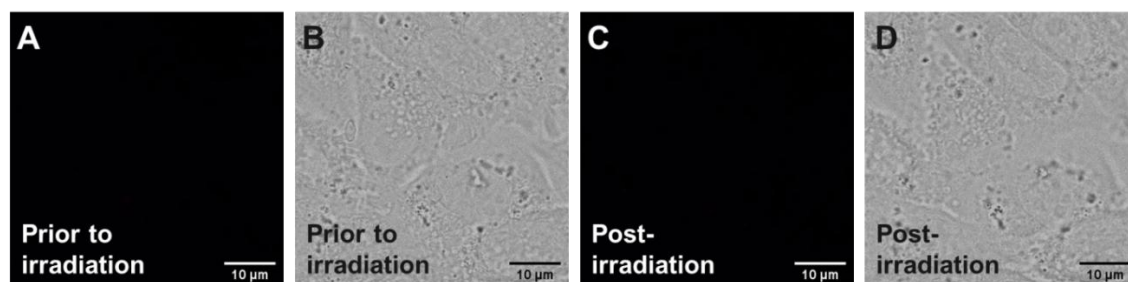


Figure S5.33 Confocal imaging of CHO cells **(A-B)** prior and **(C-D)** post irradiation confirming untreated control cells do not produce ROS under the irradiation conditions used for ROS studies with MB2PI. **(A)** and **(C)** depict the H_2DCFDA channel while **(B)** and **(D)** reflect the corresponding phase contrast image of the cells being imaged. 63x oil.

S5.4.7 Hypoxic Phototoxicity Studies

Hypoxic phototoxicity studies were performed as per routine phototoxicity protocol. Hypoxia was chemically induced using cobalt chloride ($CoCl_2$). A 1 M stock of $CoCl_2$ in sterile 1X PBS was prepared and further diluted to give a 1 mM working stock. The 1 mM stock was used to make up a 300 μM solution of $CoCl_2$ in media to induce a hypoxic environment in the cells. $CoCl_2$ was prepared fresh for each replicate. Cells were treated with $CoCl_2$ (300 μM) for 4 h prior to irradiation to ensure a hypoxic environment.

S5.4.8 3D Cell Studies: Imaging

MCF-7 cells 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 50 μM B2P or MB2PI for 17 h. The BODIPY-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 with a 40x oil immersion objective.

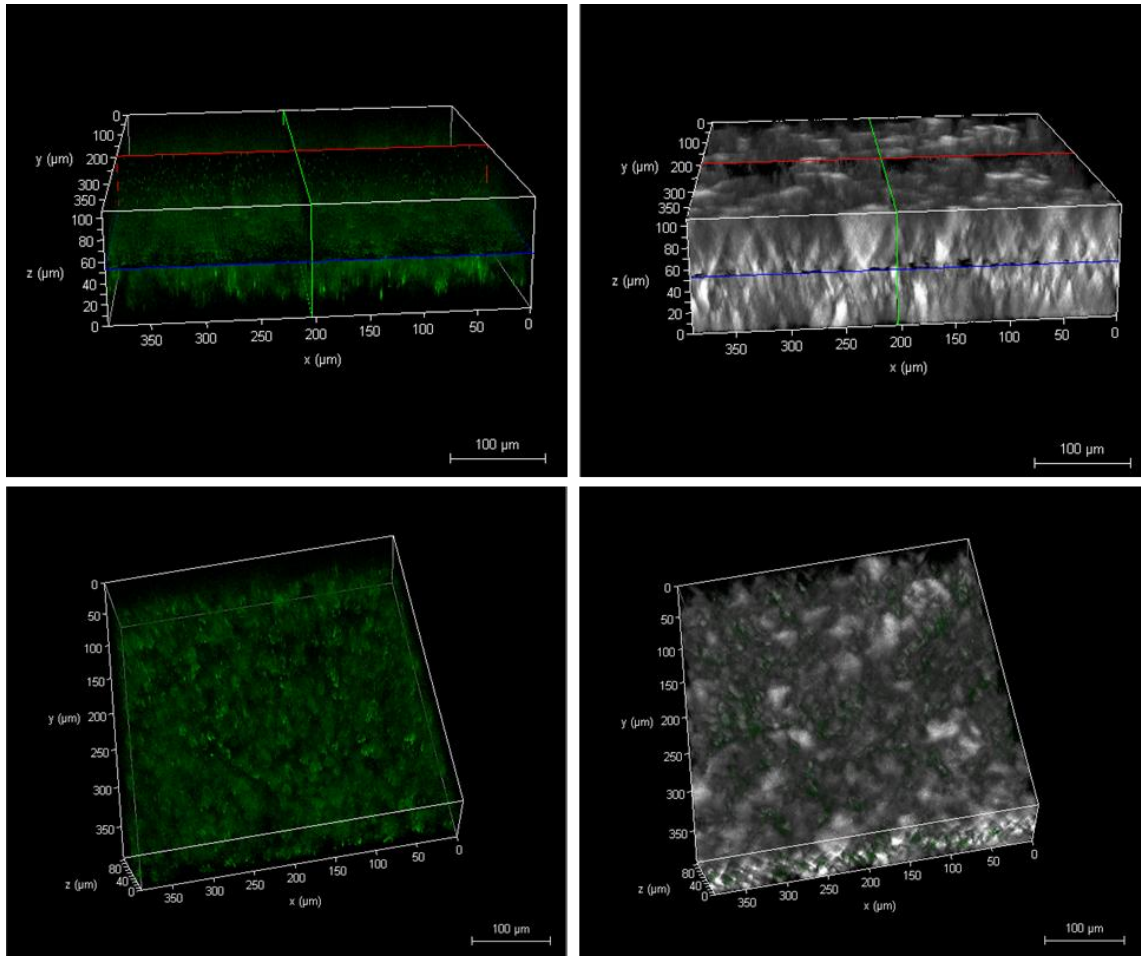


Figure S5.34 3D rendering of B2P in MCF-7 spheroid showing deep penetration throughout the entire spheroid. 40x oil objective.

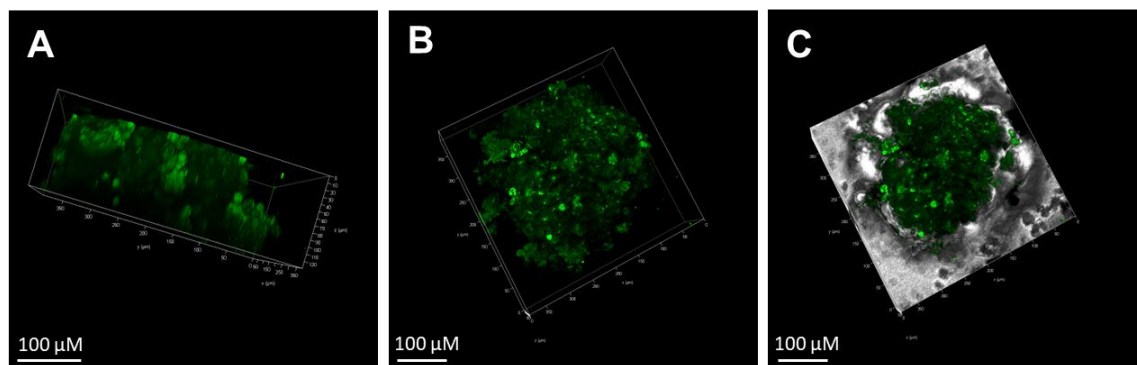


Figure S5.35 3D rendering of B2PI (50 μ M, 17 h) in MCF-7 spheroids showing deep penetration as depicted in **(A)** and **(B)** where **(C)** is the overlay of **(B)** with the corresponding phase contrast channel. 40x oil.

S5.4.9 3D Cell Studies: 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 B2P, B2PI, MB2P or MB2PI was added in a 1:1 ratio (BODIPY:media) to each well of the 96 well plate, which contained 50 μ L phenol red free media, and incubated for 24 h. Prior to irradiation 90% of the media/Ru solution was removed, replaced with fresh phenol red media to dilute the remaining BODIPY (multiple dilution steps, more required for 15 μ M and above), 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 J/cm² using a 470 nm LED. Control studies with untreated spheroids confirmed 17 J/cm² irradiation dose was toxic to spheroids, however the spheroids remained viable after 5 J/cm² irradiation. CellTiter-Glo 3D cell viability assay (Promega) was used to assess spheroid viability and performed as per the manufacturer's protocol, where the CellTiter-Glo 3D reagent was added to the spheroid wells in a 1:1 ratio.

Appendix E: Declaration of Authorship Forms

Chapter 2

Section 1: Candidate's details	
Candidate's Name	Rhianne Curley
DCU Student Number	16311483
School	School of Chemical Sciences
Principal Supervisor	Professor Tia Keyes
Title of PhD by Publication Thesis	Charge-transfer luminophores: theranostic probes of the cellular environment
Section 2: Paper details	
Title of co-authored paper included in the thesis under examination	Phototoxicity of Tridentate Ru(II) Polypyridyl Complex with Expanded Bite Angle toward Mammalian Cells and Multicellular Tumour Spheroids
List of authors as in order given on accepted/published version	Rhianne C. Curley, Dr. Christopher S. Burke, Dr. Karmel S. Gkika, Sara Noorani, Associate Professor Naomi Walsh and Professor Tia E. Keyes.
Publication Details (e.g. Year of Publication, Journal Name, Issue Number, Page Numbers, Place of Publication, DOI, ISSN, URL etc.)	<i>Inorganic Chemistry</i> 2023, 62, 32, 13089 https://doi.org/10.1021/acs.inorgchem.3c01982
Publication Status	Published
Provide a brief statement here on the disciplinary ranking of this publication and the peer-review process involved (e.g. how many reviewers, single blinded or other, initial decision, revision timeline etc and any other details that speak to the rigour of the peer review process)	Impact Factor: 4.3 (2023) Two reviewers (single blinded) No extra experiments requested by reviewers. Approx. 1 week between reviewer comments received and manuscript resubmitted.
This paper is one of 4 co-authored papers to be submitted as part of the PhD by publication thesis submitted for examination	
Section 3: Candidate's contribution to the paper	
First author of the manuscript, main contributor including literature review (introduction section), design of experiments, data interpretation, initial manuscript writing and revision of the manuscript. Main contributor to experimental work including photophysical characterisation of the complexes (absorbance spectra, emission spectra and luminescent lifetimes in aerated and deaerated conditions) and all cellular studies in both 2D and 3D cell models. Overall contribution in percentage terms: 65-70 %	

Section 4: Signature and Validation I confirm that the following statements are true: (a) the information I have provided in this form is correct (b) this paper is based on research undertaken during my candidature at DCU Signature of PhD Candidate: <u>Rhianne Curley</u> Date: <u>03/01/2025</u> I confirm that the information provided by the candidate is correct: Signature of Principal Supervisor: <u>Tia Keyes</u> Date: <u>03/01/2025</u>

Chapter 3

Section 1: Candidate's details	
Candidate's Name	Rhianne Curley
DCU Student Number	16311483
School	School of Chemical Sciences
Principal Supervisor	Professor Tia Keyes
Title of PhD by Publication Thesis	Charge-transfer luminophores: theranostic probes of the cellular environment
Section 2: Paper details	
Title of co-authored paper included in the thesis under examination	Visualising Stress Granule Dynamics with an RNA Guanine Quadruplex Targeted Ruthenium(II) Peptide Conjugate
List of authors as in order given on accepted/published version	Rhianne C. Curley ⁺ , Dr. Lorcan Holden ⁺ , Professor Tia E. Keyes
Publication Status	Submitted
This paper is one of 4 co-authored papers to be submitted as part of the PhD by publication thesis submitted for examination	
Section 3: Candidate's contribution to the paper	
<i>Joint first author of the manuscript with Dr. Lorcan Holden. Contributed equally to the literature review (introduction), data collection, analysis, conception of experiments, methodological design, and summary of findings. Completed all cellular studies.</i> <i>Overall contribution in percentage terms: 50%</i>	
Section 4: Signature and Validation	
I confirm that the following statements are true: (a) the information I have provided in this form is correct (b) this paper is based on research undertaken during my candidature at DCU Signature of PhD Candidate: <u>Rhianne Curley</u> Date: <u>03/01/2025</u> I confirm that the information provided by the candidate is correct: Signature of Principal Supervisor: <u>Tia Keyes</u> Date: <u>03/01/2025</u>	

Chapter 4

Section 1: Candidate's details	
Candidate's Name	Rhianne Curley
DCU Student Number	16311483
School	School of Chemical Sciences
Principal Supervisor	Professor Tia Keyes
Title of PhD by Publication Thesis	Charge-transfer luminophores: theranostic probes of the cellular environment
Section 2: Paper details	
Title of co-authored paper included in the thesis under examination	Targeting Mitochondrial Guanine Quadruplexes for Photoactivatable Chemotherapy in Normoxic and Hypoxic Environments
List of authors as in order given on accepted/published version	Rhianne C. Curley [†] , Dr. Lorcan Holden [†] , Dr. Giuseppe Avella, Professor Conor Long and Professor Tia E. Keyes.
Publication Details (e.g. Year of Publication, Journal Name, Issue Number, Page Numbers, Place of Publication, DOI, ISSN, URL etc.)	<i>Angewandte Chemie</i> e202408581, 2024 https://doi.org/10.1002/anie.202408581
Publication Status	Published
Provide a brief statement here on the disciplinary ranking of this publication and the peer-review process involved (e.g. how many reviewers, single blinded or other, initial decision, revision timeline etc)	Impact Factor: 16.1 (2023) Two reviewers (single blind) Completed additional cellular studies requested by reviewers (approx. 1 month from receiving reviewer comments to re-submission)
This paper is one of 4 co-authored papers to be submitted as part of the PhD by publication thesis submitted for examination	
Section 3: Candidate's contribution to the paper	
Joint first author of the manuscript with Dr. Lorcan Holden. Designed and completed all cellular studies in both 2D and 3D cell models. Participated in discussion of results and manuscript revisions. Equally involved in the literature review (introduction) of the paper, summary of findings and all aspects of the data interpretation and writing.	
Overall contribution in percentage terms: 50%	
Section 4: Signature and Validation	
I confirm that the following statements are true: (a) the information I have provided in this form is correct (b) this paper is based on research undertaken during my candidature at DCU	
Signature of PhD Candidate: <u> Rhianne Curley </u> Date: <u> 03/01/2025 </u>	
I confirm that the information provided by the candidate is correct:	
Signature of Principal Supervisor: <u> Tia Keyes </u> Date: <u> 03/01/2025 </u>	

Chapter 5

Section 1: Candidate's details	
Candidate's Name	Rhianne Curley
DCU Student Number	16311483
School	School of Chemical Sciences
Principal Supervisor	Professor Tia Keyes
Title of PhD by Publication Thesis	Charge transfer luminophores: theranostic probes of the cellular environment
Section 2: Paper details	
Title of co-authored paper included in the thesis under examination	Enhancing Phototoxicity in BODIPY-perylene Charge Transfer Dyads by Combined Iodination and Mesylation
List of authors as in order given on accepted/published version	Rhianne C. Curley, Dr. Ruben Arturo Arellano-Reyes, Dr. James N. McPherson, Professor Vickie McKee and Professor Tia E. Keyes.
Publication Details (e.g. Year of Publication, Journal Name, Issue Number, Page Numbers, Place of Publication, DOI, ISSN, URL etc.)	<i>Chemistry - A European Journal</i> e202403149, 2024 https://doi.org/10.1002/chem.202403149
Publication Status	Published
Provide a brief statement here on the disciplinary ranking of this publication and the peer-review process involved (e.g. how many reviewers, single blinded or other, initial decision, revision timeline etc)	Impact Factor: 3.9 (2023) Three reviewers (single blind) No additional experiments requested by reviewers, manuscript was revised as per reviewer comments, approx. 1 week between reviewer comments received and paper resubmission.
This paper is one of 4 co-authored papers to be submitted as part of the PhD by publication thesis submitted for examination	
Section 3: Candidate's contribution to the paper	
First author of the manuscript and main contributor to experimental work including all cellular studies in both 2D and 3D cell models. Main contributor of the literature review (introduction), experimental design (cellular work), data collection and analysis, summary of findings and revision of the manuscript. Overall contribution in percentage terms: 65%	
Section 4: Signature and Validation	
I confirm that the following statements are true: (a) the information I have provided in this form is correct (b) this paper is based on research undertaken during my candidature at DCU Signature of PhD Candidate: <u> Rhianne Curley </u> Date: <u> 03/01/2025 </u> I confirm that the information provided by the candidate is correct: Signature of Principal Supervisor: <u> Tia Keyes </u> Date: <u> 03/01/2025 </u>	