Development of new bis(thiosemicarbazonates) and investigations into their potential as molecular imaging and therapeutic agents

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Development of new
bis(thiosemicarbazonates) and investigations
into their potential as molecular imaging and
therapeutic agents

Rory Louis Arrowsmith

A thesis submitted for the degree of Doctor of Philosophy University of Bath
Department of Chemistry
February 2013

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University of Bath, February 2013
List of publications resulting from this PhD


List of talks presented during this PhD


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Abstract

Chapter 1 – Introduction, describes medical imaging modalities focussing on molecular imaging agents for SPECT and PET, where examples of dual modal imaging with either technique or theranostics (simultaneous diagnosis and therapy) are highlighted. Significant examples of imaging probes using technetium and rhenium are included with an emphasis on emerging zinc, copper, gallium and indium complexes. Development of metal complexes conjugated to agents enabling targeting of receptors is described as well as hypoxia and its diagnosis and therapy.

Chapter 2-5 describe the synthesis and characterisation of bis(thiosemicarbazonato) ligands (Chapter 2) and zinc & copper (Chapter 3), gallium (Chapter 4) and indium (Chapter 5) complexes. An interesting isomerism is explored using Density Functional Theory (DFT). The complexes are investigated for their suitability as molecular imaging probes, firstly by testing their spectroscopic properties using UV-visible and fluorescence spectroscopies. This is followed by assessing their stability, utilising the aforementioned techniques with biologically relevant media in solution and in biological cells in vitro, using fluorescence lifetime imaging microscopy (FLIM). Laser scanning confocal microscopy is used to investigate cell uptake demonstrating localisation within organelles. Furthermore, gallium and indium complexes are evaluated for their potential as hypoxia imaging agents, in vitro in addition to the investigation of indium complex nuclear and chromosomal uptake. Lastly, copper and gallium complexes are studied in nude mice with PC-3 xenografts under normoxic conditions (by the Jason Lewis Group, MSKCC, NYC).

Chapter 6. Compounds introduced in chapters 2-5 are studied and compared for their in vitro cytotoxicity using MTT and LDH assays. MTT assay is used to determine a quantitative value of toxicity, to enable a direct comparison of all compounds tested and in cancerous and non-cancerous cell lines. LDH assay on the other hand, provides insight into the mechanism of molecular action upon the cell membrane, which may explain differences obtained using MTT assay in this study and trypan blue experiments, which relies upon membrane integrity.
**Abstract**

Chapter 7 explores methods of attaching a targeting agent, a bombesin analogue, to the bis(thiosemicarbazonato) complexes. Novel complexes are characterised and subsequently investigated in cells for cytotoxicity and by confocal microscopy and FLIM.

Chapter 8 describes a summary of this thesis.

Chapter 9 contains the entire experimental data for the work of this thesis.
Acknowledgements

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Last, but certainly not least, I would like to thank my wife, Merle, who has been a great person to discuss my science with and supported me through thick and thin.
**Abbreviations**

2D two-dimensional  
3D three-dimensional  
A absorbance  
Å Angstrom  
Ac acetyl  
Ar aromatic  
ATSM/A diacetyl-2-(4-N-methyl-3-thiosemicarbazone)  
ATP Adenosine Triphosphate  
BAM biologically active molecule  
BIAN bis(imino)acenaphthene  
bipy 2,2'-bipyridine  
Bodipy boron-dipyrrromethane  
Bq Becquerel  
C Curie  
°C degrees Celsius  
cm centimetre  
CT Computed Tomography  
cyclam 1,4,6,11-tetraazacyclotetradecane  
D Emission Integral  
δ chemical shift  
DABCO 1,4-diazabicyclo[2.2.2]octane  
DCC N,N'-dicyclohexylcarbodiimide  
DCM dichloromethane  
DDQ 2,3-dichloro-5,6-dicyano-1,4-benzoquinone  
deg degree  
DIPEA N,N'-diisopropylethylamine  

DMEM Dulbecco’s Modified Eagles Medium  
DMF N,N'-dimethylformamide  
DMSO dimethyl sulfoxide  
DNA deoxyribonucleic acid  
DOTA 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid  
DPA dipicolylamine  
DTPA diethylene triamine pentaacetic acid  
EC electron capture  
eV electron volt  
EDC 1-ethyl-3-(3’-dimethylaminopropyl) carbodiimide  
EDTA ethylenediaminetetraacetic acid  
EGFR epidermal growth factor receptor  
EPR electron paramagnetic resonance  
eq equivalents  
ESI electrospray ionization  
Et ethyl  
f femto  
fab antibody binding fragment  
Φ quantum yield  
fab antibody fragment  
FDG 2-fluoro-2-deoxy-D-glucose  
FLIM fluorescence lifetime imaging microscopy  
FMISO fluoromisonidazole  
FRET Fluorescence Resonance Energy Transfer
Abbreviations

**FWHM** full width at half maximum

**g** gram

**h** hour

**η** refractive index

**HBED** $N,N'$-bis-(2-hydroxybenzyl)ethylene-diamine-$N,N'$-diacetic acid

**HBSS** Hank’s Buffered Salt Solution

**HER2** Human Epidermal Growth Factor Receptor 2

**HIF** hypoxia-inducible factor

**HOBt** hydroxybenzotriazole

**HPLC** high performance liquid chromatography

**Hz** hertz

**GLP-1** Glucagon-like peptide receptor-1

**IR** Flux Intensity

**IR** infra-red

**K** Kelvin

**k** kilo / equilibrium constant

**L** litre

**LC** Liquid Chromatography


**λ** wavelength

**λ** _ex_ excitation wavelength

**λ** _max_ maximum wavelength of absorption

**M** molar / mega

**m** milli / meter

**µ** micro

**MAG** mercaptoacetylglycylglycylglycine

**MDP** methyl diphosphonate

**Me** methyl

**MEM** minimum essential medium

**min** minute

**mol** mole

**MRI** magnetic resonance imaging

**MS** mass spectrometry

**m/z** mass-to-charge ratio

**n** nano

**NAD** nicotinamide adenine dinucleotide

**NADH** nicotinamide adenine dinucleotide phosphate

**NHS** $N$-hydroxysuccinimide

**NIR** near-infrared

**NMR** nuclear magnetic resonance

**NOTA** 1,4,7-triazacyclononane-1,4,7-carboxylic acid

**OFI** Optical Fluorescence Imaging

**ORTEP** Oak Ridge Thermal Ellipsoid Program

**P** partition coefficient

**p** pico

**PBS** phosphate buffered solution

**PDT** photodynamic therapy

**PEG** polyethylene glycol

**PET** positron emission tomography

**Ph** phenyl

**pH** $\text{-log}_{10}[H^+]$

**ppm** parts per million
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PTSM</strong></td>
<td>pyruvaldehyde-\textit{bis}(methyl-N-thiosemicarbazone)</td>
</tr>
<tr>
<td><strong>RGD</strong></td>
<td>L-Arginyl-Glycyl-L-Aspartic acid</td>
</tr>
<tr>
<td><strong>RPMI</strong></td>
<td>Royal Park Memorial Institute</td>
</tr>
<tr>
<td><strong>rt</strong></td>
<td>room temperature</td>
</tr>
<tr>
<td><strong>Rt</strong></td>
<td>retention time</td>
</tr>
<tr>
<td><strong>s</strong></td>
<td>second</td>
</tr>
<tr>
<td><strong>SPECT</strong></td>
<td>single photon emission computed tomography</td>
</tr>
<tr>
<td><strong>t_{1/2}</strong></td>
<td>half life</td>
</tr>
<tr>
<td><strong>τ</strong></td>
<td>fluorescence lifetime</td>
</tr>
<tr>
<td><strong>τ_1</strong></td>
<td>major component of fluorescence lifetime</td>
</tr>
<tr>
<td><strong>τ_2</strong></td>
<td>minor component of fluorescence lifetime</td>
</tr>
<tr>
<td><strong>τ_m</strong></td>
<td>weighted average of fluorescence lifetime components</td>
</tr>
<tr>
<td><strong>TCSPC</strong></td>
<td>Time-Correlated Single Photon Counting</td>
</tr>
<tr>
<td><strong>t-Boc</strong></td>
<td>tert-butyloxycarbonyl</td>
</tr>
<tr>
<td><strong>TETA</strong></td>
<td>1,4,8,11-tetraazacyclododecane-1,4,8,11-tetraacetic acid</td>
</tr>
<tr>
<td><strong>TFA</strong></td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td><strong>THF</strong></td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td><strong>US</strong></td>
<td>ultrasound</td>
</tr>
<tr>
<td><strong>UV</strong></td>
<td>ultraviolet</td>
</tr>
<tr>
<td><strong>vis</strong></td>
<td>visible</td>
</tr>
</tbody>
</table>
Numbering of compounds

Mono(thiosemicarbazonato) ligands

Compound ia

Compound ib

Compound ic

Compound id

Bis(thiosemicarbazonato) ligands

Compound iia

Compound iib

Compound iic

Compound iid
Zinc bis(thiosemicarbazono)- complexes

Compound 1a

Compound 1b

Compound 1c

Compound 1d

Copper bis(thiosemicarbazono) complexes

Compound 2a

Compound 2b

Compound 2c

Compound 2d
Gallium Chloride bis(thiosemicarbazonato) complexes

Compound 3a

Compound 3b

Compound 3c

Compound 3d

Indium Chloride bis(thiosemicarbazonato) complexes

Compound 4a

Compound 4b

Compound 4c

Compound 4d
Asymmetric zinc bis(thiosemicarbazonato) complexes

Compound 1aN

Compound 1bN

Compound 1cN

Compound 1dN

Compound 1bB

Compound 1dB
Asymmetric gallium bis(thiosemicarbazonato) complex

Compound 3bN
Chapter 1. Introduction

1.1. Perspective

Cancer accounts for more than a third of deaths in the England and Wales,\(^1\) (44.3 % males and 32.2 % females). Furthermore, current lifetime risk of developing a malignant tumour has recently been calculated as high as 40%, and rising, therefore the necessity for earlier cancer detection, identification and effective therapy of later cancer stages is becoming increasingly important.\(^2\) Hypoxic tissue develops under low in oxygen concentrations and has been correlated to cancer, strokes and heart disease.\(^3,4\) 

As a tumour grows the intercapillary distance increases until it becomes greater than the diffusion range of oxygen (ca. 200 µm) (Figure 1.1). Furthermore, limited perfusion and as a result erratic blood supply has also been shown to increase hypoxia.

![Figure 1.1. Illustration of relative oxygen levels in a tumour, whereby a greater distance from the blood vessel signifies a lower threshold of oxygen.\(^5\)](image)

A crucial protein in the development of this process is hypoxia inducible factor-1 (HIF-1), the mechanism of action of which is beautifully simple: under normal oxygen conditions (normoxia) the protein, HIF-1α, is hydroxylated at numerous proline residues resulting in ubiquitination and proteasomal degradation; under insufficient oxygen conditions, however, HIF-1α translocates to the nucleus and combines with protein HIF-1β to form the heterodimer, HIF-1 (Figure 1.2). This transcription factor is subsequently responsible for the upregulation of up to 100 proteins, in turn altering the cellular glucose metabolism, metastasis, cell proliferation, angiogenesis, iron metabolism and drug resistance, all of which increase the chances of cancer cell survival and decrease prognosis.6

Figure 1.2. A diagram summarising the mechanism of hypoxia inducible factor-1 release, from Ref6.

Early diagnosis of hypoxia would allow a change in treatment plan and therefore improve patient survival chances. Prognosis is often poor at this stage of cancer since traditional therapies do not reach hypoxic tissue due to isolation from capillaries. Additionally, most therapies target rapidly dividing cells, whereas under hypoxic conditions cells grow slowly due to reduced oxygen and nutrient supply. Finally, as mentioned above, hypoxia promotes genetic instability, thus increasing the likelihood of drug resistance and metastases.7,8 As a result there is growing interest in molecular imaging as a non-invasive, highly sensitive method capable of both revealing solid tumours and enhancing our understanding of the molecular basis of the disease –
essential for diagnosis, assessing tumour progression and patient prognosis. The aims of this project were to develop molecular imaging probes with therapeutic potential to be studied both in vivo, by positron emission tomography (PET) or single photon emission computed tomography (SPECT) and also in vitro, by fluorescence imaging and cytotoxicity assays.

**Table 1.1.** Summary of the characteristics of medical imaging modalities. OFI = Optical Fluorescence Imaging, A = Anatomical, P = Physiological, M = molecular, nwc = not well characterised. Adapted from references 9 and 10.

<table>
<thead>
<tr>
<th>Technique</th>
<th>Resolution</th>
<th>Depth</th>
<th>Information</th>
<th>Sensitivity/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT</td>
<td>50 µm</td>
<td>No limit</td>
<td>A, P</td>
<td>nwc</td>
</tr>
<tr>
<td>MRI</td>
<td>10 – 100 µm</td>
<td>No limit</td>
<td>A, P, M</td>
<td>$10^{-3}$-$10^{-5}$</td>
</tr>
<tr>
<td>Ultrasound</td>
<td>50 µm</td>
<td>Frequency Dependant</td>
<td>A, P</td>
<td>nwc</td>
</tr>
<tr>
<td>SPECT</td>
<td>1 – 2 mm</td>
<td>No limit</td>
<td>P, M</td>
<td>$10^{-10}$-$10^{-11}$</td>
</tr>
<tr>
<td>PET</td>
<td>1 – 2 mm</td>
<td>No limit</td>
<td>P, M</td>
<td>$10^{-11}$-$10^{-12}$</td>
</tr>
<tr>
<td>OFI</td>
<td>2 – 3 mm</td>
<td>&lt;1 cm</td>
<td>P, M</td>
<td>$10^{-9}$-$10^{-12}$</td>
</tr>
</tbody>
</table>

**Figure 1.3.** Normal human brain imaged by SPECT $^{123}$I-liomazenil (left), MRI (middle) and $^{11}$C-flumazenil (right), which correspond to the same slice.

Medical imaging techniques can provide anatomical, physiological and even molecular information (Table 1.1, Figure 1.3). Traditional imaging modalities such as X-ray and computerised tomography (CT) irradiate tissue with harmful radiation and provide anatomical information. Anatomical imaging tools have benefited from progress in spatial and temporal resolution, but they do not readily recognise
physiological abnormalities. The capacity of these methods to differentiate cancer is therefore reserved for the later stages of disease progression. Consequently there is a current trend towards developing more sensitive molecular imaging techniques utilising radionuclides for PET or SPECT, which enter diseased tissue selectively. Furthermore, healthy tissues remain unharmed due to the low concentrations required (ca. $10^{11}$ M). These modalities give both functional and metabolistic information, which not only accelerate understanding of the molecular basis of disease, but also enable accurate detection and diagnosis. Metallic radionuclides ultimately undergo uptake within cells and, while the distribution of their complexes can be determined in vivo at the 1-2 mm range of resolution, little is known of their fate once they reach the intercellular environment. This hampers the rational design of new diagnostics and therapeutics and ultimately the accurate diagnosis of cancer. However, there is growing interest in molecular imaging as a non-invasive, highly sensitive tool capable of both early diagnosis and enhancing the understanding of the molecular basis of the disease.\textsuperscript{9,10}

Intense research is currently underway for the development of new PET and SPECT agents for imaging a wide range of disease states, and of new drugs for targeted radiotherapy. Radiopharmaceuticals chosen for the purpose of diagnosis are usually positron emitters (PET) or gamma emitters (SPECT), whereas therapeutic radiopharmaceuticals usually rely upon $\beta^-$ emission and the Auger effect causing cell death. The choice of radioisotope is also made according to an optimum half-life, which at the same time minimises radiation doses whilst giving sufficient time for synthesis and accumulation.

In 2007, this was defined by the Society of Nuclear Medicine defined Molecular Imaging as a new interdisciplinary research field, which is at the interface between clinical and preclinical research. The rapid development of this key area is highlighted by the increasing demand for new imaging probes for specific biological targets.\textsuperscript{12} By the end of 2010 more than 3.2 million positron emission tomography (PET) studies had been carried out worldwide and this number continues to rise. It is widely recognised that optimal disease management can only be achieved by
monitoring patient status before, during and after therapy. PET agents offer high resolution, high sensitivity, non-invasive imaging with provision of invaluable diagnosis of biological function at agent concentrations below the pharmacological threshold, at which dosage no significant drug effect is observed.

Since no medical imaging technique is ideal for acquiring all the necessary information, the combination of modalities such as SPECT/CT, PET/X-ray, PET/MRI or, most frequently PET/CT, allows for better image quality, shorter scanning time and reduced costs. Such multimodal imaging results in more efficient use of radiopharmaceuticals (i.e. drugs containing a radionuclide) and more facile recognition of abnormalities. A combination with optical imaging enables both greater understanding of the probe both in cells and in organisms as well as enabling the identification of tumours. The advantages of combining the high sensitivity of PET or SPECT, which are not limited by tissue penetration with optical imaging presents a very useful marriage of modalities with potential to improve both scientific knowledge and patient diagnosis and therapy.

1.2. Single Photon Emission Computed Tomography

The first tomographic device for medical imaging, Single Photon Emission Computed Tomography, SPECT, was reported by Kuhl and Edwards in 1963. The technique utilises gamma emission, which occurs when a highly energetic nucleus relaxes to a ground state. This type of emission can be detected using a gamma camera, yielding a 3D image of radionuclide uptake. Commonly used radioisotopes for SPECT include $^{123}$I, $^{131}$I, $^{67}$Ga and $^{111}$In (Table 1.2). The most frequently used however is $^{99m}$Tc, which will be discussed further in section 1.4.
### Table 1.2. Relevant radionuclides for SPECT imaging

<table>
<thead>
<tr>
<th>Radionuclide</th>
<th>Half-life ($t_{1/2}$) / Mode of Decay (% branching ratio)</th>
<th>Availability</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{99m}$Tc</td>
<td>6.0 it (99.9)</td>
<td>Gen: $^{99}$Mo/$^{99m}$Tc</td>
<td>SPECT</td>
</tr>
<tr>
<td>$^{111}$In</td>
<td>67.392 ec (100)</td>
<td>Cyclotron</td>
<td>SPECT/e$^{-}$ therapy</td>
</tr>
<tr>
<td>$^{67}$Ga</td>
<td>78.281 ec (100)</td>
<td>Cyclotron</td>
<td>SPECT</td>
</tr>
<tr>
<td>$^{123}$I</td>
<td>13.2 ec (100)</td>
<td>Cyclotron</td>
<td>SPECT</td>
</tr>
<tr>
<td>$^{131}$I</td>
<td>193 $^{a}$</td>
<td>Reactor</td>
<td>SPECT/ $\beta^{-}$ therapy</td>
</tr>
</tbody>
</table>

$^{a}$ Iodine-131 has a process of six $\beta^{-}$ and fourteen $\gamma$-ray emissions

### 1.3. Positron Emission Tomography

Compared to SPECT, PET imaging has a crucial advantage in terms of sensitivity and resolution (Table 1.1). PET was first reported by Michel Ter-Pogossian in 1972 and is based on the monitoring of positron-electron annihilation. PET therefore relies on the presence of a positron emitting radionuclide where a proton is converted into a neutron. A single annihilation causes emission of two gamma rays ca. 180° apart, each with a 511 keV energy (Figure 1.4). Detectors are placed around the patient so that simultaneous gamma ray detection can be traced to the point of annihilation by following the line of response (LOR). These are subsequently reconstructed to produce a 3D image of the respective environment and characterise any abnormalities.
Use of PET was initially limited by the requirement of an on-site cyclotron, the short half-lives of available positron emitting radionuclides, and highly expensive equipment. Predominantly a research tool in the beginning, it has now become an essential diagnostic technique, with more than 2,000 PET centres worldwide.

Table 1.3. Commonly used radionuclides for PET imaging

<table>
<thead>
<tr>
<th>Radionuclide</th>
<th>Half-life (t_{1/2})</th>
<th>Mode of Decay (% branching ratio)</th>
<th>Availability</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>(^{18}\text{F})</td>
<td>1.83 h</td>
<td>(\beta^+ (97))</td>
<td>Cyclotron</td>
<td>PET</td>
</tr>
<tr>
<td>(^{11}\text{C})</td>
<td>0.34 h</td>
<td>(\beta^+ (100))</td>
<td>Cyclotron</td>
<td>PET</td>
</tr>
<tr>
<td>(^{64}\text{Cu})</td>
<td>12.701 h</td>
<td>ec + (\beta^+ (61.5))</td>
<td>Cyclotron</td>
<td>PET</td>
</tr>
<tr>
<td>(^{62}\text{Cu})</td>
<td>0.16 h</td>
<td>ec + (\beta^+ (100))</td>
<td>Gen: (^{62}\text{Zn}^{62}\text{Cu})</td>
<td>PET</td>
</tr>
<tr>
<td>(^{68}\text{Ga})</td>
<td>1.13 h</td>
<td>ec + (\beta^+ (100))</td>
<td>Gen: (^{68}\text{Ge}^{68}\text{Ga})</td>
<td>PET</td>
</tr>
<tr>
<td>(^{89}\text{Zr})</td>
<td>78.41 h</td>
<td>ec + (\beta^+ (100))</td>
<td>Cyclotron</td>
<td>PET</td>
</tr>
<tr>
<td>(^{86}\text{Y})</td>
<td>14.74 h</td>
<td>ec + (\beta^+ (100))</td>
<td>Cyclotron</td>
<td>PET</td>
</tr>
</tbody>
</table>

Figure 1.4. A representation of PET imaging, adapted with permission from reference 15.
The choice of radionuclide is dependent on availability, half-life and pharmacokinetics. The isotope $^{18}$F ($t_{1/2}$ 109.8 min) is most widely used for imaging applications, especially incorporated into 2-$[^{18}$F]-fluoro-2-deoxy-D-glucose ($^{18}$F-FDG) where there are no limitations owing to the availability of a cyclotron typically needed for radionuclide generation. $^{18}$F-FDG, the “gold standard” for PET imaging tumours / ischaemic myocardium in clinical practice, lacks selectivity for cancer and is not universally applicable for imaging all tumours: for example it does not image hypoxic tumors per se.

1.4. Radionuclides for therapy

Diagnosis using radiometals, such as $^{64}$Cu, $^{67}$Ga, $^{68}$Ga, $^{90m}$Tc, can be followed up by treatments with radiotherapeutic agents (Table 1.4) such as $^{177}$Lu, $^{90}$Y, and $^{111}$In provided that the chemical properties of the complex are not significantly changed. Therapeutic radionuclides are emitters of either $\alpha$-particles, $\beta$-particles or low energy Auger electrons.

Table 1.4. Radionuclides for therapy

<table>
<thead>
<tr>
<th>Radionuclide</th>
<th>Half-life ($t_{1/2}$)</th>
<th>Mode of Decay (% branching ratio)</th>
<th>Availability</th>
<th>Application</th>
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</thead>
<tbody>
<tr>
<td>$^{131}$I</td>
<td>8.02 d</td>
<td>$\beta^-$ (100)</td>
<td>Nuclear reactor</td>
<td>$\beta$-therapy</td>
</tr>
<tr>
<td>$^{90}$Y</td>
<td>64.00 h</td>
<td>$\beta^-$ (100)</td>
<td>Gen: $^{90}$Sr/$^{90}$Y</td>
<td>$\beta$-therapy</td>
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<tr>
<td>$^{177}$Lu</td>
<td>6.647 d</td>
<td>$\beta^-$ (100)</td>
<td>Nuclear reactor</td>
<td>$\beta$-therapy</td>
</tr>
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<td>$^{188}$Re</td>
<td>17.00 h</td>
<td>$\beta^-$ (100)</td>
<td>Gen: $^{188}$W/$^{188}$Re</td>
<td>$\beta$-therapy</td>
</tr>
<tr>
<td>$^{153}$Sm</td>
<td>46.50 h</td>
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</tr>
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<td>$^{213}$Bi</td>
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<td>$\alpha$ (2.20)</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>$\beta^-$ (97.8)</td>
<td></td>
<td>$\beta$-therapy</td>
</tr>
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<td>240 h</td>
<td>$\alpha$ (100)</td>
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<td>$\alpha$-therapy</td>
</tr>
<tr>
<td>$^{223}$Ra</td>
<td>11.4 d</td>
<td>$\alpha$ (100)</td>
<td>Gen: $^{227}$Ac/$^{227}$Th/$^{223}$Ra</td>
<td>$\alpha$-therapy</td>
</tr>
<tr>
<td>$^{67}$Cu</td>
<td>2.6 d</td>
<td>$\beta^-$ (100)</td>
<td>Cyclotron/reactor</td>
<td>$\beta$-therapy</td>
</tr>
</tbody>
</table>

Furthermore, a diagnostic agent combined with a therapeutic agent form a
‘theranostic pair’. Intrinsically cytotoxic agents can also be radiolabelled while the use of nanoparticles filled with a biologically active molecule (BAM)-targeted drug may provide a different approach to single theranostic compounds.

1.5. Radiopharmaceuticals and multimodality probe design considerations

Radiopharmaceuticals are designed to answer a specific medical need and are based on the knowledge of molecular biology. The first generation of radiopharmaceuticals involved radioactive isotopes aimed at mimicking normal biological processes, such as 2-[¹⁸F]-fluoro-2-deoxy-D-glucose and [⁹⁹mTcO₄]²⁻, which take advantage of upregulated glucose uptake by cancer cells and mimic iodine uptake by the thyroid, respectively.¹⁷ The current trend is moving towards ‘second generation’ of radiopharmaceuticals, which use a BAM, such as a peptide or antibody for specific targeting.

![Chelator structures and targeting groups frequently used with radiopharmaceuticals](image)

**Figure 1.5.** Chelator structures and targeting groups frequently used with radiopharmaceuticals
Much of the work makes use of the labelling of standard chelating agents such as 1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid (DOTA), 1,4,8,11-tetraazacyclododecane-1,4,8,11-tetraacetic acid (TETA), 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA), cyclam or cyclam variants conjugated to a BAM via a linker (Figure 1.5). Reviewed below are recent developments in luminescent zinc, copper, gallium, indium metal complexes for in vitro and/or in vivo imaging, multimodal imaging, theranostics (combined ‘all-in-one’ diagnostics and therapeutics) as well as selected examples from relevant coordination chemistry.

1.6.  Technetium and rhenium based imaging probes

\(^{99m}\)Tc is metastable, which means that it does not transmute into another nucleus upon emission and was first isolated in 1959 and initially used in 1961 for thyroid diagnosis. As previously mentioned, technetium-99m is the most regularly used SPECT imaging probe, which is owed largely to its ideal half life of ca. 6 hours and its ready availability via a \(^{99}\)Mo generator.

![Figure 1.6. Structures of selected \(^{99m}\)Tc complexes in commercial use](image)

There are numerous \(^{99m}\)Tc molecular probes in clinical use covering a wide range of imaging applications of the: brain (Ceretec\textsuperscript{TM}), heart (Cardiolite\textsuperscript{TM} and Myoview\textsuperscript{TM}), kidney (\([^{99m}\text{TcO(MAG)}_3]\)) and bone (\(^{99m}\text{Tc-MDP}\)). Technetium complexes are additionally under development as hypoxia targeting agents, with early attempts via conjugation of chelate derivatives to a nitroimidazole, such as BMS-181321 (Figure 1.7). \(^{17, 18}\) Nitroimidazoles are promising as hypoxia selective probes, such as pimonidazole hydrochloride (Hypoxyprobe\textsuperscript{TM}), which is used in
immunohistochemistry or $^{18}$F-fluoromisonidazole ($^{18}$F-FMISO), which will be discussed later. High liver uptake, due to the lipophilicity of the compound however, caused it to be unsuitable. Rey et al. however, recently developed a metronidazole based technetium complex with a good tumour to muscle ratio and low liver uptake (Figure 1.7).\(^{19}\)

\[
\text{Figure 1.7. Structures of compounds developed for hypoxia selectivity}
\]

Despite the popularity of this radiometal, it does have its drawbacks. Firstly that $^{99m}$Tc hinders the binding of organ specific pharmaceuticals, due to its non-physiological nature and secondly, there is no stable isotope of technetium.

\[
\text{Figure 1.8. Rhenium(I) complex (left), which enters the nucleoli of cells (right)}
\]

Isostructural rhenium complexes however, are often used as analogues for \textit{in vitro} luminescence studies, as well as being exciting \textit{in vitro} imaging agents in their own right and with broad applications.\(^{20}\) Rhenium(I) polypyridine complexes are of particular relevance as sensors, due to good quantum yields, especially since the high environmental sensitivity of rhenium(I) polypyridine complexes was reported.\(^{21,22}\) Rhenium(I) complexes have long luminescent lifetimes and significant
Stokes shifts, making them highly suitable as in vitro probes. Coogan et al. have developed numerous tricarbonyl polypyridyl rhenium(I) complexes for imaging cells, notably reporting in 2011 a Re(I) complex that can act as a carrier of ions such as silver and copper. The unfilled form of the complex does not enter cells; however, when filled with Ag⁺ cations it can enter the nucleoli (Figure 1.8). Interestingly, dinuclear tricarbonyl rhenium(I) complexes appended to peptide nucleic acid showed rapid cell uptake, low cytotoxicity and have the ability to discriminate between the nucleus and cytoplasm via different excitation/emission properties (Figure 1.9). Tricarbonyl rhenium complexes have also been developed by Lo et al. for in vitro metal ion sensing and displayed increased luminescence emission and a longer lifetime upon Zn(II) or Cd(II) binding. Furthermore, Alberto et al. developed tricarbonyl Re(I) and Tc(I) complexes in 2012 for in vitro and in vivo imaging of Alzheimer’s disease. The ligand contained a phenylbenzothiazole, known to have high affinity for amyloid plaques of the disease.

Figure 1.9. A bimetallic Re(I) complex (d) in the nucleus (a) and in the cytoplasm (b), where (c) is an overlay of (a) and (b).

Numerous rhenium(I) complexes suitable for bioconjugation and fluorescence imaging are currently under development. Notably, a cytotoxic folic acid-PEG derivatised Re(I) complex was investigated in A2780/AD cells, which are a multidrug resistant ovarian cancer cell line. Rhenium(I) complexes with an appended α-D-glucose were developed with the purpose of monitoring glucose uptake, which is increased in cancer and showed mitochondrial uptake as well as cytotoxicity that did not depend on cell type. Polypyridinerhenium(I) bis-biotin complexes were observed in HeLa cells by laser scanning confocal microscopy. Subsequently, rhenium complexes with polylactide conjugates displayed cell uptake in A2780 cells.
As mentioned above, isostructural Re/\textsuperscript{99m}Tc complexes can be developed for \textit{in vitro} and \textit{in vivo} investigations respectively. For example, Pelecanou \textit{et al.} designed rhenium and technetium complexes incorporating the [M(CO)\textsubscript{3}(NNO)] unit covalently attached to anticancer agent 2-(4'-aminophenyl)benzothiazole for theranostic SPECT applications.\textsuperscript{36} Recently they developed new rhenium and technetium complexes of the same family for optical and SPECT imaging respectively, which demonstrate greater uptake in cell lines of cancerous origin with respect to non-cancerous lines.\textsuperscript{37}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure110.png}
\caption{Reaction scheme of “click chemistry” (above, left), folic acid derivatised technetium complex by SPECT (below).}
\end{figure}

The first instance of substituting a well established chelator with a 1,2,3-triazole analogue for complexation of Re/\textsuperscript{99m}Tc without modifying the biological effects of the compound was reported by Mindt \textit{et al.} in 2008.\textsuperscript{38} The isostructural Re/\textsuperscript{99m}Tc folic acid analogues were synthesised using a Cu(I) catalysed cycloaddition method, known as a “click reaction” that allowed chelation and bioconjugation in one step, which the authors named “click-to-chelate” (\textit{Figure 1.10}). Moreover, Mindt \textit{et al.} designed several new imaging probes for PET, SPECT, Near Infra-red (NIR) or MRI from a single folic acid based precursor, using \textsuperscript{67}Ga, \textsuperscript{111}In and \textsuperscript{99m}Tc agents for SPECT, fluorophore Cy 5.5 for optical imaging and \textsuperscript{18}F for PET.\textsuperscript{39} The \textsuperscript{111}In-DTPA folate complex has recently been reported as with the capacity to quantify macrophage activation (\textit{Figure 1.10}).\textsuperscript{40} The authors demonstrated that the later
stages of osteoarthritis can be correlated to reduced macrophage activation, allowing monitoring of the disease activity, for which there are no clinical measures at present. Furthermore the same group has used click reactions to design tridentate di-1,2,3-triazole chelator imaging tracers as well as multifunctional $^{99m}$Tc complexes, as a platform for a broad number of potential purposes including multimodal imaging probe development.\textsuperscript{41, 42} This efficient and facile synthesis combined with uptake in folic acid receptor expressing KB (human epidermoid carcinoma) cells and tumour targetting in mice confirms the promise of this procedure.

Rhenium and technetium complexes have also been designed to enter the nucleus and bind to DNA, a significant example of which was published by Santos \textit{et al.} who synthesised tricarbonyl pyrazolyl-diamine rhenium(I) complexes that show potential for the development of future targeted radiopharmaceuticals.\textsuperscript{43} Furthermore, tricarbonyl rhenium and technetium complexes with acridine derivatives showed nuclear uptake \textit{via} fluorescence and activity based studies respectively.\textsuperscript{44} Policar \textit{et al.} designed a tricarbonyl rhenium(I) complex in 2012 to selectively enter Golgi apparatus, with its uptake in breast cancer cells followed by fluorescence microscopy and IR mapping. Already a dual-modal imaging agent, the analogous technetium complex would present potential for a tri-modal imaging.\textsuperscript{45} Another example of a dual-modal imaging probe under development was reported by Faulkner \textit{et al.} combining a Gd$^{3+}$ for MRI and a Re fluorophore for luminescence investigations.\textsuperscript{46} Furthermore, the first dinuclear rhenium/technetium complex was designed for dual-modal fluorescence/SPECT imaging with potential for therapy \textit{via} $^{188}$Re.\textsuperscript{47}

1.7. \textbf{Copper-based imaging probes}

Radionuclides of copper include $^{60}$Cu, $^{61}$Cu, $^{62}$Cu and $^{64}$Cu, all of which are positron emitters, as well as $^{67}$Cu, which is a $\beta^+$ emitter. Copper-64, with a half-life of 12.7 h, decays 41\% by electron capture, 40\% by $\beta^-$ radiation and 19\% by $\beta^+$ radiation,\textsuperscript{48} making this radioisotope highly appropriate for simultaneous diagnosis and therapy. A challenge to dual-modal imaging with copper(II) complexes is the
paramagnetic quenching caused by the d⁹ electronic configuration of the metal centre. Despite this, a number of fluorescent copper(II) complexes have been reported in the literature, most of which are conjugated to fluorophores such as fluorescein, rhodamine or BODIPY as well as BAMs for selectivity (Figure 1.11).

![Fluorescein, Rhodamine, BODIPY](image)

**Figure 1.11.** Commonly used fluorophores

With possible coordination numbers between four and six and a wide range of geometries the 3d⁹, 2+ oxidation state represents the most significant proportion of copper complexes developed as radiopharmaceuticals. Thus, there are a large number of potential chelators for this metal, which include EDTA, DTPA, NOTA, NO2A, DOTA and cyclam derivatives, all of which represent a large proportion of copper(II) research.⁴⁹ Research is ongoing to improve chelators for use as bifunctional agents, as well as to achieve better yields and very high stability with a recent example by Archibald and co-workers using cross-bridged cyclam derivatives.⁵⁰

Some examples of copper(II) complexes used in conjunction with bifunctional chelators are described below. Colorectal cancers often express receptors for the E. coli enterotoxin, STh; analogues of these show promise for imaging and therapy. A STh analogue was conjugated to DOTA, TETA and NOTA at the N-terminus and labelled with ⁶⁴Cu of which the NOTA complex gave the best tumour uptake with respect to organs, when investigated by biodistribution and PET studies.⁵¹ Similarly 4-((8-amino-3,6,10,13,16,19-hexaaazabicyclo [6.6.6] icosane-1-ylamino) methyl) benzoic acid (AmBaSar), a cage like bifunctional chelator conjugated to cyclic RGD, was synthesised in high yield and showed increased stability and tumour specificity when compared to ⁶⁴Cu-DOTA-RGD.⁵² A bombesin analogue conjugated to NO2A radiolabelled with ⁶⁴Cu also allowed visualisation of PC-3 xenografts.⁵³ Moreover, Maecke et al. developed and evaluated 4 new ⁶⁴Cu and ⁶⁸Ga complexes, chelated with
NODAGA and cross-bridged-TE2A conjugated to somatostatin antagonist p-Cl-Phe cyclo(D-Cys-Tyr-D-4-amino-Phe(carbamoyl)-Lys-Thr-Cys)D-Tyr-NH₂.\(^{54}\)

Furthermore, dual-modal MRI/PET nanoparticles have been reported by Lee et al. involving polyaspartic acid iron oxide nanoparticles conjugated to argenine–glycine–aspartic acid (RGD) peptide for integrin targeting and DOTA chelation of \(^{64}\)Cu for PET.\(^{55}\) MRI and PET both indicated selective uptake and offer the potential for higher accuracy and earlier detection of cancer. A monoclonal antibody for endoglin – an indicator of poor prognosis in cancer – was conjugated to a near infrared dye and \(^{64}\)Cu via NOTA, by Cai et al. in 2012.\(^{56}\) This dual-modal imaging agent was capable of rapidly and selectively entering endoglin expressing cells and could be useful for assessment of cancer disease progression. Furthermore a multi-modal imaging agent was developed for MRI, NIF, and SPECT or PET imaging using liposomes as a drug-delivery mechanism.\(^{57}\) \(^{64}\)Cu-DOTA and Gd-DOTA could be conjugated to liposome surface, whilst technetium, rhenium or a drug such as doxorubicin, could be loaded within. The NIRF dye (IRDye-DSPE) was postinserted into the liposomes enabling luminescence imaging. Therefore, this kind of system presents the potential advantages of the aforementioned imaging techniques combined with therapy especially if combined with a BAM.

**Figure 1.12.** a) Copper bis(thiosemicarbazono) complex, Cu[ATSM] (left). The following images are pretherapy scans of \(^{18}\)F-FDG-PET (middle left), \(^{60}\)Cu[ATSM]-PET (middle right) and CT scan. The arrow indicates lung cancer. The outside of the tumour shows greater \(^{60}\)Cu[ATSM] uptake, in contrast to the uniform uptake of \(^{18}\)F-FDG.\(^{58}\)

A significant complex for molecular imaging, aliphatic copper(II)-diacetyl-bis(N(4)-methylthiosemicarbazone) (Cu[ATSM]) has been shown to be selective for hypoxic tissue and has progressed to phase II clinical trials for cervical cancer diagnosis (Figure 1.12). \(^{18}\)F-FMISO, also in clinical trials, shows promise as a hypoxia selective agent,
but presents drawbacks such as slow body clearance and low contrast images. Cu[ATSM] has shown more rapid uptake in hypoxic cells and greater hypoxic-to-normoxic ratio than $^{18}$F-FMISO.\textsuperscript{6} The mechanism of Cu[ATSM] selectivity for hypoxic cancer cells was originally postulated to be due based on the irreversible reduction of Cu(II) to Cu(I) occurring in oxygen-poor environments.\textsuperscript{59} Dearling \textit{et al.} however, proposed that reduction occurs in both normoxic and hypoxic conditions, but and that the compound may undergo re-oxidation in normoxic cells only. As a result, the Cu(II) complex can cross the cell membrane, but is trapped within the cell and accumulates in the form of an anionic Cu(I) complex (\textit{Figure 1.13}).

![Figure 1.13. Mechanism of Cu[ATSM] hypoxia selectivity as proposed by Dearling \textit{et al.}^60](image)

The therapeutic potential of Cu[ATSM] was recently demonstrated by Fujibayashi \textit{et al.}, who reported its ability to inhibit tumour growth and malignancy.\textsuperscript{61} The mechanism of cell uptake of bis(thiosemicarbazones) can be investigated thanks to the weak intrinsic fluorescence of thiosemicarbazones, which can be enhanced by binding to a diamagnetic metal such as Zn[ATSM] or by addition of a fluorophore. The first \textit{in vitro} fluorescence study of zinc bis(thiosemicarbazone) complexes in human cancer cells was carried out in 2005 by Dilworth \textit{et al.} using the Zn[ATSM] analogue in a number of cell lines.\textsuperscript{62} As Cu[ATSM] is not fluorescent zinc analogues have been used as models for Cu[ATSM] \textit{in vitro}. Since zinc(II) and copper(II) are of comparable atomic radii and electronegativity, however there are some limitations of this model. Zinc(II) does not possess significant redox potential as does copper(II) and therefore can only represent the uptake of the complex under
normoxic conditions. Recently a series of new fluorescent zinc bis(thiosemicarbazone) complexes were reported showing in vitro uptake in cells. Copper and zinc bis(thiosemicarbazone) complexes bearing an appended fluorescent pyrene tag and reactive styrene with potential for dual modal imaging have also been synthesised by Holland et al. Moreover, the better stability attributed to copper(II) complexes, indicated by the Irving-Williams series, allows clean synthesis of a copper(II) complex via a zinc(II) complex transmetallation as well as by synthesis via a ligand precursor.

![Figure 1.14. Bis(thiosemicarbazone) complexes conjugated to glucose (left) or to a nitroimidazole (right)](image)

There are, however, limitations of Cu[ATSM], including serum instability and high liver uptake, due to its lipophilicity. It would therefore be attractive to develop similar hypoxic imaging probes, which are biologically stable, with intrinsic fluorescence and reduced lipophilicity. As a consequence, current research aims to improve upon Cu[ATSM], with recent attempts via bioconjugation. Glucose, for example, was appended to copper bis(thiosemicarbazone) complexes by Christlieb et al., the resultant complex retained hypoxia selectivity yet without the drawback of significant uptake in the heart and brain as displayed by $^{18}$F-FDG (Figure 1.14). Subsequently, bombesin conjugates were developed displaying binding to PC-3 cells, which appear suitable for PET applications. Furthermore, Bayly et al. recently coupled a nitroimidazole to a copper bis(thiosemicarbazone) complex (Figure 1.14). The conjugates displayed superior hypoxia selectivity and reduced non-target uptake when compared to propyl derivatives used as controls and CuATSM/A derivatives respectively.

It is possible to chelate a wide range of metals due to the “soft donor” characteristics of the tridentate S,N,N these include zinc and copper (as already
mentioned above), iron, nickel, rhenium, technetium, gallium, indium, platinum and palladium. Therefore, chelation of PET or SPECT radiometals such as gallium or indium with a counterion, for example chlorine could be another means of reducing the lipophilicity and therefore the uptake in the liver.

Table 1.5. Represents a broad range of bis(thiosemicarbazonato) complexes

<table>
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<td>CH₃(EtO)CH</td>
<td>H</td>
<td>Me</td>
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</tbody>
</table>

Furthermore, there is a broad scope of biological and medical activity of this family of compounds, which includes complexes and their ligand precursors. Uncomplexed ligands are active against leukaemia, TB, malaria, viruses (including HIV), bacterial cells and superoxide dismutase-like radical scavenger properties with enhanced activity displayed when bound to a transition metal. α-N-heterocyclic thiosemicarbazones (Figure 1.15), have been shown to possess anti-tumour action via inhibition of the ribonucleotide diphosphate reductase (RR) enzyme, which is involved DNA synthesis. Since pyridine-2-carboxaldehyde thiosemicarbazone anti-tumour properties were observed in 1956, a number of other heterocyclic ring systems have been synthesised showing anticancer activity and include isoquinoline-1-carboxaldehyde thiosemicarbazone, 3-aminopyridine-2-carboxaldehyde thiosemicarbazone (Triapine) and di-2pyrirylketone 4,4-dimethylthiosemicarbazone, which is the most potent (Figure 1.15).
Furthermore, Platinum(II) and palladium(II) thiosemicarbazone complexes, have shown activity against cancer cells resistant to cis-platin, one of the most commonly used anti-tumour drugs. French and Freelander assayed fifty bis(thiosemicarbazonato) ligand precursors and were the first to observe their anti-tumour properties, with determined KTS and KTSM (Figure 1.15, Table 1.5) as the most active. Moreover, it was discovered that their antitumour properties are due to their ability to chelate copper(II) ions, which lead to the investigation of chelation of other biological metal ion chelation including zinc(II), nickel(II) and iron (II/III). The potential scope therefore, of thiosemicarbazones is significant in the development of diagnostics and therapeutics, with reports of gallium(III) and indium(III) thiosemicarbazones (Figure 1.15).

1.8. Zinc

Zinc(II) is a d\(^{10}\) metal ion with coordination geometries ranging for example from tetrahedral, square pyramidal or trigonal bipyramidal thus giving rise to versatile probes for the development of luminescence imaging using transition metal complexes: some recent developments will be highlighted hereby. A series of zinc salen complexes (Figure 1.16) were designed by Xu et al. and investigated by single and two-photon microscopy in cells displaying colocalisation in the lysosome, endosome and ER as well as excellent photostability and low cytotoxicity (Figure 1.16).
A fluorescent DAQZ@2Zn$^{2+}$ complex, was reported as a sensor in live cells for oxalic acid, an inhibitor of lactate dehydrogenase (LDH), presence of which resulted in quenching (Figure 1.17).$^{79}$ Cancer cells more frequently undergo anaerobic respiration than non-cancer cells, particularly under hypoxia. Therefore oxalic acid could provide one method of treating cancer as well as indicating the necessity for this type of treatment. Additionally, a binuclear BODIPY-based fluorescent zinc complex was developed and investigated in cells by Hamachi et al.$^{80}$ Histological studies demonstrated that the complex could distinguish neurofibrillary tangles of hyperphosphorylated tau proteins and amyloid plaques in the hippocampus of a patient who had Alzheimer’s disease.

![Figure 1.17. In vitro fluorescence microscopy of a Zn$^{2+}$ complex of DAQZ ligand 7 in HeLa cells (DIC sown in image a), before (b) and after incubation with oxalic acid (c).$^{79}$](image)

Keppler et al. studied triapine, which is under investigation as an anticancer agent, by fluorescence microscopy. The corresponding zinc complex [Zn(Triapine)Cl$_2$].HCl was also synthesised and imaged in colon carcinoma cells to show uptake in nucleoli.$^{81}$ The unusual attribute of intrinsic fluorescence of this particular anticancer drug may help to facilitate personalised medicine via monitoring the uptake within patient biopsy samples and subsequent morphological changes.

In 2005, trimetallic zinc meso-to-meso ethyne-bridged tris[(porphinato)zinc(II)] complexes were designed as NIR fluorophores with potential for in vivo imaging
showing uptake by fluorescence in murine melanoma cells.\textsuperscript{82} Furthermore, NIR \textit{in vivo} fluorescence imaging of cell death was enabled by the use of a bimetallic zinc(II)-dipicolylamine (Zn-DPA) complex, conjugated to a ligand with affinity for phosphatidylinerine, which is present on dying and dead cell surfaces (\textit{Figure 1.18}).\textsuperscript{83}

\textbf{Figure 1.18.} Near-IR fluorescence image of tumour cell death after radiation therapy (a) as a result of treatment with imaging probe 14, (b). Image reproduced with permission from Ref. \textsuperscript{83}.

When compared to Annexin-Vivo 750 (a commercially available NIR fluorescence sensor for apoptosis) the complex possessed higher target to non-target ratio on account of lower bladder uptake, giving complementary information as two probes and good clearance indicating potential for radiolabelling studies due to a resultant reduced radiation exposure.\textsuperscript{84} Further, this probe shows promise for the assessment of patient response to therapy as well as evaluation of novel anticancer treatments. The probe potentially has broader applications and was shown to be capable of detecting tissue infected by \textit{S. aureus}.\textsuperscript{85} The same group developed a series of Zn-DPA complexes of which the hydrophilic variants are non-toxic to mammalian cells yet toxic to \textit{S. aureus}, including antibiotic resistant strains. Moreover, the uptake of a fluorescent analogue was imaged in \textit{E. coli} and \textit{S. aureus}.\textsuperscript{86} In 2012 Hnatowich \textit{et al.} combined \textsuperscript{111}In-DOTA-biotin with a fluorophore-conjugated Zn-DPA analogue \textit{via} a noncovalent linker and investigated the potential of Zn-DPA for assessing infection \textit{in vivo} for the first time. There was notably greater uptake in infected areas.
when compared to induced inflammation indicating that the probe could help with diagnosis of bacterial disease.\textsuperscript{87}

![FRET mechanism of Zn-DPA complexes for sensing of nucleosides](image)

\textbf{Figure 1.19.} FRET mechanism of Zn-DPA complexes for sensing of nucleosides (reproduced with permission from Ref. \textsuperscript{88})

Hamachi \textit{et al.} developed another series of Zn-DPA complexes containing four zinc nuclei and demonstrated that their D4 tag/Zn-DPATyr pair can successfully label proteins.\textsuperscript{89} Moreover, new Zn-DPA complexes were developed by Hamachi \textit{et al.} for the recognition of nucleoside polyphosphates (including ATP), whereby fluorescence is activated by the presence of the desired compound \textit{via} fluorescence resonance energy transfer (FRET) yielding a ratiometric response (\textit{Figure 1.19}).\textsuperscript{88} A zinc(II) dipicolylamine complex with ATP sensitivity and conjugated to nanoparticles with a silica core was imaged in fixed non-cancerous rat epithelial (NRK) cells with potential use in metabolic studies.\textsuperscript{90} Furthermore, targeted cancer imaging of oral epithelial cancer was reported by Menon \textit{et al. via} the conjugation of a mannose ligand to chitosan-zinc sulphide nanocrystals.\textsuperscript{91}

\section*{1.9. Gallium}

Gallium is predominantly found in the 3+ oxidation state and has a coordination number range of 3-6. A hard acidic cation, Ga(III) strongly binds to multiple anionic donor sites including oxygen and thiolates. Gallium is known to bind to chelators EDTA, DTPA, DOTA and has promising thermodynamic stability with NOTA (including under acidic conditions), DFO and HBED, and show favourable characteristics for bifunctional chelators (\textit{Figure 1.20}).
There are three gallium isotopes of interest for molecular imaging: $^{66}$Ga (9.5 hour half-life) and $^{68}$Ga (68 minute half-life), which are positron emitters and $^{67}$Ga (3.26 days half-life), a gamma emitter. The PET radioisotope $^{68}$Ga, has a major advantage in that it does not require a nearby cyclotron and is relatively simple to obtain via a commercially available $^{68}$Ge (half-life of 270.8 days) generator. Furthermore, $^{67}$Ga is used as a prognostic of non-Hodgkin’s and Hodgkin’s lymphoma as well as to detect bone infection in scintigraphy.

There have been a number of bioconjugated gallium complexes with an outlook towards theranostics, some examples of which will be presented here. A DOTA-Gly-Glu-Cyc lactam bridged cyclised $\alpha$-melanocyte stimulating hormone peptide was radiolabelled with $^{67}$Ga to allow the visualisation of primary and metastatic melanoma. There is potential for further development of this probe as a theranostic agent since the versatility of DOTA could allow chelation of a radiotherapeutic metal in the place of gallium. Furthermore, a DOTA-PEG4-BN(7-14) bombesin analogue labelled with $^{67/68}$Ga and $^{177}$Lu showed good tumour uptake in PC-3 xenografted nude mice, where $^{67/68}$Ga acted as a diagnostic radionuclide and $^{177}$Lu as a therapeutic. $^{68}$Ga DOTATOC was utilised as a diagnostic and indicated the necessity for $^{177}$Lu-DOTATATE treatment, where FDG PET had not shown any increased uptake.

Additionally, a quadruple-modality imaging probe, which can be detected by fluorescence, bioluminescence resonance energy transfer (BRET), PET and MRI, was reported by Kim et al. (Figure 1.21). The probe consisted of a cobalt–ferrite nanoparticle (for MRI), incorporating rhodamine (for fluorescence) and
conjugated to luciferase (for bioluminescence) and a \( p \)-SCN-bn-NOTA chelating group that was tagged with \( ^{68}\text{Ga} \) (a generator-produced PET radionuclide). Each imaging modality displayed dose dependence in vivo. Further functionalisation with targeting groups could enhance the potential for effective delivery to tumours.

Figure 1.21. Quadruple modal imaging: (a) bioluminescence, (b) micro-PET, (c) MRI and (d) fluorescence imaging, adapted from Kim et al. 2009. 101

1.10. Indium

Indium predominantly exists in the 3+ oxidation state when complexed and can be from 3 to 8 coordinate. Despite the possibility of the higher coordination numbers indium, like gallium, forms its most stable complexes with hexadentate chelators. In contrast to gallium, indium has a higher affinity for softer donors and therefore EDTA, DTPA and DOTA bind more securely to indium. There are a number of indium radiometals, such as \( ^{110}\text{In} \), \( ^{110m}\text{In} \) and \( ^{114m}\text{In} \), however the most commonly reported indium radioisotope is \( ^{111}\text{In} \). Indium-111 is a commercially produced radionuclide for SPECT imaging, with a half-life of 2.8 days and photon energies of 171 and 245 keV. Notable applications to date include tumour-targeted imaging. There have been a number of reported peptide/antibody targeted small molecules involving indium-111, therefore some selected examples will be reviewed here, in
particular those pertaining to multimodal imaging or theranostics.

Figure 1.22. LLP2A \( N^\prime\)-[[[2-ethylphenyl]amino]carbonyl]amino]phenyl]acetyl]-N^\prime\prime\'-6-[(2E)-1-oxo-3-(3-pyridinyl-2-propenyl)]-L-lysyl-L-2-amino hexadiol-(1-amino-1-cyclohexane)carboxamide (left) and linker (right).

LLP2A coupled to DOTA and PEG was labelled with \( ^{111}\)In and showed good tumour uptake.\(^\text{102}\) The drawback of renal retention however, would require further modification. Nevertheless the \( ^{111}\)In-(LLP2A-DOTA)\(_4\)-PEG\(_{10,000}\) displayed potential for simultaneous imaging and therapy. Glucagon-like peptide receptor-1 (GLP-1) imaging with In-[Lys\(^{40}\)(Ahx-diethylenetriaminepentaacetic acid [DTPA])NH\(_2\)-exendin-4 and \( ^{68}\)Ga DOTATATE enabled the assessment of receptor status as well as the imaging of benign insulinomas, which contrary to malignant insulinomas often lack somatostatin receptor subtype 2 (sst2) and express GLP-1 receptors.\(^\text{103}\) DOTATE radiotherapy could be applied to patients with sst2 suppressing tumours.

Ibritumomab, a monoclonal antibody, was complexed for \( ^{90}\)Y-ibritumomab therapeutic purposes was combined with \( ^{111}\)In-ibritumomab for pre-therapy scans. The \( ^{111}\)In scans proved capable of predicting disease progression and could be a useful tool to tailor therapy towards more aggressive therapeutic strategies.\(^\text{104}\) Additionally, Yoshimoto et al. radiolabelled DOTA-c(RGDfK) with \( ^{111}\)In and \( ^{90}\)Y, reporting high tumour uptake due to specificity for \( \alpha_\text{v}\beta_3 \) integrin, which is present in cancerous melanoma cells, and therefore promise as a theranostic pair.\(^\text{105}\) Indium complexes are also currently being developed for multimodal imaging. In 2006 Li et al. designed a dual-labelled probe for tumour imaging, \( ^{111}\)In-DTPA-Lys(IRDye800)-cyclic(KRGDf), which binds to integrin \( \alpha_\text{v}\beta_3 \).\(^\text{106}\) Figure 1.23 shows the uptake of the imaging probe within \( \alpha_\text{v}\beta_3 \)-positive tumours, with no uptake
observed within \( \alpha_\beta_3 \)-negative tumours, demonstrating the selectivity of this agent.

\[ \text{Figure 1.23.} \quad ^{111}\text{In-DTPA-Bz-SA-Lys(IRDye800)-c(KRGdf)} \text{ in nude mice visualised by (from left to right) white light, } \gamma \text{-scintigraphy and NIR. Arrows represent the tumours.}^{106} \]

DOTA-neurotensin (DOTA-NT) analogues labelled with \( ^{68}\text{Ga} \) or \( ^{111}\text{In} \) for PET were designed by Gruaz-Guyon et al. for the purpose of targeted radiotherapy in combination with \( ^{90}\text{Y} \) or \( ^{177}\text{Lu} \).\(^{107} \) The \( ^{68}\text{Ga} \) and \( ^{111}\text{In} \) DOTA-NT complexes showed higher tumour and renal uptake than the DOTA-LB119, with very low background in other tissue with exception of the kidneys. Despite the poor affinity of the indium DOTA-NT complex for colorectal carcinoma cell line HT29, the respective Yttrium complex possessed could bind significantly better, indicating potential for tumour targeting as a radiotherapeutic. Tumours were visualised using NIR optical imaging, with good resolution and sensitivity, which was complemented by \( \gamma \)-scintigraphy for tissue penetration. Another cyclic peptide \( c(\text{CGRRAGGSC}) \) was applied for combined near infrared fluorescence and SPECT imaging of tumours, allowing unambiguous visualisation of the tumour by both modalities.\(^{108} \) Moreover, in 2012, Häfeli et al. designed a trimodal imaging agent for SPECT, MRI and fluorescence imaging.\(^{109} \) This consisted of hyperbranched glycerols conjugated to \( ^{111}\text{In-DOTA, Gd-DOTA and the fluorescent dye Alexa Fluor. The imaging probe was excreted slowly, allowing the image contrast to be significantly enhanced with time.} \]
Figure 1.24. Trastuzumab-Cy5.5-CHX-A” (left), investigated using confocal microscopy in cells expressing HER2 (top and middle) and cells seldom expressing HER2 (bottom).

Monoclonal antibodies trastuzamab (binds to HER2 (Human Epidermal Growth Factor Receptor 2 expressing cells)) or cetuximab (selective for EGFR Epidermal Growth Factor Receptor expressing cells) were conjugated to chelators (CHX-A”) for the incorporation of radioisotopes (in this case, $^{111}$In) (Figure 1.24). The uptake of the respective ligands was followed in LS174T, EGFR expressing A431 and two strains of HER2 expressing cells (NIH3T3-HER2$^+$). Furthermore, antibodies for the targeting of tumours via epidermal growth factor receptors has been utilised by Kobayashi et al. allowing discrimination between three types of tumour to be (EGFR-1, EGFR-2 and a tumour that did not overexpress HER2). Using cetuximab or trastuzumab conjugated to a linker and to a fluorophore (Rhodamine Green, Cy5.5 or Cy7) it was possible to utilise the wavelength of in vivo emission as an indication of response to the antibody. For comparative purposes $^{111}$In-labelled antibodies were used in this study – with the fluorescent probes possessing the advantages of avoidance of ionising radiation and the potential to discriminate between up to three tumour types. Additionally Kobayashi et al. developed a probe, which can be labelled with $^{111}$In and a fluorophore, for antibody-based targeting (panitumumab and separately trastuzumab) to enable dual modal optical and SPECT imaging. The fluorescent probes were activated upon internalisation by the cell,
thus providing a clear visualisation of tumours, which was well complemented by the “always on” of nuclear imaging.

Small molecules containing copper-64 and indium-111 have been targeted using peptides/antibodies, allowing for the possibility of both PET and SPECT. Recently an $^{111}$In labelled antibody binding fragment (fab) for c-kit (a proto-oncogene significant for many malignancies) was tested for *in vitro* binding and by cellular internalisation assays, whilst $^{64}$Cu c-kit fab was evaluated *in vivo* by PET, allowing clear tumour visualisation. This makes it a promising tool for enabling an informed decision to be made before beginning c-kit targeted therapy. $^{64}$Cu and $^{111}$In DOTA-HSA-Z(HER2:342) were also studied by PET and SPECT respectively, both displaying high tumour and liver uptake. This ligand is likely to be suitable for labelling with radionuclides such as $^{90}$Y and $^{177}$Lu, which could open the door to usage as a theranostic pair. Furthermore, a diabody (a bivalent single-chain antibody dimer fragment) was attached to a DOTA-PEG, labelled with $^{125}$I, $^{111}$In and $^{64}$Cu and investigated by biodistribution and $^{64}$Cu PET in athymic mice, indicating tumour uptake and low kidney uptake. Furthermore, visualisation of the tumour was possible after 22 h, which suggests potential as a theranostic pair in combination with $^{125}$I.

1.11. Summary

Medical imaging provides essential diagnosis for a multitude of diseases including cancer, whilst nuclear imaging can offer highly sensitive molecular and physiological information, enabling rapid detection of early stages of disease. The combination of imaging modalities (optical/PET and or SPECT/ MRI) can allow for more accurate diagnoses thanks to the specific advantages in terms or resolution and sensitivity of each technique, with optical imaging enabling a crucial insight into cellular behaviour of a probe under development. One of the crucial research areas is that of hypoxia detection, which is a symptom present in numerous diseases and is highly significant in the development of cancer. There is currently no clinically
approved hypoxia imaging probe, therefore the necessity for further development remains. Although there has been a recent increase in the development of agents capable of both detection and therapy. Thus, the progression of current research moves towards a future of personalised medicine and as a result promises an improved patient response to therapy.

1.11.1. Aims of thesis

1. Synthesise copper, gallium and indium acenaphthenequinone bis(thiosemicarbazones) with intrinsic fluorescence, which can be rapidly and cleanly radiolabelled and have selectivity for cancer cells and hypoxic tissue.

2. Investigate the stability, cell uptake and cytotoxicity of bis(thiosemicarbazonato) complexes to allow in vivo studies to commence. Usage of computational studies to study electronic configuration and thermodynamic stability of these complexes in gas phase and solution models.

3. Explore acenaphthenequinone bis(thiosemicarbazonone) complexes for selective dual modal imaging and/or enabling of targeted nanomedicines with potential for therapy.
Chapter 2. Synthesis, characterisation and spectroscopic investigation of mono(substituted) and bis(substituted) thiosemicarbazonato ligand precursors

2.1 Overview

The objective of this project was to synthesise new complexes based upon the design of Cu[ATSM], but with improved kinetic stability, reduced liver and other non-target organ uptake and increased intrinsic fluorescence. The design was modified by introducing a bis(imino)acenaphthene (BIAN) backbone, which is likely to provide superior kinetic stability and fluorescence. The BIAN ligand itself possesses redox activity, which conceivably could affect the hypoxia selectivity.\textsuperscript{116, 117} Moreover three ligands containing an acenaphthyl moiety were recently shown to display greater uptake \textit{in vitro} under hypoxia than normoxia by fluorescence microscopy.\textsuperscript{118} The compounds were designed to work on the same principle as many fluorescent hypoxia probes based on 2-nitroimidazole, whereby bioreduction of the nitro group under hypoxia results in increased fluorescence. This BIAN compound did not contain a nitroimidazole, however, and instead was based upon 6-aminoquinoxaline and acenaphtho[1,2-b]quinoxaline substituted with a nitro group, for bioreduction. Redox properties of the BIAN backbone therefore, did not impede the hypoxia selectivity.

The aim of this chapter was to synthesise and characterise proligands that could firstly act as potential precursors to analogous metal complexes. Secondly, the comparison between ligand precursors and their metal complexes is essential for assessing the stability of the latter in biologically relevant conditions as well as \textit{in vitro}. The next objective was to investigate these compounds by UV-visible and fluorescence spectroscopy, followed by the determination of their fluorescence lifetime in solution and \textit{in vitro}, to allow comparisons with their corresponding metal complexes described in later chapters.
2.2 Ligand precursor synthesis and characterisation

Mono(substituted) ligand precursors were synthesised by refluxing one equivalent of thiosemicarbazide (incorporating R substituents as peripheral functionalities such as methyl, ethyl, phenyl or allyl), with one equivalent of acenaphthenequinone, in presence of acid as a catalyst. These mono(thiosemicarbazone) proligands present an opportunity to synthesise asymmetric bis(substituted) compounds and will be discussed in Chapter 7. To synthesise the bis(thiosemicarbazone) ligand precursors, three or more equivalents of thiosemicarbazide were added to the diketone starting material using the same procedure, in ethanol with ten drops of concentrated hydrochloric acid (Figure 2.1). In both cases the suspension was filtered after three hours and the solid product washed with methanol.

![Figure 2.1. Reaction scheme of ligand synthesis method.](image)

Isolated ligand precursors were yellow solids, with the exception of phenyl-substituted compound ic which was pale orange. The yields of the mono(substituted) ligand precursors ia – id were good (72 - 85 %). Mass spectrometry and NMR spectroscopy were used for their characterisation, whereby the mono(substituted) ligand precursor m/z resonances were found in agreement with calculated [M + H]^+ as 292.05, 306.07, 332.09, and 318.11 (ia, ib, ic and id respectively). All bis(substituted) ligand precursors were isolated in moderate to good yields of (64 - 85 %). ESI-MS were confirmed in negative ionisation mode as 355.08, 383.12, 479.12 and 407.11, for the bis(substituted) ligand precursors iia, iib, icc and iid respectively.

^1^H-NMR spectroscopy indicated the high purity of the ligand precursors and that the bis(substituted) compounds showed an asymmetric conformation. Figure 2.2 shows ^1^H-NMR spectra of proligands ib and iib in d_6-DMSO. The asymmetric structure of the
bis(substituted) proligands is characteristic of an \((E,Z)\) isomerism at the imine functionality and has been structurally confirmed using X-ray crystallography (\textit{vide infra}). Moreover, when \(H_2\)ATSM was optimised by DFT (Density Functional Theory), the conformer possessing the \((E,Z)\) isomerism was found to be the most energetically favourable.\(^{119}\)

Distinctive nitrogen-bonded protons (referred to as \(NH\)) for both mono(substituted) and bis(substituted) ligand precursors appear between 8.8 and 12.9 ppm. The \((E,Z)\) \(NH\) resonances can be assigned accurately for the bis(substituted) ligand precursor, due to the characteristic resonances corresponding to the mono(substituted) proligand, which has been determined crystallographically to represent the \(Z\) geometry.

![Figure 2.2. \(^1H\) NMR (300 MHz, \(d_6\)-DMSO) spectroscopy of the aromatic and amino \(NH\) shifts of the mono-ethyl substituted and bis-ethyl substituted ligand precursors in the 7.5 to 13 ppm region.](image)

In the case of compounds \(\text{iia, iib and iid}\) bis(substituted) proligand resonances analogous to the mono(substituted) ligand precursor for between 12.5 and 12.7 ppm are representative of the \(Z\) hydrazone proton, with shifts between 9.1 and 9.4 ppm corresponding to external \(Z\) proton. This indicates that shifts of the \(E\) nitrogen-bound hydrogen atoms are upfield of their \(Z\) counterparts, with resonances between 11.2 and
11.3 ppm (hydrazonal) and 8.8 and 9.1 ppm (external). Structural data and DFT calculations (*vide infra*) suggest that the Z hydrazone proton is involved in hydrogen bonding to the ketone or the imine of the respective mono(substituted) and bis(substituted) ligand precursors. This would likely result in deshielding and the observed downfield shift of Z hydrazone proton. When comparing the methyl and ethyl-substituted proligands (ia to ib or iia to iib) the NH chemical shifts were almost identical, as expected from the similar nature of these alkyl substituents. In comparison the id and iid allyl-substituted ligand precursor external NH shifts were slightly downfield, for example 9.32 ppm and 9.02 ppm for iid and 9.19 ppm and 8.80 ppm for iib. This signifies the reduced shielding due to the electron-withdrawing functionality. In comparison with other functionalities there was a significant downfield shift observed for the two NH resonances of ic (12.79 ppm and 10.93 ppm) and the four NH resonances of ic (12.95 ppm, 11.54 ppm, 10.74 ppm and 10.68 ppm). This can also be attributable to the electron-withdrawing nature of the phenyl substituent.

### 2.3 Structural investigations by X-ray crystallography and DFT modelling

Yellow crystals of bis(thiosemicarbazonato) ligand precursor of iid and of a sulphur-sulphur dimerised form of iib (iib S-S) suitable for X-ray crystallographic analysis were grown from DMSO solutions. The proligands was an (E,Z) isomer, whereby the literature reflects a scope of geometries for such compounds. For similar compounds such as bis(alklimino)acenaphthene (alkyl-BIAN) ligand precursors the (E,E) geometry would be expected, however Cowley *et al.* reported (E,Z) alkyl-BIAN proligand isomers. Authors suggested that the observed geometry was due to the substituents steric effects, imino-nitrogen lone pair repulsion and crystal packing. It would be reasonable to consider that the isomerism displayed by compound iid and the iib dimer are of similar origins. Structures were reported by Besenyei *et al.* also reported that aryl(BIAN) derivatives may be observed as either (E,E) (major isomer) or (E,Z) isomers depending on the solvent. Furthermore, Dilworth *et al.* reported a benzil bis(phenylthiosemicarbazone) ligand precursor and a benzil bis(methylthiosemicarbazone) proligand, which display an (E,E) and a (Z,Z) geometry respectively.
The results of X-ray diffraction experiments are presented in Figure 2.3, with selected bond lengths presented in Table 2.1. This allows comparisons to be made with the
mono(substituted) thiosemicarbazonato ligand \textit{ib}. Almost all corresponding bonds of \textit{ib}, \textit{iib} and \textit{iid} are similar therefore little discussion structural is possible, but allows for later comparisons with analogous metal complexes. The only exception is the carbon-sulphur bond of \textit{iib} involved in the sulphur bridge, which is a single bond and therefore significantly longer than the corresponding bonds of \textit{iid} or \textit{ib}. This may have been formed by tautomerisation, followed by deprotonation as illustrated in Figure 2.4. Both crystal structures display an \((E,Z)\) geometry, which is consistent with their NMR spectra.

Table 2.1. Selected bond lengths of the ligand precursor crystal structures and optimised structures of \textit{iic} and \textit{iic}' by B3LYP 6-31++ (d,p) IEFPCM, DMSO, in Angströms.

<table>
<thead>
<tr>
<th>Compound/Molecular Parameters</th>
<th>\textit{ib} \textsuperscript{123}</th>
<th>Sulphur-bridged \textit{iib}\textsubscript{S,S} dimer</th>
<th>\textit{iic} optimised DFT structure</th>
<th>\textit{iic}' optimised DFT structure</th>
<th>\textit{iid}</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-N</td>
<td>1.352(2)</td>
<td>1.391(7)</td>
<td>1.356</td>
<td>1.359</td>
<td>1.367(4)</td>
</tr>
<tr>
<td>C=N or DFT structure</td>
<td>1.392(6)</td>
<td>1.356</td>
<td>1.348</td>
<td>1.361(4)</td>
<td></td>
</tr>
<tr>
<td>C=O for \textit{ib} DFT structure</td>
<td>1.294(2)</td>
<td>1.280(7)</td>
<td>1.293</td>
<td>1.297</td>
<td>1.297(4)</td>
</tr>
<tr>
<td>C=S</td>
<td>1.224(2)</td>
<td>1.311(8)</td>
<td>1.293</td>
<td>1.303</td>
<td>1.306(4)</td>
</tr>
<tr>
<td>*C-S</td>
<td>1.6780(19)</td>
<td>1.683(6)</td>
<td>1.691</td>
<td>1.692</td>
<td>1.678(4)</td>
</tr>
<tr>
<td>*C-S</td>
<td>1.807(6)*</td>
<td>1.691</td>
<td>1.692</td>
<td>1.674(4)</td>
<td></td>
</tr>
</tbody>
</table>

\begin{figure} [h]
\centering
\includegraphics[width=\textwidth]{figure2_4.png}
\caption{Proposed dimerisation of proligand \textit{iib}.}
\end{figure}
It is notable that a dimerised structure of the ethyl-functionalised bis(substituted) ligand precursor has been formed via sulphur-sulphur bridging under aprotic conditions. The capacity for DMSO to form disulphide bonds of peptides is well-known. All previously reported thiosemicarbazone disulphide-bridged ligand precursors were formed by oxidation via a metal-based catalyst. It has been well documented that Cu(II) and Fe(III) oxidise organo-thiols to produce sulphur-sulphur bonds.\textsuperscript{124} Cu(II) and Mn(II) catalysed thiosemicarbazone sulphur-bridged proligand were reported by López-Torres\textit{ et al.} and Bermejo\textit{ et al.} respectively (Figure 2.5).\textsuperscript{125} The S-S bond lengths of these published ligand precursors were 2.0335(11) Å and 2.039(1) Å correspondingly, which is somewhat shorter than the disulphide bond of the iib dimer, 2.051(2) Å. This suggests that the iib dimer bond may be labile and the dimerisation reversible. Literature show that the resultant disulphide may be formed intentionally via metal-based catalysis or by aerial oxidation in DMSO, which in this case is the latter.\textsuperscript{126}
2.3.1 Density functional theory calculations

To provide insight into the geometry of the bis(imino) ligand-precursor \((E,Z)\) and \((E,E)\) isomers were optimised by DFT in the gas phase and in solution. Phenyl-functionalised compound optimisations were used as representatives of the bis(imino) ligand-precursors and were based on available crystal structures.

![Figure 2.6](image1.png) Optimised in solution of asymmetric \(\text{ii}c^*\) by B3LYP 6-31++ (d,p) IEFPCM, DMSO. Where grey = carbon, white = hydrogen, blue = nitrogen.

![Figure 2.7](image2.png) Optimised in solution structures of symmetric \(\text{ii}c\) by B3LYP 6-31++ (d,p) IEFPCM, DMSO. Where grey = carbon, white = hydrogen, blue = nitrogen.
Following the method used by Holland et al., the ligand precursors were initially optimised in the gas phase using B3LYP 6-31G(d,p), followed by B3LYP 6-31++ (d,p) IEFPCM, DMSO to obtain an optimised structure in solution (Figures 2.7 and 2.8). Bond lengths of the optimised structures were comparable to crystallographic data acquired for compounds iib and iic (Table 2.1). In the gas phase the calculated energy difference is 4.17 kJ mol\(^{-1}\), indicating that the asymmetric structure iic' is more stable than the symmetric structure iic. Likewise, when solvent effects were considered in DMSO, the asymmetric structure was more energetically favourable when compared to the symmetric by 2.04 kJ mol\(^{-1}\), confirming what was observed by NMR spectroscopy and X-ray crystallography. The (E,Z) structure iic' is likely stabilised by internal hydrogen-bonding, whilst the (E,E) structure iic may be more favourable for complexation as it offers more space for a metal. For example, the alkyl-BIAN ligand precursor reported by Cowley et al. as the (E,Z) isomer was metallated to form a zinc complex, which possessed the (E,E) geometry. Likewise, the 1,2-cyclohexanedione bis(4-methylthiosemicarbazone) ligand precursor reported in the above study possessed an (E,Z) isomeric structure, with its analogous copper complex possessing the (E,E) structure.

![Figure 2.8. DFT calculations on H\(_2\)ATSM by Holland et al., which show (E,Z) isomer, 0.0 kJ mol\(^{-1}\) (left) to be more energetically favourable than the (E,E) isomer, 16.6 kJ mol\(^{-1}\) (right). (Numbers to the left represent the relative energy of the calculated structure in kJ mol\(^{-1}\).)](image)

Furthermore, the study by Holland et al. demonstrated that the most stable conformer of H\(_2\) ATSM is the (E,Z) geometry. The energy difference between the two conformers of H\(_2\) ATSM examined was 16.6 kJ mol\(^{-1}\). The low energy difference between the (E,Z) and the (E,E) structures is an encouraging result indicating that metal chelation may be possible.
2.4 Fluorescence Spectroscopy

Light emitted from a fluorophore is detectable via fluorescence spectroscopy and microscopy. These spectroscopic and imaging techniques allow the monitoring of a fluorescent compound in solution and in biological cells. The naphthyl backbone of ligand precursors above provides electron-rich π-bonds and facilitates fluorescence emission.\(^{118}\) This bypasses the necessity of conjugating a commercially available fluorophore, such as fluorescein or BODIPY which are expensive and may significantly affect the biological properties of the compound under investigation. Appropriate biological assays and microscopy conditions can be chosen once the fluorescent properties of the ligand precursors are understood. Therefore fluorescence scans between 200-800 nm were obtained in 100 μM DMSO solutions, allowing maxima of excitation (\(\lambda_{\text{ex-max}}\)) and emission (\(\lambda_{\text{em-max}}\)), useful information for fluorescence microscopy. Furthermore ranges of absorption and emission were assessed using 2D contours, which are significant to the choice of cytotoxicity assays and fluorescence imaging settings. Since relevant cytotoxicity assays use absorbance readings at 570 nm, it is preferable that there should be minimal or no excitation at this wavelength. This was confirmed for each ligand precursor making them suitable for later in vitro studies. In general the fluorescence of the bis(substituted) ligand precursors was very weak, with the overall range of absorption for these compounds approximately between 260 nm and 550 nm and an emission range of 450 nm and 680 nm (Table 2.2, Figures 2.9 and 2.10).

<table>
<thead>
<tr>
<th>Compound</th>
<th>(\lambda_{\text{ex-max}} / \text{ nm})</th>
<th>Excitation range / nm</th>
<th>(\lambda_{\text{em-max}} / \text{ nm})</th>
<th>Emission range / nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>id</td>
<td>490</td>
<td>240-615</td>
<td>554</td>
<td>454-735</td>
</tr>
<tr>
<td>iia</td>
<td>470</td>
<td>260-610</td>
<td>618</td>
<td>432-800</td>
</tr>
<tr>
<td>iib</td>
<td>490</td>
<td>380-530</td>
<td>550.5</td>
<td>496-663</td>
</tr>
<tr>
<td>iic</td>
<td>490</td>
<td>270-560</td>
<td>545.5</td>
<td>476.5-715</td>
</tr>
<tr>
<td>iid</td>
<td>480</td>
<td>260-560</td>
<td>547</td>
<td>499.5-695</td>
</tr>
</tbody>
</table>

The wavelengths resulting in maximum excitation (\(\lambda_{\text{ex-max}}\)) were found between 470 to 490 nm, indicating that compounds would be appropriate for excitation via a standard 488 nm confocal microscopy laser. With \(\lambda_{\text{em-max}}\) lying between 545 and 555 nm the
emission can be expected in a standard green channel of a confocal microscope. The notable exception of methyl substituted ligand iiia with the $\lambda_{\text{max}}$ of emission at 618 nm and a significantly broader emission range of 432 nm to 800 nm is likely to be observable in both green and red channels of a standard confocal microscope. The mono(substituted) ligand precursor was only weakly fluorescent compared to the corresponding bis(substituted) compound. As a representative example the allyl-substituted mono(substituted) ligand precursor showed broader excitation and emission (240 nm to 615 nm and 454 nm to 735 nm respectively). While $\lambda_{\text{ex-max}}$ and $\lambda_{\text{em-max}}$ were 490 nm and 554 nm respectively for id, were comparable to the maxima of iid (Table 2.2).

Considering that the fluorescence of their analogous complexes is likely to be significantly greater, due to metal to ligand charge transfer (MLCT) and ligand to metal charge transfer (LMCT) this is a positive result. Encouraged by these preliminary results a study of the ligand precursors was conducted in vitro, in order to allow comparisons with their corresponding metal complexes in later chapters.

![Excitation/emission map of compound iiic](image)

*Figure 2.9. Excitation/emission map of compound iiic.*
Figure 2.10. Excitation/emission map of compound iid
2.5 Laser scanning confocal microscopy

Experiments were carried out to ascertain if the weak fluorescence of the ligand precursors was sufficient to be observed in vitro. Cells were cultured using standard protocols, analogous to earlier investigations on fluorescent thiosemicarbazones in the group.\textsuperscript{127, 128}

![Image](image1.png)

**Figure 2.11.** Single-photon confocal microscopy images of ia in HeLa cells, incubated with lysotracker 100 μM, 1% DMSO, 20 minutes, at 37°C. a) Differential Interference Contrast (DIC) image. b) Micrograph of cells after excitation at 488 nm, green channel, showing localisation of the compound ia. c) Micrograph of cells after excitation at 543 nm, red channel, showing localisation of the lysotracker. d) Overlay of a), b) and c). Scalebar: 10 μm.
Cell viability prior to experiments and over their course was monitored by optical microscopy, and validated at the start of the experiments by standard trypan blue tests. The compounds were imaged in HeLa (cervical carcinoma), PC-3 (prostate carcinoma), MCF-7 (breast cancer) and FEK-4 (non-cancerous fibroblast) cell lines using standard confocal fluorescence microscopy with one photon excitation at 488 nm.

**Figure 2.12.** (a – b) Single-photon confocal microscopy of compound iid in FEK-4 at 50 μM, 0.5 %. DMSO incubated for 20 minutes, washed then iid was incubated at 100 μM, 1% DMSO and incubated for 20 minutes, (c – d) iid at 100 μM, 1% DMSO, after 20 minutes in HeLa. (a, c) DIC image. (b, c) Fluorescence images at 488 nm excitation. Scalebar 20 μm.
The imaging studies were performed using concentrations of 100 µM in a DMSO: RPMI (Royal Park Memorial Institute) cell medium 1:99 solvent mix, whereby the final DMSO concentration on the imaging plate was lower than 1%. The fluorescence of the compounds was very weak, therefore such high concentrations of the ligand precursors were necessary to assess uptake within cells. Compound \textit{ia}, the methyl substituted mono(substituted) thiosemicarbazone ligand was incubated in HeLa cells and was found to possess a good colocalisation with lysotracker (\textit{Figure 2.11}), suggesting that this compound is likely to enter the lysosome.

\textit{Figure 2.13}. Single-photon confocal microscopy of \textit{ic} at 100 µM in PC-3, 1% DMSO, incubated for 20 minutes. a) Micrograph of DIC. b) Red channel, excitation 405 nm. c) Red channel, excitation 488 nm. d) Red channel, excitation 543 nm.
Further investigations with colocalisation dyes demonstrated that the ligand precursor did not localise in the mitochondria or nucleus (Appendix Figures G.2-3). Experiments showed that bis(substituted) ligand precursor **iii** possessed weak uptake in HeLa cells, which was barely detectable when incubated in FEK-4 cells, under the same conditions (Figure 2.12). The latter result suggests that there could be a preference for the compound to enter cancerous cell lines over non-cancerous cells (Figure 2.12 and Appendix Figures G.4-8). Optimal imaging conditions were found to be at 405 nm rather than at 488 nm excitation and this emission was observed maximally in the green channel for methyl, ethyl and allyl substituents (Appendix G). A blue shift in absorbance can be expected in a more polar solvent such as water. A higher level of solvation of a lone pair lowers the n-orbital energy, resulting in a larger energy \( n \rightarrow \pi^* \) transition and thus absorption at a lower wavelength. Absorption of the phenyl bis(substituted) ligand precursor was most favourable when using the 405 nm laser, however, the emission was maximised in the red channel (Figure 2.13). A comparison between the fluorescence observed in the 2D spectra in DMSO solution and within cells shows a blue shift in the \( \lambda_{\text{ex-max}} \) from ca. 480 nm to ca. 405 nm, with a redshift in emission. This redshift can be understood as a solvent effect, whereby a more polar solvent reduces the energy difference between the excited and ground states. The process, known as solvent relaxation occurs when solvent molecules stabilise and decrease the excited state energy level, therefore resulting in a higher wavelength emission.

Rather than being a hindrance, the weak fluorescence of the ligand precursors within cells is a significant advantage in being able to assess the stability of their respective metal complexes *in vitro* as will be demonstrated in subsequent chapters. Moreover, stability in cells was subsequently investigated using fluorescence lifetime imaging microscopy (FLIM), as a quantitative means of stability assessment. In addition to a qualitative evaluation using standard fluorescence microscopy this may provide another powerful means of evaluating complex stability *in vitro*.
2.6 Two-photon excitation and fluorescence lifetime imaging microscopy

Two-photon absorption occurs when the combined energy of two simultaneous photons results in molecular excitation. The first photon to interact generates what is known as a virtual state, which has no classical analogue and only exists transiently (generally for femtoseconds) in which time a photon can travel ca. 1 μm. Arrival of the second photon must occur before the virtual state decays (dephases), which will occur if the laser intensity is high and focussed. The probability of single photon absorption is proportional to the intensity of light and is in contrast to two-photon absorption, which is dependent on both spatial and temporal coincidence (Equations 2.1 and 2.2). Therefore, where $NA_x$ is the number of photons absorbed per second, $I$ is the intensity, $\sigma_x$ is the cross-section and $x$ is 1 or 2 photon absorption.

Single-photon absorption: \[ NA_1 = \sigma_1 I \] (Equation 2.1)
Two-photon absorption: \[ NA_2 = \sigma_2 F \] (Equation 2.2)

An advantage of two-photon excitation therefore, is that imaging occurs only from the focal plane. This is contrasting with single-photon excitation microscopy, in which the image is often distorted as it may occur outside the focal plane. Furthermore, despite the requirement of high-powered lasers, two-photon microscopy has been found to cause less damage to biological cells than single-photon microscopy, as well as to decrease photo-bleaching and augment imaging depth.

Two-photon absorption has been used in the development and application of two-photon fluorescence confocal microscopy since 1990 and provides significantly greater control from a clinical perspective, with ongoing research seeking to enable an in-depth understanding of biological processes. In turn this allows for selection of individual cells and as a consequence, the capacity to improve treatments of sensitive tissues such as the retina.

Recent advancements have resulted in the design of two-photon fluorescence lifetime imaging (FLIM), which combined with highly sensitive single-photon counting lifetime measurements poses an attractive method for investigating small molecule fluorescence. Fluorescence lifetime is often considered as a means of distinguishing between different
fluorophores, which possess sufficiently different lifetimes and can also be achieved using spectrally resolved microscopy.

FLIM is frequently used to separate different portions of the same fluorophore, for purposes such as quenching of luminescence by intracellular ions (e.g. Ca\(^{2+}\) or Cl\(^{-}\)) or by oxygen. Furthermore, this technique can be used to monitor binding of and even distance from a fluorophore, with a conformational change is likely to result in a different rate of internal non-radiative decay and, therefore, a modification of the fluorescence lifetime.\(^{133, 134}\) pH effects can also be studied, since protonated and non-protonated forms of a molecule may possess different lifetimes. In addition investigations of aggregation, viscosity, proximity to metal surfaces and nanoparticles (due to their long luminescent lifetimes) are carried out.\(^{133}\) There has, however, been little use of FLIM for the purpose of assessing the stability of sodium\(^{135}\) and magnesium\(^{136}\) complexes \textit{in vitro}, but thus far no reports of use of the stability evaluations technique with transition metal complexes.

Consequently, if the fluorescence lifetime of the complex is significantly different to that of the ligand precursor, FLIM could be utilised to assess the complex stability \textit{in vitro}. Two-photon excitation experiments were performed at the Rutherford Appleton Laboratory following the methodology described in Botchway \textit{et al.} 2008\(^{137}\) and in our recent publication (Pascu \textit{et al.} 2011\(^{138}\)) with assistance of Dr S Botchway, Dr P Burgos, Mr A. Mckenzie and Mr A. Henman.

\textbf{Figure 2.14.} Control experiment at 810 nm, in HeLa cells where \textbf{a)} is the colour image corresponding to the two-photon fluorescence lifetime map of the $\tau_m$ (the weighted average fluorescence lifetime), \textbf{b)} is the distribution curve of the $\tau_m$ where the colour represents the lifetime of \textbf{a}).
Figure 2.15. Control experiment at 910 nm, two-photon fluorescence lifetime map ($\tau_m$) in HeLa cells showing weak autofluorescence where a) is the colour image corresponding to the two-photon fluorescence lifetime map of the $\tau_m$, b) is the distribution curve of the $\tau_m$ where the colour represents the lifetime of a).

In order to be able to quantify compound fluorescence the background autofluorescence of cells had first to be assessed. The 810 nm control possessed the fluorescence lifetimes of 0.812 ns ($\tau_1$, 79 %, 0.214 ns FWHM) and 3.140 ns ($\tau_2$, 21 %, 0.647 ns FWHM). Figure 2.11b and 2.12b represent the mean lifetime of both components in HeLa cells when modelled to two components. Autofluorescence when excited at 910 nm, however, was negligible therefore all compounds displaying significant fluorescence at 910 nm were tested at the latter wavelength (Figure 2.14). It is in agreement with literature that autofluorescence is less at 900 nm than 800 nm. The background fluorescence can be attributable to biological fluorophores; at 910 nm these include flavin adenine dinucleotide (FAD) and lipoamide dehydrogenase (LipDH), which also absorb at 810 nm. Biological molecules such as reduced nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH) absorb 810 nm light, but not 910 nm, therefore contributing to the difference in autofluorescence intensity observed at the respective wavelengths.

The full width at half maximum (FWHM), calculated from the lifetime distribution curve within the focal area was used to assess the error. The percentage of components $\tau_1$ (major) and $\tau_2$ (minor) in cells was from the respective amplitudes $a_1$ and $a_2$. 
calculated using SPCIImage software, which models the data for each individual pixel to Equation 2.3, where \( F \) is fluorescence, \( a_0 \) is background and \( t \) is time:

\[
F(t) = a_0 + a_1e^{-t/\tau_1} + a_2e^{-t/\tau_2}
\]  
(Equation 4.3)

2.6.1 Ligand precursor fluorescence lifetime investigation

For each bis(substituted) ligand precursor tested by Time-Correlated Single Photon Counting (TCSPC) two fluorescence lifetime components could be determined in DMSO at 10 mM, with the \( \tau_1 \) accounting for at least 94% in each case. The \( \tau_1 \) values were very similar for compounds iia, iib and iic when excited at 810 nm (0.226 ns with \( \chi^2 \) of 1.88, 0.183 ns with a \( \chi^2 \) of 1.63 and 0.158 ns with a \( \chi^2 \) of 1.71 respectively). The excitation wavelength did not appear to have a significant effect on the fluorescence lifetime. For example when iic was excited at 910 the major component was 0.156 ns. Similarly the \( \tau_1 \) of iid was comparably shorter (0.020 ns at 810 nm and 0.021 ns at 910 nm), indicating no observable difference between the lifetimes when exciting at the respective wavelengths in DMSO solution.

The minor component (\( \tau_2 \)) of the fluorescence lifetime of the ligand precursors in DMSO at 10 mM did not follow the same pattern. While compounds iia and iic had a \( \tau_2 \) of ca. 2.5 ns as the minor component for iib was 3.969 ns. By contrast the minor component for iid was one order of magnitude longer, with \( \tau_2 \) values of 0.274 ns and 0.468 ns for excitation with 810 nm and 910 nm respectively. These numbers, however, are to be taken with caution as the errors are much larger than for \( \tau_1 \) due to the weakness of this second component (< 5%). Excitation at 810 nm produced considerably higher quality data than at 910 nm for the ligand precursors in cells (Figures 2.16-2.17), which is why in vitro studies were also conducted at 810 nm excitation wavelength.
Figure 2.16. Two-photon mean fluorescence lifetime distribution curve ($\tau_m$) in HeLa cells of iia, iib, iic and iid at 100 µM at a) 810 nm and b) 910 nm.

The fluorescence lifetime of the compounds were obtained from the FLIM distribution curve in cancer cells (HeLa, MCF-7 and PC-3 cells). Standard conditions used were 1% DMSO 100 µM, with an incubation time of 20 minutes. The majority of cellular studies could be modelled to two fluorescence lifetime components, one of which was short (ca. 0.2 ns) with the other being long (ca. 2.5 ns). The solution data were within the FWHM, which represents the error for in vitro data and thus confirms their comparability.

There were some exceptions however, such as the long components of iib in PC-3 cells (0.434 ns, 0.209 ns FWHM) and of iid in HeLa and PC-3 cells at 910 nm (0.436 ns, 0.653 ns FWHM and 0.418 ns, 0.455 ns FWHM, respectively). Other notable exceptions were the major components observed for iia and iib when excited at 810 nm, with lifetimes of 0.903 ns and 0.868 ns, correspondingly. These are within the FWHM of the control, which suggests that most of the fluorescence observed at this wavelength for these two compounds is as a result of cellular autofluorescence. This could indicate the weakness of the fluorescence of the proligand or simply that the conformation of the compound within the cell and in turn the fluorescence lifetime has been significantly altered as opposed to in DMSO.
The minor components nevertheless, could be correlated to the ligand precursor, confirming the weak fluorescence of the compound in cells. The fluorescent lifetime measurements of the mono(substituted) ligands were similar to their bis(substituted) analogues, possessing in general a short, major component of the order of 0.4 ns and a long, minor component in the region of 3 ns. Unlike the bis-thiosemicarbazonato ligands, this was consistently observed in cells when excited at 910 nm. Different molecular orientations would result in a change in fluorescent lifetime, which could suggest that the mono(substituted) proligand geometry was less variable than its bis(substituted) counterpart. Interestingly, although consistency of data was observed in cells, the respective short, major component is slightly shorter (ca. 0.2 ns), which suggests that the complexity of the cellular environment does have impact on the lifetime. There appeared to be a more limited effect on the mono(substituted) than the bis(substituted) ligand precursor, which displayed greater variation of lifetime within cells. Notably, the fluorescence of the mono(substituted) ligand precursors was very weak and comparable to the bis(substituted) ligands.

Figure 2.17. Two-photon fluorescence lifetime map ($\tau_{\text{m}}$) of **a** in PC-3 cells with excitation at 910 nm and **b** in HeLa cells with excitation at 810 nm.
2.7 Summary to Chapter 2

Mono(thiosemicarbazonato) and bis(thiosemicarbazonato) ligand precursors were successfully synthesised and characterised by ESI-MS and NMR spectroscopy. Furthermore their fluorescence properties were analysed, showing weak, but detectable emission in solution and in cancerous and non-cancerous cells at 100 μM concentration. Fluorescence of respective complexes of these proligands would be expected to be higher due to MLCT and LMCT, therefore strong fluorescence at this concentration or lower would provide a qualitative indication of complex stability. Furthermore, the fluorescence lifetime components of these compounds were determined in solution and in cells with characteristic data that proved distinguishable from cellular autofluorescence. This means that if a complex decomposes in the cell by deconvoluting the various fluorescence lifetime components, provided that these are sufficiently dissimilar to allow discrimination between them.
Chapter 3. Synthesis, characterisation, spectroscopic, *in vitro* and *in vivo* analysis of zinc and copper bis(thiosemicarbazonato) complexes

3.1 Overview

In this Chapter, the transmetallation reactions to generate new copper complexes are described. Zinc bis(thiosemicarbazonato) complexes precursors provide a clean means of synthesising other metal complexes (such as copper) *via* transmetallation. The fluorescent analogue of the known tracer Cu[ATSM] is its zinc analogue Zn[ATSM]. Therefore a means of comparison can be provided by synthesising zinc bis(thiosemicarbazonato) complexes containing a acenaphthenequinone based backbone, which were first synthesised in the group.\(^{123, 127, 128}\) Subsequently the aims were to synthesise zinc and copper bis(thiosemicarbazonato) complexes, acquire and discuss new structural data and distinguish, for the first time, their stability in biological cells.

3.2 Zinc complex synthesis

![Figure 3.1. Synthesis of acenaphthenequinone zinc bis(thiosemicarbazones)](image)

Earlier studies in the group, showed that acenaphthenequinone zinc bis(thiosemicarbazone) complexes can be made in a one-pot synthesis by reflux in acetic acid, followed by filtration and washing of the product with diethyl ether. Terminal functionalities in this study included methyl, ethyl, phenyl and allyl groups (*Figure 3.1*).\(^{123, 127, 128}\) The complexes were obtained as orange or red solids in moderate to good yields of (49% to 68%). The compounds (1a, 1b, novel complex 1c and 1d) were characterised by \(^1\)H and \(^{13}\)C NMR spectroscopy and ES-MS, giving peaks at m/z 419.01, 447.04, 543.04 and 471.03 respectively, which represent [M + H]\(^+\).
3.3. Copper complex synthesis

The zinc compounds (1a-d) act as precursors to the analogous copper complexes in transmetallation reactions. One equivalent of the respective zinc complex and two equivalents of copper acetate were stirred in methanol at room temperature for 24 hours and the product isolated as a brown solid by filtration, after washing with water and methanol the yields were good (53% to 77%) for 2a, 2b, 2c and 2d (Figure 2).\textsuperscript{123,127}

![UV trace of transmetallation from 1c to 2c](image1)

![Radio trace of transmetallation from 1c to 2c](image2)

**Figure 3.3.** HPLC of transmetallation from 1c to 2c showing a) the UV trace (blue) and b) the radio trace (pink).

LC-MS was used to characterise these compounds since NMR spectroscopy was not informative due to the paramagnetic nature of copper, giving m/z values of 418.01, 446.04, 542.32 and 470.03, which represent [M + H]\. Complexes 2a and 2b were first reported in Reference 123 for which the writer of this thesis is a co-author. Furthermore, EPR spectroscopy of novel complex 2c gave comparable spectra to 2a, 2b and 2d and confirms the presence of a paramagnetic species (Appendix I). Moreover it was possible to cleanly and rapidly radiolabel complex 1c with $^{64}$Cu using $^{64}$Cu(OAc)$_2$ at Oxford Siemens Molecular Imaging Laboratory under the supervision of Dr Phillip A. Waghorn and Prof Jon Dilworth.
The partition coefficient \( \log P \), which is an indicator of lipophilicity, were calculated with \( 2a, 2b \) and \( 2c \) possessing values of \( 1.209 \pm 0.076, 1.188 \pm 0.043 \) and \( 1.436 \pm 0.062 \) respectively. The \( ^{64}\text{Cu} \) compound was added to a mixture of equal volumes of water and octanol, followed by vortexing and centrifugation for 5 minutes. The activity of each phase was measured and the \( \log P \) was calculated as \( \log(\text{octanol [radioactivity]} \div \text{water [radioactivity]}) \), a value above 1 meaning that the compound is lipophilic.\(^{141}\) The value for Cu[ATSM] under the same conditions was found to be 1.48, which indicates that uptake of these copper complexes, in particular for \( 2c \) is likely to occur in the liver \textit{in vivo}, as does Cu[ATSM].\(^{142}\)

Cyclic voltammetry of complexes \( 2a \) and \( 2b \) was carried out by Dr Phillip A. Waghorn. The compounds were found to possess reduction potentials of -0.517 V and -0.536 V, respectively.\(^{123}\) Since these are close to the reduction potential of Cu[ATSM] (-0.581 V) under comparable conditions it appears that the additional acenaphthene ligand backbone has not significantly affected the reduction potentials. Surprisingly, despite displaying a \( \log P \) and reduction potential equivalent to that of Cu[ATSM] a preliminary hypoxia study by Dr Waghorn showed that complex \( 2b \) did not possess hypoxia selectivity within one hour. It can be speculated that longer incubation times may be required to observe selectivity.

![Synthesis of acenaphthenequinone copper bis(thiosemicarbazones) via the free ligand](image)

\( \text{Figure 3.4. Synthesis of acenaphthenequinone copper bis(thiosemicarbazones) via the free ligand} \)

Another method of synthesising the copper complexes was attempted \textit{via} the free ligand by stirring in methanol for 24 h with 3 equivalents of copper acetate (\textit{Figure 3.4}). Despite demonstrating some success, this synthesis method did not prove reliable as significant batch-to-batch variations in the product were found, frequently producing a black powder, which did not possess the desired spectroscopic properties, as opposed to a brown solid expected for compounds in this family. This could be explained by the
dimerisation observed by X-ray crystallography (Chapter 2.3), which could cause formation of a copper-based polymeric compound which proved to be intractable. Therefore the transmetallation method was chosen in preference to the free ligand process.

3.4 X-ray crystallography

Crystals suitable for X-ray diffraction were obtained from DMSO or THF:pentane solvent mixtures. The crystals were grown slowly by solvent diffusion techniques at room temperature. Data will be compared to the previous crystallographic results reported in references 123, 127 to 128 and work carried out during this project. The bond lengths and angles below of the newly acquired phenyl-substituted crystal structures are discussed below. The structures here will be discussed in terms of how their structure is affected by functionality, whereby the effect of the metal centre will be discussed in Chapter 5.

It is notable that all of the copper complex crystal structures were determined to be an (E,E) the symmetrically bound isomer. Whereas zinc complexes 1a, 1b and 1d were found to possess the asymmetric structure. In previous studies in the group, the Zn(II) complexes only yielded suitable crystals for X-ray determinations when they were co-crystallised with DABCO and possessed (E,Z) isomerism. Here, it was possible to obtain X-ray crystallographic data for the phenyl-substituted zinc complex without co-crystallisation. This structure contained a DMSO adduct has been isolated showing that ligand may also be bound as the an (E,E) isomer in these Zn(II) complexes (Figures 3.5-3.6).
Figure 3.5. ORTEP representation of 1c, ellipsoids drawn at 30% probability, hydrogens omitted for clarity.

Figure 3.6. ORTEP representation of 2c, ellipsoids drawn at 30% probability, hydrogens omitted for clarity.

For the zinc and the copper complexes there was little variation in the M-N bond lengths, however when considering the longest of the two Zn-N bonds it is possible to note that phenyl-substituted and the ethyl-substituted zinc complex bond was longer than those of the methyl and allyl-substituted bond. Furthermore, the functionality of the copper complex did not appear to greatly affect the bond lengths, whereby the N-N bonds were similar as were the C=N bonds, with little differences observed in Cu-S bonds where 2b and 2d possessed comparable data, which were longer than for the similar Cu-S bond lengths of 2a and 2c. A greater degree of variation was observed when comparing zinc complexes of different functionality (Tables 3.1-3.2). The length of the Zn-S bond pattern was the reverse when compared to those of Zn-N, with 1d the longest, followed by 1a and with 1c possessing the shortest (Table 3.1). Meanwhile there was little variation in C=N bond lengths according to functionality and N-N bond lengths with respect to the substituent group.
Notably the C-C bonds in both the zinc and copper complexes (here for 1c and 2c this bond is C8-C19, (Figures 3.5-3.6) were shortest for the methyl-functionalised compounds. The length of this bond was similar for the other substituents in the case of the copper complexes, with the methyl substituted zinc complex possessing a shorter C-C bond than 1b and 1d. It had previously been noted that the asymmetric (E,Z) zinc structures possessed longer C-C bonds than their analogous (E,E) copper and nickel structures. Here there was no significant difference between the C-C bond of the zinc compound of various functionalities. Indeed a closer look at one (E,E) isomer (the phenyl-substituted zinc complex 1c) suggested that the dissimilarity observed was caused by the nature of the metal centres employed rather than that of the isomer. This hypothesis will be further explored in subsequent chapters.

The bond angles around the zinc atom showed small variations with different substituents. For example, the N-M-N angle of complex 1b, 75.5(3)° is smaller than the respective angles of the other zinc complexes, of which complex 1d possessed the largest N-M-N angle of 88.55(15)°. Conversely the corresponding angles of 1a and 1c are comparable, 85.87(17)° and 86.6(1)°. The inverse was observed for the S-M-S angle, whereby the largest angle was 118.49(13)°, of complex 1b and the smallest angle of 110.69(5)° was possessed by compound 1d. Interestingly complex 1b possessed greater symmetry in comparison to the other zinc complexes in that the angles around the zinc atom within the same molecule were similar (for example the S-M-N angles, 79.7(3)° and 79.6(2)°, S-M-A angles, 100.30(19)° and 100.4(2)° and N-M-A angles, 98.4(3)° and 98.0(3)°.) Allyl-substituted complex 1d possessed similar S-M-A angles (103.43(9°) and 103.16(10)°) and N-M-A angles (107.36(13)° and 107.40(15)°), yet different S-M-N angles (83.88(10)° and 60.55(12)°). Meanwhile with comparably less symmetry, complex 1a had equivalent N-M-A angles (105.16(14)° and 105.13(14)°), with disimilar S-M-N (83.47(12)° and 64.89(13)°) and S-M-A angles (102.94(10)° and 99.15(10)°). Complex 1c, which was an (E,E) isomer was interestingly found to possess the least symmetry whereby its respective S-M-N (83.25(8)° and 80.27(4)°), S-M-A (104.31(4)° and 102.01(4)°) and N-M-A (101.11(5)° and 93.77(5)°) angles within the same compound were different.
Table 3.1. Selected bond lengths (Å) and bond angles (°) for compounds 1a-1d, determined by X-ray diffraction, where A is the adduct atom.

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<th>1b. DABCO (E,Z) isomer</th>
<th>1c. DMSO (E,E) isomer</th>
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<td>64.89(13)</td>
<td>79.6(2)</td>
<td>80.27(4)</td>
<td>60.55(12)</td>
</tr>
<tr>
<td>N-M-N</td>
<td>85.87(17)</td>
<td>75.5(3)</td>
<td>86.6(1)</td>
<td>88.55(15)</td>
</tr>
<tr>
<td>S-M-A</td>
<td>102.94(10)</td>
<td>100.30(19)</td>
<td>104.31(4)</td>
<td>103.43(9)</td>
</tr>
<tr>
<td></td>
<td>99.15(10)</td>
<td>100.4(2)</td>
<td>102.01(4)</td>
<td>103.16(10)</td>
</tr>
<tr>
<td>N-M-A</td>
<td>105.16(14)</td>
<td>98.4(3)</td>
<td>101.11(5)</td>
<td>107.36(13)</td>
</tr>
<tr>
<td></td>
<td>105.13(14)</td>
<td>98.0(3)</td>
<td>93.77(5)</td>
<td>107.40(15)</td>
</tr>
</tbody>
</table>

The copper complexes, in comparison showed much greater symmetry with respect to the bond angles around the metal centre, for example the equivalent angles of S-M-N of 2c, which were 83.72(15)° and 83.64(16)° respectively. Little functionality-dependent variation was observed for example with similar angles for complexes 2b and 2c for S-M-S (111.12(4)° and 110.99(7)° respectively) and N-M-N (82.45(14)° and 81.62(2)° correspondingly). Interestingly, the S-M-S and N-M-N angles of the bis(acenaphthenequinone) copper complexes were significantly larger in comparison with Cu[ATSM], 105.25(5)° and 76.09(6)° respectively.
Table 3.2. Selected bond lengths (Å) and bond angles (°) for compounds 2a-2d, determined by X-ray diffraction

<table>
<thead>
<tr>
<th>Compound/Molecular Parameters</th>
<th>Cu[ATSM]$_{123}^{123}$</th>
<th>2a (E,E) isomer$_{123}^{123}$</th>
<th>2b (E,E) isomer$_{123}^{123}$</th>
<th>2c (E,E) isomer</th>
<th>2d (E,E) isomer$_{127}^{127}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-N</td>
<td>1.9590(14)</td>
<td>1.983(4)</td>
<td>1.987(3)</td>
<td>1.967(5)</td>
<td>1.9855(17)</td>
</tr>
<tr>
<td></td>
<td>1.9616(13)</td>
<td>1.977(4)</td>
<td>1.980(3)</td>
<td>1.976(5)</td>
<td>1.9779(17)</td>
</tr>
<tr>
<td>M-S</td>
<td>2.2462(4)</td>
<td>2.2539(14)</td>
<td>2.2575(11)</td>
<td>2.2508(17)</td>
<td>2.2620(6)</td>
</tr>
<tr>
<td></td>
<td>2.2367(4)</td>
<td>2.2562(13)</td>
<td>2.2693(11)</td>
<td>2.2598(18)</td>
<td>2.2699(6)</td>
</tr>
<tr>
<td>C-C</td>
<td>1.478(2)</td>
<td>1.470(6)</td>
<td>1.493(5)</td>
<td>1.481(8)</td>
<td>1.476(3)</td>
</tr>
<tr>
<td></td>
<td>1.3673(18)</td>
<td>1.364(5)</td>
<td>1.359(5)</td>
<td>1.382(6)</td>
<td>1.358(2)</td>
</tr>
<tr>
<td></td>
<td>1.3697(15)</td>
<td>1.358(5)</td>
<td>1.360(5)</td>
<td>1.384(6)</td>
<td>1.360(2)</td>
</tr>
<tr>
<td>N-N</td>
<td>1.299(2)</td>
<td>1.309(6)</td>
<td>1.289(5)</td>
<td>1.286(7)</td>
<td>1.291(3)</td>
</tr>
<tr>
<td></td>
<td>1.2961(2)</td>
<td>1.321(6)</td>
<td>1.294(5)</td>
<td>1.293(7)</td>
<td>1.297(2)</td>
</tr>
<tr>
<td>S-M-S</td>
<td>109.234(17)</td>
<td>110.11(5)</td>
<td>111.12(4)</td>
<td>110.99(7)</td>
<td>111.52(2)</td>
</tr>
<tr>
<td>S-M-N</td>
<td>85.20(4)</td>
<td>83.69(12)</td>
<td>82.92(10)</td>
<td>83.72(15)</td>
<td>83.01(5)</td>
</tr>
<tr>
<td></td>
<td>85.08(4)</td>
<td>83.45(12)</td>
<td>83.51(10)</td>
<td>83.64(16)</td>
<td>83.30(5)</td>
</tr>
<tr>
<td>N-M-N</td>
<td>80.62(5)</td>
<td>82.95(16)</td>
<td>82.45(14)</td>
<td>81.6(2)</td>
<td>82.28(7)</td>
</tr>
</tbody>
</table>
3.5 Density functional theory calculations

The crystal structure of the above complexes were first optimised by B3LYP 6-31G(d,p) and then by B3LYP 6-31++ (d,p) IEFPCM, DMSO, following the method of Holland et al.\textsuperscript{119} Whereby an unrestricted spin was used for the optimisation of the copper(II) complex due to its paramagnetic nature (Figure 3.7).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.7.png}
\caption{Optimised in solution structures by B3LYP 6-31++ (d,p) IEFPCM, DMSO, where a) is symmetric 1c and b) is asymmetric 1c', c) is symmetric 2c and d) is asymmetric 2c'. Where grey = carbon, white = hydrogen, blue = nitrogen, red = oxygen, green = zinc, orange = copper.}
\end{figure}

In the gas phase the asymmetric isomers of the zinc and the copper complexes were more energetically favourable with energy differences in energy of 6.18 kJ mol\textsuperscript{-1} for 1c and 4.68 kJ mol\textsuperscript{-1} respectively for 2c. In solution however, the (E,E) symmetric form was more stable for each complex with a difference of -0.96 kJ mol\textsuperscript{-1} and -4.37 kJ mol\textsuperscript{-1}, for 1c and 2c correspondingly. This is in agreement with similar structures as discussed
in Chapter 2.3 that a ligand structure possessing \((E,Z)\) geometry would re-orientate to the \((E,E)\) conformation to allow more space for metal chelation.

![Diagram](image1)

**Figure 3.8.** Calculated electronic distribution for the molecular orbitals of complex 2c involved in the 13th excitation state, in which the most significant absorption occurs, where a) is 137\(\alpha\) (HOMO -2), b) is 139\(\alpha\) (HOMO), c) is 140\(\alpha\) (LUMO), d) is 137\(\beta\) (HOMO-1), e) 138\(\beta\) (HOMO) and f) is 140\(\beta\) (LUMO +1).

It is notable that the copper complex symmetric structure was more energetically favourable when compared to its corresponding asymmetric structure, in agreement with the observation that all the crystal structures of the copper acenaphthenequinone bis(thiosemicarbazonato) complexes determined by in the group have been determined as the symmetric isomer. The difference in the energy between the symmetric and the asymmetric isomers is relatively low and since the asymmetric has slightly lower energy in the gas phase it can be speculated that the asymmetric form may exist in
solution in small proportions. Subsequently, TD-DFT was calculated for the first 24 excited states using uB3LYP 6-31++ (d,p) methodology, of which excited state 13 had the most significant oscillator strength, \( f \) (Figure 3.8, Appendix I). Since the molecular orbitals involved the ligand (including the phenyl substituent) and the metal it is very likely that both contribute to the fluorescent properties. This could explain the observed similarity between the fluorescent lifetimes of complexes in this family of compounds as well as the small metal dependency.

3.6 Fluorescence spectroscopy

Fluorescence spectra were recorded in DMSO for initial scans between 200-800 nm, in order to obtain information about intrinsic fluorescence and \( \lambda_{em-max} \) using solutions of 100 µM. The ranges of absorption and emission were selected to be of relevance with regards to future cytotoxicity assays and fluorescence imaging, were assessed using the 2D contours as a guideline (Figures 3.9-3.10). Relevant cytotoxicity assays use absorbance readings at 570 nm, therefore there should be minimal or no excitation at this wavelength, which was true for zinc and copper complexes analysed in this manner, with very low absorbance for each.

![Excitation/emission map of compound 1c](image)

**Figure 3.9.** Excitation/emission map of compound 1c.
Figure 3.10. Excitation/emission map of compound 2c.

Initial spectroscopic data for 1a, 1b and 1d and 2a, 2b and 2d have been already studied by Pascu et al. [123, 127, 128]. The spectroscopy of compounds 1c and 2c is described below. The absorption range was similar when comparing the zinc and copper complexes, however the $\lambda_{\text{max}}$ of absorption was significantly red shifted in the case of the copper complex, whilst the emission maxima was slightly lower for 2c, indicating a reduction in the Stokes shift. Interestingly, the range of emission was larger for 2c with respect to 1c. As was expected, 2c possessed significantly reduced fluorescence when compared to 1c, which can be explained by paramagnetic quenching.

Table 3.3. Fluorescence measurements of complexes 1c and 2c.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$\lambda_{\text{em-max}}$ / nm</th>
<th>Excitation range / nm</th>
<th>$\lambda_{\text{ex-max}}$ / nm</th>
<th>Emission range / nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1c</td>
<td>440</td>
<td>250-590</td>
<td>581.5</td>
<td>535-723.5</td>
</tr>
<tr>
<td>2c</td>
<td>490</td>
<td>250-590</td>
<td>576.5</td>
<td>514.5-737</td>
</tr>
</tbody>
</table>
3.7 Kinetic stability testing

*In vitro* assays to determine the stability of complexes for use as medical imaging probes is very desirable. Demetallation could result in non-target tissue uptake and poor image contrast *in vivo*. Stability is therefore essential for the intended function of these complexes and a series of assays with excess of numerous chemicals found in biological systems have been carried out up to 24 hours. These include Glutathione (GSH), L-cysteine, L-histidine, L-methionine, citric acid, ethylenediaminetetraacetic acid (EDTA), biomimetic eagle’s modified essential media (EMEM) (both with and without foetal calf serum) and biologically relevant pH buffers. Indeed, stability of these complexes in a range of pHs is significant due to the tendency of cancer cells to be slightly more acidic than non-cancerous cells. UV-visible spectroscopy was utilised therefore to assess the kinetic stability of complexes and fluorescence to provide further insight whilst in the presence of biological media.

**Table 3.4.** Summary of estimated complex remaining from UV-visible data at 1:1 DMSO:assay agent at 15 minutes and 24 h where Citric Acid, EDTA, L-Cys, L-His, L-Met and GSH were in MilliQ water.

<table>
<thead>
<tr>
<th>Assay</th>
<th>15 min*</th>
<th>24 h*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DMSO</strong></td>
<td>89.1</td>
<td>88.3</td>
</tr>
<tr>
<td><strong>H₂O</strong></td>
<td>72.2</td>
<td>63.1</td>
</tr>
<tr>
<td><strong>PBS</strong></td>
<td>80.3</td>
<td>65.3</td>
</tr>
<tr>
<td>5% FCS, MEM</td>
<td>92.1</td>
<td>92.4</td>
</tr>
<tr>
<td>SFM, MEM</td>
<td>67.9</td>
<td>47.3</td>
</tr>
<tr>
<td>Citric Acid</td>
<td>83.2</td>
<td>79.0</td>
</tr>
<tr>
<td>EDTA</td>
<td>69.5</td>
<td>55.9</td>
</tr>
<tr>
<td>L-Cys</td>
<td>82.4</td>
<td>81.9</td>
</tr>
<tr>
<td>L-His</td>
<td>69.2</td>
<td>68.4</td>
</tr>
<tr>
<td>L-Met</td>
<td>61.1</td>
<td>32.7</td>
</tr>
<tr>
<td>GSH</td>
<td>91.0</td>
<td>59.1</td>
</tr>
</tbody>
</table>

Tests were carried out at room temperature at 100 μM in a 1:1 DMSO: assay solution to ensure sufficient solubility and at a concentration providing detectable fluorescence. Stability of the allyl-derivatised copper complexes in solution was previously observed in solution but only some very preliminary studies were undertaken prior to this
work.\textsuperscript{123,127} This investigation utilised both UV-visible absorption (at 10 µM and at 100 µM) and fluorescence emission at 100 µM, with 1:1 DMSO:challenger to limit precipitation.

This allowed a % stability to be estimated \textit{via} UV-Visible spectroscopy at 15 minutes and 24 hours as calculated by (100 % - percentage converted to free ligand) (\textit{Table 3.4}). The first assays carried out in DMSO, water, PBS, 5% FCS EMEM and SFM EMEM enabled complex stability to be understood in media likely to be encountered in a cell biology experiment.

\textbf{Figure 3.11.} Water assays (50% DMSO, 50% water) for compound 1d at 100 µM, by fluorescence spectroscopy (above) UV/Vis (below) monitored at time intervals up to 24h at room temperature.
The zinc complex 1d, demonstrated very good stability in each solvent with 88.3 %, 63.1 %, 65.3 % 92.4 % and 47.3 % estimated complex remaining after 24h in DMSO, water, PBS, 5% FCS EMEM and SFM EMEM respectively (Appendix Figures D.1-3). Whilst displaying encouraging results in DMSO, water and 5% FCS complex (88.2 %, 39.6 % and 79.1 % after 24 h respectively) (Figures 3.11 and Appendix Figures D.1-3). The stability of the complexes was tested against Eagle’s Minimum Essential Medium Serum Free Medium (SFM), which complexes are often incubated in for cell imaging.

As may be expected a decrease in fluorescence was observed with time, when complex 1d was assayed with DMSO, and SFM. Conversely, when assayed with FCS and PBS the complex showed an increase in fluorescence emission at ca. 450 nm and 460 nm (Appendix Figures D.1-9). This could suggest an interaction with the media perhaps via binding to a component of the solvent for example a protein within serum or phosphate respectively. Binding of an anion such as a phosphate, however would result in a small shift in absorbance, which was not observed in either case. Nevertheless, the fluorescence observed with each solvent (above) was significantly greater than that of the proligands, this combined with the presence of the characteristic UV-vis band of the zinc complex is highly indicative of complex stability.
3.7.1 Investigation testing stability using biologically relevant agents

Biologically relevant agents were chosen to provide a better insight into the stability of these complexes (*Figure 3.12*).\(^\text{145}\) For example, L-methionine and L-cysteine can act as S-donor models and L-Histidin as a N-donor model. Furthermore glutathione, a tripeptide containing glycine, cysteine and glutamate can function as both an S- and N-model. Moreover, cysteine and GSH via the thiol have potential to reduce the metal, which would likely result in decomplexation. Citric acid can act as a weak organic acid in addition to as a chelating agent, with possibility to form salts such as gallium or indium citrate. In addition EDTA, also a chelator was tested for its ability to demetallate zinc, gallium and indium complexes.

![Structures of biologically relevant assay agents used hereby.](image)

Complexes were tested in the presence of 2 equivalents of citric acid, L-methionine, Glutathione (GSH), L-cysteine, L-histidine, and EDTA. Zinc complex 1d displayed an increase in fluorescence and no significant shift in $\lambda_{\text{max}}$ in each scenario under each of these conditions at 100 $\mu$M, which is indicative of stability, since the proligands possess much weaker fluorescence.
Figure 3.13. Citric acid assays (50% DMSO, 50% water, 2 eq of citric acid) for compound 1d at 100 µM, by fluorescence spectroscopy (above) UV/Vis (below) monitored at time intervals up to 24h at room temperature.
Figure 3.14. EDTA assays (50% DMSO, 50% water, 2 eq of EDTA) for compound 1d at 100 µM, by fluorescence spectroscopy (above) UV/Vis (below) monitored at time intervals up to 24h at room temperature.

The characteristic spectrum of Compound 1d was observed when assayed with each agent with the exception of EDTA, therefore allowing speculation that zinc leaves the bis(thiosemicarbazonato) ligand in preference for chelation by EDTA (Figure 3.14). Conversely 1d in EDTA solution possessed notable absorbance at 478 nm at 24 h where the free ligand does not, signifying that some zinc complex remains in the solution. The
zinc complex can therefore be considered most likely stable under these conditions tested.

### 3.7.2 pH stability estimation

The stability of complexes at biologically relevant pH is an important factor in this study. Cancer cells are slightly more acidic than non-cancerous cells, therefore it is important that the molecular probes are stable at lower pH.\(^{146}\) All fluorescence scans were carried out with an excitation wavelength of 400 nm and at a concentration of 100 μM, with a 1:1 DMSO:buffer solution.\(^{146}\) The stability of complexes at biologically relevant pH is an important factor in this study.

Following the above preliminary study allyl-substituted compounds were selected as a representative of the other functionality to assess the stability of newly synthesised gallium and indium complexes using UV-visible and fluorescence spectroscopy both at 15 minutes and 24 hours, with 1:1 DMSO:buffer solution. The pH of the samples was measured and found to have no significant difference to the blank, however with a notable variance from that of the original pH solution in each case (Appendix Table D.1.).

![Fluorescence spectroscopy buffer assay](image)

**Figure 3.15.** Fluorescence spectroscopy buffer assay 1c pH study where: pH 2 > pH 3 > pH 5 > pH 9 ≥ pH 7 > pH 9.4 ≥ pH 10 ≥ pH 1.1

Complex 1d displayed the best stability in neutral solutions, with dissociation more likely under acidic conditions, which is consistent with data acquired in the MChem thesis of Brett Kennedy\(^6\) using citric acid based buffers (Figure 3.15). The copper
complex, however showed the most intense fluorescence using weakly acid buffers of pH 5 and pH 3 (Figure 3.16).

![Fluorescence spectroscopy buffer assay](image)

**Figure 3.16.** Fluorescence spectroscopy buffer assay 2c pH study where: pH 5 > pH 3 > pH 7 ≥ pH 9 > pH 9.4 > pH 1.1 ≥ pH 2 > pH 10.

In a pH 1.1 and pH 2.0 buffer the UV-visible and fluorescence spectra of complex 1d closely resembled that of iid, indicating that most of the complex had decomposed to form the proligand (Figure 3.17, Appendix Figures D.50 and D.51). After 24 hours however, the complex appeared to have decomposed entirely since the absorption spectra resemble neither iid nor id. Within the pH 3 buffer the absorbance and emission of 1d decreased with a blueshift ca. 5 nm in the fluorescence spectrum after 24 h, suggesting some conversion to the bis-substituted ligand, yet with a significant proportion of the complex remaining.

When assayed with a Sodium phosphate Monobasic / Sodium phosphate Dibasic based pH 7 buffer, the UV-visible spectrum of 1d at both 15 minutes and 24 hours was similar to that of iid implying that most of the complex has formed the ligand precursor (Figure 3.17). Conversely 1d displayed significantly higher fluorescence than iid, suggesting that some complex was still remaining. Furthermore it is unlikely that the complex has poor stability at this pH due to the data acquired for SFM (pH 7.0 – 7.4) and PBS (pH 7.4). It therefore is more probable that the apparent decomposition of 1d was due to interaction with the buffer components rather than due to the pH.
Figure 3.17. Buffer assays (50% DMSO, 50% water, pH 1.1 buffer, blank measured pH 1.6) for compound 1d at 100 µM by fluorescence spectroscopy (above) and UV/Vis (below) monitored at 15 minutes at room temperature.

Meanwhile, in buffers of pH 5, pH 9 and pH 10 complex 1d showed an increase in fluorescence intensity after 24 h incubation, with a decrease in absorbance, but no shift in either absorption or emission spectra indicating good stability of this complex in these buffers. It can therefore be considered from this study that zinc complex 1d is stable within the range of pH 4.4 and pH 12.7 (the measured pH of buffer:DMSO 1:1 for buffers of pH 3.0 and pH 10.0). This indicates that the complex is stable over a very broad range of pHs in a biological capacity, with complex presence as long as after 24 h and can be considered representative of the other zinc complexes.
3.7.3 Two-photon fluorescence lifetime investigations

The two-photon investigations were first introduced in Chapter 2 and all studies were performed at the Rutherford Appleton Laboratory under the supervision of Dr S. Botchway. Zinc complexes 1b-d were tested in both DMSO and in cells. In solution, 1b was found to possess a fluorescence lifetime with 2 components, at 810 nm the lifetime of the major component was 0.261 ns and representative of 98.2% of the lifetime, with the minor component 0.597 ns at 10 mM ($\chi^2 = 1.52$). This was highly comparable to the single exponential decay with the excitation wavelength of 910 nm, 0.275 ns, with a $\chi^2$ of 1.25. Furthermore, at 910 nm the phenyl- and allyl-substituted complexes possessed similar major component lifetimes of 0.30 ns and 0.28 ns respectively in solution.

If the complex were to demetallate within cells there could potentially be four components, two corresponding to the free ligand and two representing the complex. The long component (ca. 2.5 ns) of the bis(substituted) proligands which was often observed in HeLa and PC-3 cells is significantly different to the short major component and long minor component of the zinc complexes and would therefore allow facile resolution. Moreover, the amount of data necessary to enable resolution of the short components (in solution ca. 0.2 ns and 0.28 ns for ligand and complex respectively) to be sufficiently spaced would require acquisition times of many minutes. It would be possible however, to evaluate dissociation modelling the analysis to up to two decays only, compiling the longer and shorter components, with the longer providing insight into the presence of free ligand.
Figure 3.18. a) Two-photon fluorescence lifetime map ($\tau_m$) of compound 1b incubated in HeLa cells b) is the distribution curve of the $\tau_m$ where the colour represents the lifetime of a).

When 1b was incubated in cancer cells (Figure 3.18a) the lifetime observed at 910 nm was 0.217 ns, 0.098 ns FWHM (major component) and 0.505 ns, 0.821 ns (minor component), which was very close to and within the FWHM of what was observed in solution. When excited at 810 nm, the lifetimes were 0.152 ns, 0.093 ns FWHM and 0.433 ns, 0.625 ns FWHM major and minor components respectively. This indicates that in cells the lifetime was not significantly different (within the FWHM) when
excited by the two respective wavelengths. Moreover the lifetimes observed in cells at 910 nm and 810 nm closely matched data acquired for compound 1b at 10 mM, 810 nm excitation, importantly so in the case of the minor component of ca. 0.5 ns. Furthermore, the lifetimes in solution of 1c and 1d, for example 0.302 ns and 0.283 ns for the corresponding compound, were similar to those observed in cells 0.284 ns (0.087 ns FWHM) and 0.266 ns (0.146 ns FWHM) respectively.

In cells the major components of these zinc complexes were all short (in the region of 0.15 to 0.28) and their respective long components were either less than 1.5 ns or not present, this is highly indicative that no significant portion of the zinc complexes have dissociated to free ligand for which the long component would be far in excess of this value (1.5 ns). These data, therefore are in strong support of the stability of this family of zinc complexes, especially due to the high correlation between the lifetime in solution and in cells, including the second component, which not comparable with the very long component observed for the free ligand.

Copper complexes tested in solution and in cells (50 μM, 0.5 % DMSO, 3 h incubation) showed very similar τ₁ values confirming their stability under these conditions (Figure 3.19). The major component observed both in DMSO solution and in cells was in the region of 0.3 ns for 2c and 2d was a minor component that was not observable within the cell, of ca. 1 – 1.9 ns, perhaps suggesting a preference for the major conformation within the cell. As was discussed regarding the zinc complex data, the presence of a long component of ca. 2.5 ns within the cell would be indicative of dissociation.
Therefore, since there was no presence of a long component in any significant proportion when complex 2c was incubated in cells it can be considered that little demetallation has occurred. Furthermore, due to the high correlation between solution and cell data these investigations signify that the copper complexes are stable within cells and the timeframe of these experiments.

Figure 3.19. a) Two-photon fluorescence lifetime map ($\tau_m$) of 2c in HeLa cells with excitation at 910 nm b) is the distribution curve of the $\tau_m$ where the colour represents the lifetime of a).
3.8 Laser scanning confocal microscopy

Cells were cultured using standard protocols, analogous with earlier investigations of fluorescent thiosemicarbazones. Cell viability prior to experiments and over the course of experiments was monitored by optical microscopy, and validated at the start of the experiments by standard trypan blue tests and MTT assays. Complexes were imaged in HeLa (cervical carcinoma), PC-3 (prostate carcinoma), MCF-7 (breast cancer) and FEK-4 (non-cancerous fibroblast) cell lines, cultured as described in the Experimental section by confocal fluorescence microscopy using one photon excitation at 488 nm with the emission long-pass filtered at 515 nm.

The imaging studies were performed using concentrations of 50 µM compound in a DMSO: RPMI 0.5:99.5 cell medium solvent mix, (RPMI = Royal Park Memorial Institute), whereby the final DMSO concentration on the imaging plate was lower than 1%. It was found that complexes 1b-1d yielded near-identical results to each other under analogous conditions yet displayed significantly different uptake to Zn[ATSM], which had notable uptake in the nucleoli. The consistently higher fluorescence intensity of the zinc complexes 1b-1d allowed the procedure to be optimised for better comparisons with the respective free ligand and indication of stability. It was found that concentrations of 50 µM and incubation times of 20 minutes could be used, which had previously been predominantly 100 µM, with longer incubations. An interesting effect was observed when incubating HeLa cells with complex 1c, a significant phototoxicity could be observed with cell health visibly diminishing after 15 minutes and was not observed in absence of compound (Figure 3.20).
Copper complexes were initially incubated for longer periods of time of 2 hours to ensure uptake. To investigate the mechanism of uptake copper complex 2b, the compound was incubated in HeLa cells at both at 4 °C and 37 °C, whereby if uptake was only observed at 37 °C it would be highly suggestive that the complex enters the cell by an active means (Figure 3.21). The experiment showed that uptake occurred under both conditions and it can therefore be considered as entering by passive diffusion. The fluorescence was slightly less intense at 4 °C, which could be explained by a lower rate of diffusion.
3.9 In vivo imaging

Normoxic PC-3 xenografts were grown on the right shoulder of nude mice in Memorial Sloan-Kettering Cancer Center (MSKCC), New York by Dr Brian Zeglis. M = $^{64}$Cu, R = Et and Ph were labelled from their respective precursor in ethanol achieving high radiochemical yield by a 5 minute microwave-assisted reaction, followed by semi-preparative HPLC purification. MicroPET and biodistribution data collection were carried out for $^{64}$Cu these complexes at MSKCC by Zeglis and Pascu. Welsh et al. investigated the biodistribution of $^{64}$Cu [ATSM] in 1998. After 40 minutes, Welsh and co-workers found significant radioactivity in non-target organs of the lungs, liver, spleen, kidney and intestines, with uptake in the hypoxic tumour (4.17 %ID/g ± 1.03 SD). The preliminary normoxic experiments of this study using radiolabelled complexes...
2b and 2c were 0.34 %ID/g ± 0.04 SD and 0.44 %ID/g ± 0.18 SD respectively (Figures 3.22 and 3.23). It is desirable that retention within normoxic tumours is low, since it would allow a hypoxic tumour to be distinguished more readily.

Furthermore, non-target organ uptake of compounds 2b and 2c was lower when compared to $^{64}$Cu[ATSM] in the liver, kidneys, brain, heart, intestines, bone and in the blood. Retention levels in the lung were comparable (19.17 %ID/g ± 6.55 SD, 23.33 %ID/g ± 7.73 SD, respectively) and biodistribution of compound 2b was similar to that of Cu[ATSM] in the spleen. Spleen uptake levels of phenyl-substituted copper-64 labelled complex were the only non-target organ with significantly higher uptake than $^{64}$Cu[ATSM], with biodistribution levels of 14.48 %ID/g ± 2.80. Moreover, good renal
clearance was observed, therefore the reduced uptake within vital organs combined with limited retention in the normoxic tumour represents promising results for probes designed for hypoxia selectivity

3.10 Summary to Chapter 3

Zinc and copper bis(thiosemicarbazonato) complexes were synthesised and characterised in this study. New crystallographic and computational data were acquired for the phenyl-substituted complexes, with X-ray diffraction results compared to those of different functionality, showing interesting differences in structure. Furthermore, DFT calculations allowed insight into the isomeric exchange observed in solution by NMR spectroscopy for 1c, as well as for paramagnetic 2c, each indicating that in solution the symmetric isomer was more stable. The kinetic stability of these complexes was demonstrated in solution and importantly in cells. Moreover complexes 2b and 2c were radiolabelled with $^{64}$Cu and assessed in vivo displaying limited tumour uptake in nude mice under normoxic conditions, indicating that a thorough study of hypoxic selectivity of these complexes could prove useful. If significantly better uptake is observable in hypoxic cells, the copper complexes possess potential as PET molecular imaging probes for the purpose of diagnosing hypoxia.
Chapter 4. Synthesis, characterisation, spectroscopic, *in vitro* and *in vivo* analysis of gallium bis(thiosemicarbazonato) complexes

Despite the majority of copper bis(thiosemicarbazonato) complex studies focussing on their hypoxia selectivity these complexes are receiving growing interest as bifunctional chelators (discussed in greater detail in Chapters 1 and 2). However, there remains high liver uptake of the copper complex *in vivo*, with attempts to lower uptake by introducing hydrophilic groups on the exocyclic structure having some, yet limited success.\(^{67}\) Therefore, another method of reducing liver accumulation is by way of using gallium(III) and indium(III) (*vide infra*, Chapter 5) complexes. Gallium complexes hold potential for the development of new molecular imaging probes largely due to the readily available \(^{68}\)Ga positron emitting radioisotope that is generated from \(^{68}\)Ge and therefore does not require a nearby cyclotron. Therefore the aim of this chapter was to synthesise new intrinsically fluorescent gallium complexes that could act as agents capable of detecting cancer, whilst understanding their physical and chemical properties in solution, *in vitro* and *in vivo*.

4.1. Gallium complex synthesis

\[
\begin{align*}
\text{acenaphthenequinone} & + \text{GaCl}_3 \\
\text{Me} (3a), \text{Et} (3b), \text{Ph} (3c), \text{Allyl} (3d) & \text{Methanol, reflux, 6 h.}
\end{align*}
\]

*Figure 4.1. Synthesis of acenaphthenequinone gallium bis(thiosemicarbazones)*

Transmetallation served as a reliable, rapid and clean means of synthesising the gallium complex by the following method. The acenaphthenequinone gallium(III) chloride bis(thiosemicarbazone) complexes was successfully synthesised by reflux in methanol in excess of GaCl\(_3\) for 20 minutes, with 5 equivalents of GaCl\(_3\) sufficient when refluxed for 6 hours (*Figure 4.1*). Methyl, ethyl, phenyl and allyl analogues were prepared using this method and isolated by filtration after reaching room temperature as
orange-red powders in moderate yield (36% to 67%). Characterisation proceeded by NMR spectroscopy, ES-MS and X-ray crystallography. Crystals suitable for X-ray crystallography for each of these compounds were grown by vapour diffusion method of THF:Hexane and the molecular structure proved the connectivity of ligand to the [Ga Cl] in each case. Mass spectrometry (ESI) gave peaks for [M-H]$^-$ or [M+H]$^+$ at m/z = 458.96, 484.99 and 580.99, 502.94 and 508.99 for 3a, 3b, 3c, 3d respectively, all values being within 0.1% error of those calculated.

### 4.1.1 NMR spectroscopy

Studies in the group had indicated that the 1d complex displays a dynamic equilibrium between a symmetric and an asymmetric isomer in solution.\textsuperscript{127, 128} This is strongly shifted towards an asymmetric conformation when wet DMSO was bound, or in the presence of DABCO or bipy. $^1$H NMR spectroscopy of 3a-d showed similar splitting of proton resonances between an asymmetric and a symmetric form. Proportions of the different isomers were calculated by comparing the integrations of the $H$-3 or $H$-1 resonances for both isomers, which appear as two distinct (1H) doublets for the asymmetric version and a single (2H) doublet for the symmetric isomer (Figure 4.2) and (Table 4.1).

<table>
<thead>
<tr>
<th>Table 4.1. Proportions of symmetric and asymmetric isomers in the gallium complexes.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Proportion of isomers at 25°C in d$_6$-DMSO</strong></td>
</tr>
<tr>
<td>Symmetric ca.</td>
</tr>
<tr>
<td>58%</td>
</tr>
<tr>
<td>Asymmetric ca.</td>
</tr>
<tr>
<td>42%</td>
</tr>
</tbody>
</table>

Figure 4.2 shows the splitting of $^1$H resonances in the aromatic region for the 3a complex, which is representative of the 3b and 3d spectrum for this part of the spectrum.
Figure 4.2. The proton numbering of complex 3a (above), $^1$H NMR spectroscopy of complex 3a, where green indicates the (E,E) isomer and red indicates the (E,Z) isomer (below).

The methyl signals around 3.10-3.16 ppm, displayed interesting feature. The groups are represented as doublets due to the proximity of exocyclic NHs, therefore, if there were a mixture of symmetric and asymmetric only three doublets would be expected rather than the four that appear in this spectrum. Furthermore, using the approximate ratio of the number of expected protons of 1.9:2.5 a total integration of 13.5 would be expected for these methyl groups. Yet, an integral of 14.5 is observed, therefore it is possible that there are in fact 3 species: 2 symmetric and 1 asymmetric, where the abnormal symmetric isomer is of low proportion (ca. 5%), with another possibility being the formation of [Ga-DMSO]$^+$ Cl$^-$ (Figures 4.3 and 4.4). The isomerism was investigated further by VT-NMR spectroscopy to identify possible isomers and their equilibrium constants and will be discussed later (Section 4.3.3).
Figure 4.3. $^1$H NMR spectroscopy of 3a complex δ 3.3 ppm. In red, the asymmetric shifts, in green the symmetric resonances and in blue the third possible isomer.

$~ 52 \%$

$~ 43 \%$

$~ 5 \%$

Figure 4.4. Proposed isomeric exchange for the gallium methyl complex

3b presented proportions of the symmetric and asymmetric isomer that were calculated as 49% and 51% respectively. The calculated integral for the CH$_3$(Et) groups would be ca. 12.2H and for the CH$_2$(Et) groups ca. 8.1H, which is close to the actual integrals of 12.1H and 8.4H respectively. The resonances overlap significantly and are likely due to the expected 3 overlapping triplets for the CH$_3$ and 3 doublets of doublets.

For 3c, the proportions of asymmetric isomer appeared low, as demonstrated by the features observed in the $^1$H NMR spectroscopy (Figure 4.5). It was however difficult to estimate the actual proportion, which is about 25% of asymmetric isomer. This was due to overlapping and poor resolution of the asymmetric signals, only those corresponding to the symmetric isomer were fully assigned.
Moreover, complex 3d displayed both the asymmetric and symmetric isomers with a ratio of 2:1 asymmetric:symmetric. Interestingly, the integral of the CH\textsubscript{2} group is 6H, which is what would be expected as calibrated below, appears as two overlapping triplets and so does not indicate the presence of a third species (Figure 4.6).

In summary, the gallium complexes were characterised by \textsuperscript{1}H-NMR and \textsuperscript{13}C-NMR spectroscopy confirming their high purity and indicating an interesting isomeric exchange.
4.2 Radiolabelling

Zinc precursors and free ligand were radiolabelled under the supervision of Prof Eric Aboagye by Frazer Twyman at Hammersmith Hospital, London (Figure 4.7 and Appendix Figures B.6-8). A $^{68}$Ga generator was used to elute 10 mL of 0.1 M HCl, ca. 6 mCi of $^{68}$Ga, which was trapped on a 30 mg/1 mL Strata X-C cartridge and eluted with 700 µL of 0.02 M HCl (98% acetone). This was subsequently dried for 15 minutes under a nitrogen stream at 110°C. Next the of bis(thiosemicarbazonato) compound was added in dry DMSO (2 mg/mL) and 2 mL of injectable ethanol. This was heated at 90 °C for 30 minutes and injected into a radio-HPLC. Conversion to the respective gallium complex occurred rapidly (within 30 minutes), however with remaining $^{68}$GaCl$_3$ indicating that radiolabelling had not gone to completion.

![Figure 4.7](image)

Figure 4.7. Overlay of UV-HPLC trace (black) of aromatic zinc bis(thiosemicarbazonato) precursor 1d (R = allyl) with the radio-HPLC trace (blue) of aromatic $^{68}$GaCl bis(thiosemicarbazonato) complex.

Radiolabelling with the zinc complex via transmetallation was successful showing highly comparable data when considering the UV-HPLC spectrum of the ‘cold’ gallium compound and the Radio-HPLC of the ‘hot’ Ga-68 complex. The process was made more efficient using microwave-assisted reactivity with the Lewis group in the Memorial Sloan-Kettering Cancer Center, New York, for the radiolabelling of 3b with the achievement of >95% radiochemical yield in ethanol.
4.3 Structural studies

4.3.1 Crystallographic analyses

Crystal structures of 3a-d have been obtained and were grown in THF:hexane or THF:pentane either by standard liquid diffusion or gas diffusion methods, with the crystals of 3d grown by Dr. Phillip A. Waghorn (Figures 4.8-10). In agreement with the NMR spectroscopy 3c was solved as a symmetric structure, whereas 3b and 3d were asymmetric, interestingly 3a displayed the symmetric and asymmetric form within the same asymmetric unit. It is noticeable that all symmetric compounds possess a plane of symmetry across the backbone and symmetric binding to the metal, yet the terminal functionalities (Me, Ph and Allyl) are not symmetric. All complexes display a distorted square pyramidal structure around the metal centre with the Cl in the axial position with the base formed by the two nitrogen and two sulphur atoms.

Figure 4.8. Ellipsoids drawn at 30% probability. Hydrogens omitted for clarity. For 3a two molecules (left) 3a and (right) 3a’ were present in the asymmetric unit, only one is represented here.

It was notable in Reference 127 that the C-C bond (C3-C14 of 3a, C1-C2 of 3b, C8-C19 of 3c and C2-C3 of 3d) of asymmetric zinc compounds are longer than for symmetric nickel complexes. However, there was no significant difference in bond length when varying the substituent group indicating that the previously observed pattern is most likely metal-dependant rather than isomer-based. Furthermore, the fortuitous occurrence of 3a demonstrating both the symmetric and the asymmetric isomer in the same asymmetric unit allow for more facile comparisons between the isomers to be made.
Table 4.2. Selected bond lengths (Å) and bond angles (°) for compounds 3a-3d, determined by X-ray diffraction.

<table>
<thead>
<tr>
<th>Compound/ Molecular Parameters</th>
<th>[GaCl(ATSM)] (^{138})</th>
<th>3a ((E,E)) isomer (^{138})</th>
<th>3a' ((E,Z)) isomer (^{138})</th>
<th>3b' ((E,Z)) isomer</th>
<th>3c ((E,E)) isomer</th>
<th>3d' ((E,Z)) isomer (^{138})</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-Cl</td>
<td>2.415(5)</td>
<td>2.2121(19)</td>
<td>2.2121(19)</td>
<td>2.230(3)</td>
<td>2.2096(14)</td>
<td>2.237(6)</td>
</tr>
<tr>
<td>M-N</td>
<td>2.0538(15)</td>
<td>2.004(6)</td>
<td>2.004(6)</td>
<td>2.028(11)</td>
<td>2.069(4)</td>
<td>2.033(15)</td>
</tr>
<tr>
<td>M-S</td>
<td>2.3009(5)</td>
<td>1.924(11)</td>
<td>2.13(3)</td>
<td>1.947(11)</td>
<td>2.075(4)</td>
<td>1.926(16)</td>
</tr>
<tr>
<td>C-C</td>
<td>2.3065(5)</td>
<td>2.17(2)</td>
<td>2.537(9)</td>
<td>2.474(4)</td>
<td>2.3171(16)</td>
<td>2.528(6)</td>
</tr>
<tr>
<td>N-N</td>
<td>1.483(2)</td>
<td>1.468(12)</td>
<td>1.468(12)</td>
<td>1.469(18)</td>
<td>1.488(6)</td>
<td>1.480(2)</td>
</tr>
<tr>
<td>C=N</td>
<td>1.363(2)</td>
<td>1.332(9)</td>
<td>1.332(9)</td>
<td>1.278(14)</td>
<td>1.372(5)</td>
<td>1.360(2)</td>
</tr>
<tr>
<td>C=N</td>
<td>1.367(2)</td>
<td>1.32(5)</td>
<td>1.39(3)</td>
<td>1.340(15)</td>
<td>1.371(5)</td>
<td>1.380(2)</td>
</tr>
<tr>
<td>S-M-S</td>
<td>1.300(2)</td>
<td>1.356(9)</td>
<td>1.356(9)</td>
<td>1.394(16)</td>
<td>1.383(7)</td>
<td>1.30(2)</td>
</tr>
<tr>
<td>S-M-N</td>
<td>1.292(2)</td>
<td>1.30(3)</td>
<td>1.37(2)</td>
<td>1.307(16)</td>
<td>1.369(7)</td>
<td>1.30(2)</td>
</tr>
<tr>
<td>N-M-N</td>
<td>105.25(5)</td>
<td>107.1(3)</td>
<td>103.96(19)</td>
<td>106.38(13)</td>
<td>105.11(5)</td>
<td>104.8(2)</td>
</tr>
<tr>
<td>N-M-N</td>
<td>82.47(4)</td>
<td>83.5(2)</td>
<td>83.5(2)</td>
<td>84.4(3)</td>
<td>81.42(12)</td>
<td>84.9(5)</td>
</tr>
<tr>
<td>N-M-N</td>
<td>82.19(5)</td>
<td>84.0(8)</td>
<td>67.2(5)</td>
<td>68.0(3)</td>
<td>81.43(11)</td>
<td>68.1(1)</td>
</tr>
<tr>
<td>N-M-N</td>
<td>76.09(6)</td>
<td>94.1(7)</td>
<td>67.2(4)</td>
<td>88.4(5)</td>
<td>77.33(15)</td>
<td>68.0(5)</td>
</tr>
<tr>
<td>S-M-N</td>
<td>105.13(2)</td>
<td>108.34(8)</td>
<td>108.34(8)</td>
<td>106.7(1)</td>
<td>108.56(6)</td>
<td>109.06(5)</td>
</tr>
<tr>
<td>N-M-N</td>
<td>108.891(19)</td>
<td>111.0(5)</td>
<td>101.1(2)</td>
<td>102.6(1)</td>
<td>108.38(6)</td>
<td>102.14(6)</td>
</tr>
<tr>
<td>N-M-N</td>
<td>99.31(5)</td>
<td>99.3(2)</td>
<td>99.3(2)</td>
<td>98.8(3)</td>
<td>99.7(1)</td>
<td>101.72(14)</td>
</tr>
<tr>
<td>N-M-N</td>
<td>101.45(4)</td>
<td>102.2(7)</td>
<td>106.5(4)</td>
<td>107.1(4)</td>
<td>98.4(1)</td>
<td>106.84(14)</td>
</tr>
</tbody>
</table>

The M-S(1) and M-S(2) bond lengths are different for all asymmetric complexes whereas, for symmetric 3c both M-S bonds are similar. Furthermore, where the bond angles for S-M-N are dissimilar for asymmetric complexes they are similar for all symmetric complexes. It is also noticeable that both Ga-(N) bonds are shorter for the asymmetric 3a than for both other symmetric gallium compound. Interestingly, the N-M-Cl bond angle of the \((E,Z)\) isomer of complex 3a, 106.5(4)°, is larger than that of the \((E,E)\) isomer, 102.2(7)°. Moreover the same angle of the 3a \((E,Z)\) isomer is equivalent to that of the \((E,Z)\) isomers of 3b and 3d, indicating that the increase in this angle is largely an isomeric effect. For compounds 3a, 3b and 3d, the molecular structures
identified an asymmetric binding mode of the ligand compared to the structures obtained for the aliphatic compound.

Figure 4.9. ORTEP representation of 3b, ellipsoids drawn at 30% probability. Hydrogen atoms were omitted for clarity.

Figure 4.10. ORTEP representation of 3c, ellipsoids drawn at 30% probability. Hydrogens were omitted for clarity. For 3c two molecules were present in the asymmetric unit, only one is represented here.

For 3a, X-ray structure determination further showed that both symmetric and asymmetric binding modes were possible for this class of Ga(III) compounds supporting NMR spectroscopy analyses. All bond lengths and angles of 3a - 3d are within the range expected for this class of compounds, and close to those determined for their reported Zn(II) precursors. It is likely that the rigid backbone and tight control of the ligand bite angles by the presence of the aromatic naphthyl substituents in complexes 3a – 3d may account for the significantly higher kinetic stability of aromatic gallium complexes with respect to aliphatic analogues. This trend is consistent with what was found for the aromatic Cu(II) analogues.
4.3.2 DFT optimisations

To provide insight into isomeric exchange of the gallium complexes with the phenyl functionality the were optimised from their crystal structures, following the method used by Holland et al.\textsuperscript{119} Initially the compounds were optimised in the gas phase using B3LYP 6-31G(d,p), followed by B3LYP 6-31++ (d,p) IEFPCM, DMSO to obtain an optimised structure in solution (Figure 4.11). In the gas phase the calculated energy difference is 4.99 kJ mol\textsuperscript{-1}, indicating that the asymmetric structure 3c’ is more stable than the symmetric structure 3c. Likewise, when solvent effects were considered in DMSO, the symmetric structure was more energetically favourable when compared to the asymmetric (the energy difference is -4.34 kJ mol\textsuperscript{-1}), which is in agreement with \textsuperscript{1}H-NMR spectroscopy data.

\textbf{Figure 4.11.} Optimised in solution structures by B3LYP 6-31++ (d,p) IEFPCM, DMSO, where (above) is symmetric 3c and (below) is asymmetric 3c’. Where grey = carbon, white = hydrogen, blue = nitrogen, red = oxygen, green = chlorine, deep pink = gallium.
4.3.3 Variable temperature NMR spectroscopy

To investigate the isomerism further 3a was studied in $d_8$-THF, by Variable Temperature -NMR spectroscopy (400 MHz) (*Figures 4.12-13*). Change in enthalpy, entropy and Gibbs free energy were calculated from the VT-NMR spectroscopy data using Van’t Hoff analysis and the equation:

$$ln(K) = \frac{\Delta H}{RT} + \frac{\Delta S}{R}$$

Complex 3a displayed some observable fluxional behaviour in solution. Considering Figure 4.12, $k$ is ratio of symmetric / asymmetric isomer at each temperature, i.e. the equilibrium from asymmetric to symmetric isomer. $\Delta H$ was calculated to be $-3626.65$ J/mol, whilst $\Delta S$ equals $-12.0412$ J/K/mol, with $\Delta G$ at 298 K calculated as $-38.3824$ J/mol, indicating that there is a small preference towards the symmetric isomer.

![Figure 4.12](image)

*Figure 4.12.* Proposed isomeric exchange for the aromatic bis(thiosemicarbazone) complexes studies hereby where M = Zn, Cu, Ga or In, R = Me, Et, Ph or allyl, X = DMSO, THF, DABCO, bipy, and $K$ is the exchange constant.
Figure 4.13. $^1$H NMR spectroscopy showing resonances corresponding to 3a at 232.1 K, 245.3 K, 258.8 K and 299.9 K in $d_8$-THF.

4.4 Spectroscopy

Fluorescence spectroscopy was performed on DMSO solutions, using initial scans for excitation wavelengths between 200-800 nm. This provided information about the intrinsic fluorescence of the complexes and the corresponding $\lambda_{em-max}$ using solutions of 100 $\mu$M. Ranges of absorption and emission, which are important with regards to cytotoxicity assays and fluorescence imaging, were also assessed using the 2D contours as a guideline. Relevant cytotoxicity assays use absorbance readings at 570 nm, therefore there should be minimal or no excitation at this wavelength, which was true for all complexes except 3c (Figure 4.14b).

Compounds generally emitted between 520 and 675 nm, whereas excitation mainly occurred between 250 and 570 nm (Table 4.3, Figure 4.14 and Appendix Figure C.2). All compounds displayed intrinsic fluorescence. $\lambda_{em-max}$ was found to vary for gallium complexes depending on the functionalities: 510 nm, 400 nm, 450 nm and 520 nm for
3a, 3b, 3c and 3d complexes respectively. Quantum yields were calculated using the standard solution of [Ru(bipy)$_3$](PF$_6$) in water as a reference and utilising the equation:

$$\Phi_S = \Phi_R \cdot \left(\frac{D_S}{D_R}\right) \cdot \left(\frac{A_R}{A_S}\right) \cdot \left(\frac{I_R}{I_S}\right) \cdot \left(\frac{\eta_S}{\eta_R}\right)^2$$

Where $\Phi =$ quantum yield, $D =$ integrated area under emission band, $S =$ sample, $A =$ absorbance of solution at excitation wavelength, $R =$ reference, $\eta =$ refractive index of solvent, $I =$ maximum intensity of excitation band [Ru(bipy)$_3$][PF$_6$]$_2$ in water was used as a reference, with a quantum yield of 0.042. Importantly, the quantum yields indicate sufficient fluorescence imaging in biological cells, with similar values for each gallium compound.

**Table 4.3.** Fluorescence quantum yield measurements of complexes 3a-3d.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$\lambda_{\text{ex-max}}$ / nm</th>
<th>Excitation range / nm</th>
<th>$\lambda_{\text{em-max}}$ / nm</th>
<th>Emission range / nm</th>
<th>Quantum Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>3a</td>
<td>410</td>
<td>250-560</td>
<td>551</td>
<td>495-685</td>
<td>0.11</td>
</tr>
<tr>
<td>3b</td>
<td>500</td>
<td>250-570</td>
<td>556</td>
<td>500-685</td>
<td>0.19</td>
</tr>
<tr>
<td>3c</td>
<td>450</td>
<td>250-610</td>
<td>599</td>
<td>550-740</td>
<td>0.08</td>
</tr>
<tr>
<td>3d</td>
<td>520</td>
<td>250-550</td>
<td>554</td>
<td>490-690</td>
<td>0.12</td>
</tr>
</tbody>
</table>
Figure 4.14. Excitation/emission map at 100 µM in DMSO of compound 3b (above) compound 3c (below)
4.5 Kinetic stability testing in an aqueous environment

Gallium complexes \(3a\) and \(3c\) (Figures 4.15-4.16) had an unexpected intensity increase with time up to 8h. The compounds remained fluorescent at 24h, but at a lower intensity than at 8h. Preliminary assays with media in the absence of serum displayed a gradual decrease in fluorescence with time, which is consistent with the zinc complexes \(1d\) in serum and serum free media. The difference between these two assay types is protein content, indicating that the gallium compounds likely interact with the protein. Interestingly, compound \(3c\) showed that the fluorescence band maxima shifts from ca. 590 nm at \(t = 0\) h to ca. 530 nm at \(t = 2\) h, indicating that the complex may bind to a component of the serum (Figure 4.16).

Figure 4.15. FCS stability assay by fluorescence spectroscopy for compound \(3a\) in FCS: DMSO, 1: 1 ratio
Due to the significantly greater fluorescence of the gallium complexes in comparison to its respective proligand, fluorescence intensity observed is indicative of complex stability. However, since the free ligand does possess some fluorescence, it is important to consider the UV-Visible spectra of complexes to confirm stability under these conditions. In conjunction with a study allowing comparisons with [GaCl(ATSM)], which was found to form the corresponding ligand precursor within 5 minutes in 99% FCS, 1% DMSO, the gallium complex 3d was tested under analogous conditions (Figure 4.17).

Whilst changes in the UV/Vis spectra for the gallium complex 3d were observed over an incubation of 24 h in serum (Figure 4.17), it is apparent that the product of the assay has not completely converted to free ligand iid. UV/Vis spectroscopy studies on 3d suggested that little decomposition occurs up to 3 h although some deteriation of the gallium species is evident after 18 h with LCMS analysis at this time suggesting the presence of both the parent chloride complex and some of the analogue in which the chloride ion is replaced by hydroxide.
Figure 4.17. Serum assay (99% FCS, 1% DMSO) by UV/Vis for compounds 3d at 100 µM, monitored at time intervals up to 24h at room temperature and filtered using a 0.45 µm PTFE microfilter. Compound [Ga-Cl(ATSM)] (shown above) was found not to be stable under comparable conditions.\(^{138}\)

This investigation utilised both UV-visible absorption (at 10 µM and at 100 µM) and fluorescence emission at 100 µM, with 1:1 DMSO:[assay agent] to limit precipitation. This allowed a % stability to be estimated via UV-Visible spectroscopy at 15 minutes and 24 hours as calculated by (100 % - percentage converted to free ligand) (Table 4.4). The first assays carried out in DMSO, water, PBS, 5% FCS EMEM and SFM EMEM enabled complex stability to be understood in media likely to be encountered in a cell biology experiment. UV-visible spectroscopy data for each of the above assays are described first, followed by the analysis of their corresponding fluorescence studies, which were carried out in parallel.

By UV-vis complex 3d displayed encouraging results in DMSO, water and 5% FCS (88.2 %, 39.6 % and 79.1 % after 24 h respectively) (Figures 4.17 and 4.20) complex 3d was not stable during a 24 h incubation in PBS (Figure 4.18) or in SFM (Figure 4.19) and showed contrastingly high stability in SFM after 15 minutes (64.3 %) and limited
remaining complex in PBS (10.9% after 15 minutes.) Moreover, 3d demonstrated 75.3 % estimated stability in DMSO:water 1:1 after 15 minutes, but with a significant decrease in absorbance representing 39.6 % remaining complex after 24 h.

**Table 4.4.** Summary of estimated complex remaining from UV-visible data at 1:1 DMSO:biologically relevant agent at 15 minutes and 24 h where Citric Acid, EDTA, L-Cys, L-His, L-Met and GSH were in MilliQ water.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Compound 3d, 100 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15 min a</td>
</tr>
<tr>
<td>DMSO</td>
<td>92.1</td>
</tr>
<tr>
<td>H2O</td>
<td>75.3</td>
</tr>
<tr>
<td>PBS</td>
<td>10.9</td>
</tr>
<tr>
<td>5% FCS, MEM</td>
<td>75.4</td>
</tr>
<tr>
<td>SFM, MEM</td>
<td>64.3</td>
</tr>
<tr>
<td>Citric Acid</td>
<td>80.0</td>
</tr>
<tr>
<td>EDTA</td>
<td>87.1</td>
</tr>
<tr>
<td>L-Cys</td>
<td>80.0</td>
</tr>
<tr>
<td>L-His</td>
<td>44.6</td>
</tr>
<tr>
<td>L-Met</td>
<td>58.1</td>
</tr>
<tr>
<td>GSH</td>
<td>52.8</td>
</tr>
</tbody>
</table>

For the fluorescence spectra, there was no significant change in intensity of complex 3d in DMSO over a period of 24 h, which had showed a small decrease in fluorescence for the zinc complex 1d (Appendix Figures D.11-15 and Chapter 3). Subsequently complexes were tested and compared to the ligand precursor iid in a 1:1 MilliQ water:DMSO solution over a period of 24 h (Figure 4.17). Under these conditions the gallium complex displayed a significant decrease in fluorescence after 1 day of incubation and a blue shift in fluorescence maxima of 13 nm after 24 h from 559.5 nm at 15 mins, indicative of some conversion to free ligand (a λmax of 542 nm at 15 mins) (Figure 4.8). The fluorescence intensity observed was sufficient to indicate the presence of some remaining gallium complex after 1 day, in agreement with UV-visible data.
Fluorescence data in PBS were significantly weaker than in water, in combination with a blue shift in absorbance is indicative of decomposition into bis-substituted free ligand (Figures 4.17-4.18). Most of the gallium complex appears to have become ligand within 15 minutes as confirmed by the estimated 10.9 % remaining complex calculated using UV-visible measurements and possessed very similar $\lambda_{\text{max}}$ of 537.5 nm for 3d at 24 h and 538 nm for compound iid at 15 mins.
Chapter 4

The stability of the complexes was tested against Eagle’s Minimum Essential Medium Serum Free Medium (SFM), which complexes are often incubated in for cell imaging. The fluorescence of each complex decreases over the time period studied at the $\lambda_{\text{max}}$, however complexes 1d and 3d interestingly show an increase in fluorescence at ca. 450 nm and 460 nm (Figures 4.20–4.21). This could suggest that a new species is being formed or interaction with the media perhaps via binding to a component of the solvent for example amino acids or glucose. The $\lambda_{\text{max}}$ of iid under these conditions is 531 nm indicating that the new band does not correspond to free ligand, however the $\lambda_{\text{max}}$ of id of 469 nm implies that the new band may be similar to the mono-substituted free ligand.

Figure 4.19. PBS assay (50% DMSO, 50% PBS) for compound 3d at 100 µM, by fluorescence spectroscopy (above) UV/Vis (below) monitored at time intervals up to 24h at room temperature.
Figure 4.20. EMEM SFM assay (50% DMSO, 50% SFM) for compound 3d at 100 µM, by fluorescence spectroscopy (above) UV/Vis (below) monitored at time intervals up to 24h at room temperature.

With λ_max of 3d 536 and 537 nm at 15 minutes and 24 h respectively it is likely that a significant proportion of complex has rapidly become free ligand. An augmentation of absorbance and fluorescence was also observed in the case of complex 3d after incubation of 24 h and retaining a band in the UV-visible spectrum at ca. 500 nm not observed in either free ligand id or iid (Figure 4.20).
This is not surprising since FCS is known to increase background absorbance; interestingly, there was no fluorescence increase at ca. 450 nm or 460 nm as was observed in the absence of serum. This shows that presence of FCS is significant with regards to interaction of media and likely causes formation of the zinc and gallium complexes in an orientation or binding mode unlike that produced in EMEM alone.
4.5.1 Stability assays to estimate kinetic stability against common biologically-relevant agents

Biologically relevant agents were chosen to provide a better insight into the stability of these complexes, as were introduced in (Chapter 3). Complexes were tested in presence of 2 equivalents of citric acid, L-methionine, Glutathione (GSH), L-cysteine, L-histidine, and EDTA. Complex 3d, however displayed less promising stability. In citric acid 3d demonstrated significantly reduced fluorescence and absorbance in milliQ water whereby the complex appears stable at 15 minutes, at 24 h both fluorescence and UV-visible spectra were not characteristic of the allyl functionalised gallium complex (Appendix Figure D.1). When incubated in PBS and citric acid rapid conversion to free ligand was observable due to similarity of the 3d spectrum to distinctive absorption spectrum of iid under the same conditions (Figure 4.22).

Conversely it is apparent that the 3d UV-vis spectrum is not identical to iid at 15 minutes, with significantly greater fluorescence at this timepoint showing that some gallium complex is remaining in PBS with citric acid. It is likely that 3d has almost completely converted to iid after 24 h of incubation attributable to the comparable spectra. In aqueous solutions of L-methionine, L-cysteine, L-histidine, or EDTA, 3d displayed similar properties, each with the characteristic 3d absorbance spectrum at 15 minutes followed by a significant decrease in absorbance at ca. 480 nm, but not the formation of data corresponding to that of the iid free ligand (Figures 4.23-4.24 and Appendix Figure D.12-15).
Figure 4.22. Citric acid assays (50% DMSO, 50% PBS, 2 eq of citric acid) for compound 3d at 100 µM, by fluorescence spectroscopy (above)  UV/Vis (below) monitored at time intervals up to 24h at room temperature.

This combined with the greater fluorescence of 3d after 24 h incubation as compared with iid at 15 minutes is indicative that not all of the complex has formed free ligand. The 24 h spectrum of 3d in GSH is very similar to that of iid at 15 minutes, displaying a very similar absorbance spectrum, indicating that most of 3d has been converted to compound iid under these conditions (Figure 4.24).
Figure 4.23. EDTA assays (50% DMSO, 50% water, 2 eq of EDTA) for compound 3d at 100 µM, by fluorescence spectroscopy (above) UV/Vis (below) monitored at time intervals up to 24h at room temperature.

It can be observed that there is a much higher fluorescence intensity for 3d at 24h than for iid at 15 minutes, indicating that although most complex may have converted to free ligand, it is likely that not all of the complex has. In contrast to the data acquired for L-cysteine, L-methionine and L-histidine, in which 3d appeared much more stable, it can be speculated that this combination of three peptides, containing both N- and S- groups presents a much more potent biomolecule for the purpose of demetallating the complex.
Figure 4.24. Glutathione assays (50% DMSO, 50% water, 2 eq of Glutathione) for compound 3d at 100 µM, by fluorescence spectroscopy (above) UV/Vis (below) monitored at time intervals up to 24h at room temperature.

4.5.2 pH study

The stability of complexes at biologically relevant pH is an important factor in this study. Cancer cells are slightly more acid than non-cancerous cells it is important that the molecular probes are stable at lower pH. All fluorescent scans were carried out with an excitation wavelength of 400 nm and at a concentration of 100 µM, with a 1:1 DMSO:buffer solution. An initial preliminary study was carried out using fluorescence spectroscopy for the gallium complexes.
The gallium compounds all displayed maximum fluorescence at pH 2, with particularly intense fluorescence for 3a and 3b at this pH. It is interesting to notice that 3a and 3b are very fluorescent at pH 2, but very little fluorescent at pH 3. This could be explained by the nature of the species involved in the buffer. Buffer pH 2 contained HCl and KCl, whereas the pH 3, pH 5 and pH 7 buffers contained citric acid. Holland et al. have recently shown that citric acid can be used to remove zinc from zinc bis(thiosemicarbazonato) complexes, which may have happened in the preliminary study, since dissociation of the metal centre would cause a decrease in fluorescence.
Therefore an initial study where citric acid was used was carried out (prior to the assays described above) followed by another investigation that did not include citric acid as part of the buffer systems, a means to monitor if the nature of the buffer had a significant effect. These gallium complexes are therefore very pH sensitive, for example, 3c is fluorescent at pH 1 and pH 2, indicating that it can readily be protonated, but shows little fluorescence under more alkaline conditions (Figure 4.25).

Following the above preliminary study, the allyl-substituted compounds were selected as a representative of the other functionalities to assess the stability of newly synthesised gallium and indium complexes using UV-visible and fluorescence spectroscopy both at 15 minutes and 24 hours, with 1:1 DMSO:buffer solution with the assistance of Johan Stojanović, technician student from ESCOM, France. The pH of the samples was measured and found to have no significant difference to the blank, however with a notable variance from that of the original pH solution in each case (Table 4.5).

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Measured pH</th>
<th>% stability at 15 mins</th>
<th>% stability at 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 1.1</td>
<td>1.6</td>
<td>68.6</td>
<td>NS</td>
</tr>
<tr>
<td>pH 2</td>
<td>2.5</td>
<td>85.5</td>
<td>53.9</td>
</tr>
<tr>
<td>pH 3</td>
<td>4.4</td>
<td>86.9</td>
<td>NS</td>
</tr>
<tr>
<td>pH 5</td>
<td>6.9</td>
<td>73.7</td>
<td>51.7</td>
</tr>
<tr>
<td>pH 7</td>
<td>9.4</td>
<td>5.0</td>
<td>NS</td>
</tr>
<tr>
<td>pH 9</td>
<td>9.35</td>
<td>6.2</td>
<td>NS</td>
</tr>
<tr>
<td>pH 10</td>
<td>12.7</td>
<td>6.8</td>
<td>NS</td>
</tr>
</tbody>
</table>
Figure 4.26. All buffer assays (50% DMSO, 50% water) for compound 3d at 100 µM by fluorescence spectroscopy (above) and UV/Vis (below) monitored at 24 hours at room temperature.

Furthermore all gallium complexes (Figure 4.26 and Appendix Figures D.16, D.20-27) appeared most stable at either pH 2 or pH 3 after 15 minutes or less, which is also in agreement with data acquired where citric acid was a component of the buffer solutions. By UV-visible spectrometry compound 3d seems to decompose completely after 24 hours when incubated in a pH 1.1 buffer, yet appears to remain intact at 15 minutes, as its spectrum has formed the shape characteristic of iid in this time, fluorescence however appears to be fully quenched at 15 minutes (proligand iid buffer study Appendix Figures D.50 and D.51). It is likely that stability of the gallium complex is poor at this pH. In pH buffers 2.0, 3.0 and 5.0 there is complex presence indicated by both absorption and emission spectra at 15 minutes, however a clear decrease in
fluorescence intensity and absorbance including a blue shift for pH 3.0 and pH 5.0 buffers are indicative of instability of this compound at longer incubations.

As was observed with 1d, 3d spectra closely resembled those of compound iid when incubated with a pH 7.0 buffer, however due to the high stability observed in the 5% FCS assay the apparent lack of stability could be explained by interaction with the components of the buffer rather than the pH itself. When considering the measured pH of the pH 7.0 buffer blanks 1:1 DMSO:buffer is 9.4 (a Sodium phosphate Monobasic / Sodium phosphate Dibasic based buffer), as was the measured pH when incubating with the pH 9.0 buffer was pH 9.35 (a glycine / sodium hydroxide based buffer) and that spectra were comparable the most likely outcome is in fact that the complex has poor stability under alkaline conditions, which are seldom observed in a biological environment.

Whilst spectroscopic (fluorescence and UV-vis) data acquired with the pH 7.0 and pH 9.0 buffers were similar, in both cases it was observable that conversion to iid was not complete, indicating a small proportion of complex remaining. UV-visible and Fluorescence spectra of 3d when incubated with a pH 10.0 buffer (measured pH with blank pH 12.7) displayed spectra more closely resembling the free ligand iid, indicating that the stability was poorer under more alkaline conditions. The range of good stability of this complex when considering the measured pH with the blank is pH 2.5 to pH 6.9, however under no conditions tested using the pH buffers was complete conversion to iid after 15 minutes, indicating that short-term experiments in biological environments are valid with this compound, especially with the stability observed after 24 h in 99% FCS for this complex, with a blank pH of 7.35. In summary the spectra acquired support the legitimacy carrying out cell uptake experiments, where incubations would be less than 20 minutes, whereby the complex would most likely intact under these biomimetic conditions.
4.3 Two-photon fluorescence lifetime study

The two-photon investigations were first introduced in Chapter 2 and all studies were performed at the Rutherford Appleton Laboratory under the supervision of Dr S Botchway. Gallium complexes possessed two components in solution with $\tau_1$ comparable to the $\tau_1$ of the ligand precursor under the same conditions, yet slightly longer for each complex. The $\tau_2$, on the other hand was significantly lower at ca. 1 – 1.5 ns (Appendix Figure E.3., Table E.5). This combined with the stability study in solution using UV-vis and fluorescence signifies that this similarity is most likely due to the ligand-dependant nature of the fluorescence (Chapter 3) according to TD-DFT calculations carried out for the copper complex 2c, rather than decomposition to free ligand in solution. The presence, therefore of a long component by FLIM in vitro of more than 2 ns may thus be the best indication of complex becoming proligand using this technique.

In cells when exciting at 910 nm results were similar to those observed within solution for all major components, and most minor components under standard conditions. Gallium complexes were incubated at 50 μM, 0.5% DMSO, for 20 minutes, unless stated otherwise in the text. The $\tau_2$ (minor component) are highly comparable to the lifetimes observed for the free ligand 3a HeLa (0.588 ns, 0.406 ns FWHM), 3b PC-3 (0.475 ns, 0.094 ns, FWHM) and 3c MCF-7 (0.567ns, 0.228 ns FWHM), for example the major component of iic at 910 nm was 0.531 ns, 0.508 ns FWHM. Equally, this could signify some conversion to free ligand 4%, 8% and 20% respectively) or that the gallium complex forms similar interactions for the cell as its ligand counterpart.

Further investigations, using excitation of 810 nm showed that $\tau_2$ was higher than expected for the complex in HeLa cells 3b and 3c (2.026 ns 0.865 ns FWHM and 2.523 ns, 1.562 ns FWHM respectively), therefore making some ligand formation the more probable of the two above mentioned outcomes. These minor components respectively accounted for 11% and 16 %, thus indicating that most of the complexes are stable within cells under standard conditions. In each case (at 810 nm or 910 nm) the fluorescence intensity was significantly greater than observed for the proligand, indicating complex stability, with FLIM providing quantitative means of evaluating...
stability and demetallation. The combined use therefore of UV-Visible spectroscopy, fluorescence spectroscopy and FLIM offer a powerful method of investigating small molecule stability in both solution and in cells and confirm the stability of these complexes.

![Image](image_url)

**Figure 4.27.** Two-photon FLIM ($\tau_m$) at 910 nm in HeLa cells after 20 minutes of incubation of a) ii b at 100 µM, 1% DMSO, b) 3b at 50 µM, 0.5% DMSO c) 4b at 50 µM, 0.5% DMSO

### 4.6 Laser scanning confocal microscopy

Cells were cultured using standard protocols, analogous with earlier investigations on fluorescent thiosemicarbazones. Cell viability prior to experiments and over the course of experiments was monitored by optical microscopy, and validated at the start of the experiments by standard trypan blue tests and MTT assays.\(^{127, 128}\) Complexes 3a-3d were imaged in HeLa (cervical carcinoma), PC-3 (prostate carcinoma), MCF-7 (breast cancer) and FEK-4 (non-cancerous fibroblast) cell lines, cultured as described in the Experimental section by confocal fluorescence microscopy using one photon excitation at 488 nm with the emission long pass filtered at 515 nm.

The imaging studies were performed using concentrations of 50 µM compound in a DMSO: RPMI 0.5:99.5 cell medium solvent mix, whereby the final DMSO concentration on the imaging plate was lower than 1%. The solutions were left to incubate with the adherent cancer cell lines for 20 or 60 min, with preliminary investigations incubating up to 3 hours and at concentrations of 100 µM, at 4 °C or 37 °C. The cells were carefully washed with Phosphate Buffered Saline (PBS) pre-warmed to 37°C, which was replaced by FCS-free medium to remove the non-
internalised fluorescent dispersion prior to fluorescence imaging. As before, the imaging work was carried out in the presence of minimum amount of serum to avoid potential background fluorescence and for suitability for investigation via colocalisation dyes.$^{123, 127, 128}$

Figure 4.28. Single-photon confocal microscopy images of 3a PC-3 cells, 50 μM, 0.5% DMSO, 20 minutes, at 37°C (a) DIC image, (b) excitation at 488 nm, emission $>$505 nm, (c) excitation at 405 nm, emission 420-480 nm (d) is an overlay of (a), (b) and (c). Scalebar: 20 μm.
Figure 4.29. Complex 3b, 50 μM, 0.5% DMSO, 20 minutes, at 37°C irradiation experiment at 488 nm in MCF-7 cells: (a – e) before irradiation and (f – j) after irradiation for ca. 10 minutes, where DIC image (a, f), micrograph of cells after excitation at 405 nm (b, g), micrograph of cells after excitation at 488 nm (c, h, compound, green channel), micrograph of cells after excitation at 543 nm (d, i, compound, red channel). Image (e) is an overlay of (a), (b) and (c) images, whereas (j) is an overlay of images (f – i). Scalebar: 20 μm.

The necessity to use concentrations as high as 50 μM in these studies was a result of the rather weak fluorescence emissions (by comparison with organic, commercial dyes). Control experiments prior to incubation of cells with the compounds of interest were obtained by fluorescence imaging (Appendix G) to ensure that the cell morphology remains unaltered prior to the imaging experiments. No significant changes in cell morphology were observed by optical microscopy after 20 min incubation with respect
to control. After 60 min incubation at 37 °C with compounds 3a–3d the cell morphology gave evidence of stress thus suggesting that these metal complexes are toxic at the 1 h timepoint. The following figures below depict representative confocal fluorescence microscopy images in HeLa and PC-3 cells for these complexes.

![Figure 4.30](image)

**Figure 4.30.** 2-photon FLIM ($t_m$) of 3b in MCF-7 a) before irradiation, b) after irradiation for 9 minutes at 488 nm, c) fluorescence lifetime distribution curve ($t_m$) at 910 nm before and after irradiation.

Co-localisation dyes and cells are shown and were recorded a minimum incubation of 20 minutes and a maximum of up to 3 hours. The observation of fluorescence in these images suggests that substantial amounts of these complexes remain intact inside the cell. Within the first 20 minutes there is widespread distribution of the gallium complexes within the cell cytoplasm (Figure 4.28).

Co-localisation with commercially available dyes (e.g. Mitotracker Red and Green, Hoechst, Lysotracker Red and Green) were used to identify regional uptake within the cell, investigating whether the gallium complexes enter the mitochondria, lysosomes, the nucleus and lipid-rich regions of the cell, which may in turn shed light upon the nature of compound activity.

Additionally, complex 3b displayed interesting photoinduced changes in intracellular localisation within cells and was studied in MCF-7 and PC-3 cells, showing comparable data in each cell line (Figure 4.29 and Appendix Figure G.12). After irradiation with 488 nm light the green fluorescence was less evenly distributed within the cell with a more punctuated appearance. It was noticeable that the cell morphology was dramatically altered resulting in rounder and larger cells indicating occurrence of blebbing, which occurs during the process of apoptosis (programmed cell death). This
signifies that the photoactivity of the complex results in greater cytotoxicity implying a potential for photodynamic therapy using these compounds. The effect of light on the complex was further investigated in solution by NMR spectroscopy, using LEDs producing light broadly around 500 nm with the assistance of Mr Christopher Woodall.

Interestingly, after irradiation for 9 minutes a significant change in fluorescent lifetime was observed when incubating 3b in MCF-7 cells (Figure 4.30), resulting in a fluorescence lifetime of 2.001 ns, 1.118 ns FWHM. It can be speculated that the metabolism of this particular cell line has caused formation of free ligand, since no change in fluorescence lifetime was observed in PC-3 under the same conditions.
4.6.1 $^1$H NMR spectroscopy investigation

The study was carried out using a 400 MHz NMR spectroscopy and 5 mg of 3b in 0.5 mg/mL of $d_6$-DMSO at room temperature. An initial control was carried out keeping the solution in the dark at room temperature for 6 hours measuring a NMR spectrum before and after the required time to investigate if the spectrum changed without irradiation. The spectra were identical, indicating that there is no significant change observable by NMR spectroscopy when the solution is stored in the dark (Figure 4.31).

Moreover, the solution was irradiated using LEDs that produce light broadly around 500 nm for 5 minutes. This resulted in the appearance of new resonances at 9.38 ppm, 8.10 ppm and 7.71 ppm. The solution was then irradiated a further 14 hours, a spectrum taken and subsequently protected from light followed by spectra taken after an additional 45 minutes and another 10 hours (Table 4.6). The ratio of the first asymmetric N(H) proton at 9.60 ppm (which will be referred to as ‘resonance 1’) to the new resonance at 9.38 ppm (‘resonance 2’) was measured for each of these spectra integrating between 9.662 - 9.501 ppm and 9.418 and 9.350 respectively allowing direct comparisons to be made. The ratio was also calculated for the spectrum before
irradiation to give an indication of a ‘background’ in which there was no visible triplet resonance, which accounted for 2.21%. It is apparent that a new species is formed upon irradiation, which is produced rapidly after just 5 minutes with resonance 2 representing 10.6 % of a resonance 1. Further irradiation did not increase this ratio (with the slight decrease to 9.79 % after a further 14 hours of irradiating indicating that the new species) signifying that the species very quickly reaches its maximum proportion whilst using these LEDs.


<table>
<thead>
<tr>
<th>Irradiation details of 3b in d₆-DMSO</th>
<th>9.38 ppm resonance % integral of 9.60 resonance</th>
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<tbody>
<tr>
<td>Before irradiation</td>
<td>2.21</td>
</tr>
<tr>
<td>&lt; 5 min after irradiation for 5 min</td>
<td>10.6</td>
</tr>
<tr>
<td>&lt; 5 min after irradiation for 14 h</td>
<td>9.79</td>
</tr>
<tr>
<td>45 min after irradiation</td>
<td>9.7</td>
</tr>
<tr>
<td>10 h after irradiation</td>
<td>5.95</td>
</tr>
</tbody>
</table>

This implies that more highly powered light producing instruments, such as lasers may be able to produce a more significant fraction of this species and should be investigated using high-powered lasers. Despite the fast production of the new species, conversion back to produce the original spectrum was not as rapid since after 45 minutes in the dark the proportion was 9.7 %.

After a further 10 hours protected from light the percentage was reduced to 5.95 % indicating that the process is slowly reversible. One possibility is that the new species is the alternative symmetric isomer as discussed above for complex 3a (Section 4.1.1). This is plausible since two of the new resonances are triplets, one in the region expected for nitrogen bound protons and the other in the aromatic region. If this is correct each of the new resonances represent two protons of the alternate symmetric isomer as compared to the one proton of the asymmetric isomer and so meaning that there is ca. 5% of the isomer in solution.
Currently, it can be hypothesised that high power lasers would be required to achieve the difference observed within cells. Another possibility however, is that the new species can enter different organelle(s) more rapidly than its other respective isomers, which upon entry begin to convert back to its more usual forms, which are thus effectively trapped within the cell. Likewise as the alternate symmetric isomer outside the organelle is produced it is removed from the cytoplasm, forcing the uptake of the complex to appear as a punctuated shape. To explore the nature of organelle in question
MCF-7 cells were incubated with Nile Red dye, which is a marker for lipid-rich regions of the cell, which showed very strong correlation as to that of the uptake of 3b after irradiation (*Figure 4.32*).

![Image](image1.png)

**Figure 4.33.** Single-photon Confocal fluorescence imaging: Complex 3d, 50 µM, 0.5% DMSO, at 37°C in HeLa cells incubated for 1 hour: (a – b) the cells were irradiated with 488 nm light for less than 5 minutes, where DIC image is represented by (a) and micrograph of cells after excitation at 488 nm by (b, compound, green channel). Scalebar: 20 µm.

Interestingly, complex 3d when incubated for 60 minutes, punctuation was also observed during the process of finding a representative area to image, indicating that the effect is also rapid with the allyl functionality (*Figure 4.33*). A further study was carried out incubating HeLa cells with 3d for 3 hours at 100 µM, 1% DMSO to determine if this effect occurs without irradiation when incubation is longer and more compound is present. This effect was not observable in absence of irradiation indicating that this process does not occur with time alone. Additionally, the study was carried out at 37°C and 4°C, to investigate if the uptake occurred by passive diffusion alone, since mechanisms such as endocytosis do not occur at low temperatures (*Figure 4.34*). 3d entered cells both at 4 °C and 37 °C, implying that cell uptake occurs by passive diffusion alone in fixed cells with the nucleus stained with DAPI. Nuclear uptake was observed at 37 °C, but not at 4 °C, signifying that the process did not occur by passive diffusion.
Figure 4.34. Single-photon confocal microscopy images of complex 3d and DAPI in HeLa (fixed cells), incubated for 3h, 100 µM, 1% DMSO where (a) – (b) at 37°C and (c) – (d) at 4°C; (a) and (c) represent the micrograph with excitation at 488 nm, emission >505 nm, (b) and (f) represent an overlay of the micrograph of excitation at 405 nm, emission 420-480 nm (a) and (c) respectively and the DIC image.

The study was further carried out with 3b with more standard incubation times of 20 minutes and 50 µM 0.5% DMSO, to determine if the same effect is observed for shorter incubations and lower concentrations, which indicated no nuclear uptake at 4°C and very little complex within the nucleus at 37°C signifying that entry to the nucleus is only significantly achieved at longer time-points as above. Interestingly it appears that the fluorescence of 3b was red-shifted at 4°C as well as being taken up by HeLa cells (Figure 4.35).
Figure 4.35. Single-photon confocal microscopy images of (a-d) Complex 3b, 50 μM, 0.5% DMSO, 20 minutes and Hoechst (nuclear stain) in HeLa cells at 37°C: DIC image (a), micrograph of cells after excitation at 488 nm (b, compound, green channel), micrograph of cells after excitation at 543 nm (c, compound, red channel). Image (d) is an overlay of (a), (b), (c) and the micrograph from excitation at 405 nm. (e-h) 3b, 50 μM, 0.5% DMSO, 20 minutes in HeLa cells incubated at 4 °C, (e) DIC image, (f) excitation at 488 nm, emission >505 nm, (g) after excitation at 543 nm and (h) is an overlay of (e), (f) and (g). Scalebar: 20 μm.

Complex 3d was additionally incubated in FEK-4 cells, a non-cancer cell line, which displayed similar properties as to in the cancer cell lines tested with the majority of uptake in the cytoplasm and little in the nucleus. Likewise, longer incubation times of 3 hours and 100 μM concentrations resulted in nuclear uptake in FEK-4 cells (Figure 4.36).
Moreover, strong red emission with weak green emission was also observed for 3c when incubated at 37°C. This is in agreement with the fluorescence data in solution described above. Thus, imaging is suggesting that this complex unsuitable for investigation via green and red colocalisation dyes, however staining with Hoechst
demonstrated that this complex does not enter the nucleus within 20 minutes at 50 µM (Figure 4.37).

\[ \text{Figure 4.37. Complex 3c, 50 µM, 0.5\% DMSO, 20 minutes at 37°C in HeLa cells incubated with} \]
\[ \text{Hoechst dye, where DIC image (a), micrograph of cells after excitation at 405 nm (b), micrograph of cells} \]
\[ \text{after excitation at 488 nm (c, compound, green channel), micrograph of cells after excitation at 543 nm (d,} \]
\[ \text{i, compound, red channel). Image (e) is an overlay of (a), (b) and (c) images. Scalebar: 20 μm.} \]

Initial tests on the co-localisation of \textbf{3a} and \textbf{3d} with the mitochondrial stains Mitotracker Red and lysosome stain Lysotracker Red(Invitrogen) were carried out and results show mitochondrial (Figure 4.39 and Appendix Figures G.16) and lysosomal colocalisation in line with earlier observations for their Zn(II) precursors\textsuperscript{127, 128} Data were highly comparable for \textbf{3a} and \textbf{3d} in both HeLa and PC-3 cells. (Figure 4.39 and Appendix Figures G.17-18)

In summary, this family of gallium complexes enter both cancer and non-cancer cells. Localisation appears to occur in many organelles and indicates that, if cytotoxic these compounds may have many modes of action. Long incubations saw the complex \textbf{3d} enter the nucleus indicating a possibility DNA damage during prolonged exposure, whereas rapid uptake was observable in the lysosome and the mitochondria, meaning that these complexes may possess potential to disrupt these organelles. Data suggest that the complex can enter cells by passive diffusion, yet enters the nucleus \textit{via} other means. Moreover, the photoactivity of \textbf{3b} presents a prospect of photodynamic therapy, with
the evolution of a new species in a reversible process as well as localisation in lipid-rich regions of the cell.

Figure 4.38. (a – b) Complex 3a, 50 μM, 0.5% DMSO, 20 minutes at 37°C and Mitotracker in PC-3 cells: (a) micrograph of cells after excitation at 488 nm, (b) is an overlay of (a), DIC image and micrograph of cells after excitation at 543 nm. Images (c-d). Complex 3d, 50 μM, 0.5% DMSO, 20 minutes at 37°C and Lysotracker in HeLa cells, (c) micrograph of cells after excitation at 488 nm, (d) is an overlay of (c), DIC image and micrograph of cells after excitation at 543 nm. Scalebar: 20 μm
4.7 Preliminary In vivo imaging

Normoxic PC-3 xenografts were grown on the right shoulder of nude mice in Memorial Sloan-Kettering Cancer Center, New York by Dr Sofia I. Pascu and Dr Brian Zeglis. MicroPET was carried out for $^{68}$Ga complex 3b, good renal clearance was observed, indicating suitability of these probes (Figure 4.39). Furthermore, no tumour uptake under these normoxic conditions was observed for all cases, with some limited uptake in the lungs, liver and spleen and clear bladder localisation/excretion. This represents a promising result for probes designed for hypoxia selectivity, signifying that it would be beneficial for an in vitro hypoxia study to be carried out data processing is in process reveal the exact biodistribution.

![MicroPET images of in nude mice, M = 68-Ga, R = Et, where a, b, c and d are at timepoints 30 mins, 1h, 2h and 4h respectively.]

**Figure 4.39.** MicroPET images of in nude mice, M = 68-Ga, R = Et, where a, b, c and d are at timepoints 30 mins, 1h, 2h and 4h respectively.

4.8 Hypoxia selectivity testing

An investigation to assess the hypoxia selectivity of these complexes was carried out by the collaborating group of Professor Eric Aboagye (Dr Israt S. Alam) in EMT6 (murine breast carcinoma) and PC-3 cells, grown under standard conditions described in Experimental Section.

Compound 3d was incubated in cells at 50 µM, 4% DMSO, with normoxic conditions of 20.7% $O_2$ and 5% $CO_2$ at 37 °C, with hypoxic samples pre-incubated for 20 minutes
at 1% O$_2$, 5% CO$_2$ at 37 °C before complex addition. Following addition of the compound cells were incubated for a further 20 minutes and subsequently washed three times with PBS before being returned to serum free media and imaged immediately.

![Confocal micrographs of 3d in FEK-4 (top) and EMT6 (bottom) cells under normoxic (left) and hypoxic (right) conditions.](image)

**Figure 4.40.** Confocal micrographs of 3d in FEK-4 (top) and EMT6 (bottom) cells under normoxic (left) and hypoxic (right) conditions.

Using confocal imaging the compound preference for hypoxia was not easily deducible (*Figure 4.40*), however, flow cytometry and radioactive cell uptake studies, provided quantitative means to assess the selectivity (conditions used are within the experimental section).
Figure 4.41. Flow cytometry studies under normoxia and hypoxia of 3d (a) in EMT6 and (b) in PC-3 cells.

Median fluorescence intensities (MFU) obtained using flow cytometry under hypoxic conditions were lower than those of the normoxic cells (12% lower for EMT6 (*P<0.05) and 21% lower in PC-3 cells (**P<0.001)) (Figure 4.41). Although this decrease in fluorescence may be understood as lower uptake, it is more likely that incubation under hypoxic conditions causes reduction of the complex, which would result in demetallation and therefore loss of fluorescence. It consequently is difficult using this technique alone to determine if the lower fluorescence is due to less uptake, conversion to ligand and lower uptake or conversion to ligand and increased uptake.

Figure 4.42. Radioactive cell uptake studies under normoxia and hypoxia of 3d in EMT6 cells.

To investigate this issue a preliminary radioactive cell uptake experiment was carried out, showing that $^{68}$Ga uptake was greater in hypoxic cells than normoxic cells (64% and 49% higher at 30 and 60 minutes respectively) (Figure 4.42). This therefore indicates that the most likely cause of the reduction in fluorescence is due to the
combination of increase in uptake as well as conversion to free ligand, signifying that the gallium complexes possesses selectivity for cells under hypoxic conditions.

4.9 Summary to Chapter 4

New intrinsically fluorescent gallium complexes were synthesised and characterised using ES-MS, NMR spectroscopy and X-ray crystallography. The complexes displayed a symmetric and an asymmetric isomer in solution, which were further explored using VT-NMR spectroscopy and DFT for complexes 3a and 3c respectively each indicating a small preference towards formation of the symmetric isomer. Laser scanning confocal microscopy studies showed that the gallium complexes enter cancer and non-cancerous cells and rapidly localise in the lysosome and mitochondria, whilst nuclear uptake occurs more gradually. Entry to cells occurred at 4°C, indicating the process occurs by passive diffusion, however penetration into the nucleus was only achieved at 37°C meaning that that this did not occur by passive diffusion alone.

Complex 3b demonstrated photoactivity and appears to form a new species upon irradiation with light around 500 nm, causing a change in localisation in the cell and initiation of cell death via apoptosis. In vivo experiments demonstrated suitability for 3b in that there was good renal clearance and seldom any uptake in the tumour under normoxic conditions – an essential feature of a hypoxia imaging agent. Furthermore, an investigation to discover the hypoxia selectivity of these complexes has been carried out indicating a preference for hypoxic cells of ca. 20%. Further experiments should be carried out to ascertain if complex uptake occurs under hypoxic conditions in vivo, which would confirm the suitability of these complexes as hypoxia imaging agents.
Chapter 5. Synthesis, characterisation, spectroscopic, *in vitro* and *in vivo* analysis of indium(III) bis(thiosemicarbazone) complexes

Indium complexes were designed and synthesised since they present an alternative method of reducing liver uptake of bis(thiosemicarbazone) complexes, with the radioactive isotope In-111 an attractive agent for SPECT, by way of its gamma decay and for radiotherapeutics due to its auger electron emission. Aromatic indium bis(thiosemicarbazone) complexes are likely to be sufficiently intrinsically fluorescent, due to metal to ligand charge transfer, to enable their progress to be monitored *in vitro* and potentially for dual-modal imaging. The main objective of this chapter was therefore to develop new indium(III) bis(thiosemicarbazone) complexes that would be highly stable in biological media, rapidly and cleanly radiolabelled and capable of entering cancer cells.

5.1 Indium complex synthesis

![Figure 5.1. Synthesis of acenaphthenequinone indium bis(thiosemicarbazones)](image)

As was found with the gallium complexes transmetallation provided a clean means of obtaining the indium complexes. Indium(III) compounds 4a, 4b, 4c and 4d were successfully synthesised in methanol under reflux for 6 hours, *via* the zinc precursor and vast excess of InCl₃ *(Figure 5.1).* Following filtration and washing with diethyl ether the product was isolated as the solid as a red coloured powder for 4a, 4b, and 4d, with 4c isolated from the filtrate, which was rotary evaporated and subsequently washed with diethyl ether. NMR spectroscopy and ES-MS were used for characterisation, with crystals suitable for X-ray crystallography obtained by liquid diffusion method in
THF:Hexane. Mass spectrometry (ESI) gave peaks for [M-H]− at m/z = 502.94, 530.97, 556.98 and 626.97 4a, 4b, 4c and 4d respectively, all values being within 0.1% error of those calculated.

Unlike the gallium complexes discussed in the previous chapter the indium complexes did not display the same kind of isomeric mixture, with only the symmetric isomer present in a significant proportion (Figure 5.2).

Figure 5.2. ¹H NMR spectra of 3d (above) and 4d (below) in d6-DMSO, 4.0 ppm to 10.0 ppm.
5.2 Density functional theory calculations

The isomerism was investigated further for the indium complexes with the phenyl substituent in an analogous study to the previous chapter by Density Functional Theory, following the method used by Holland et al. An initial optimisation was carried out where the basis set was 6-31G(d,p) for S, Cl, C, N, H and SDD for In in the gas phase, with a final optimisation of with a basis set of B3LYP 6-31++ (d,p) IEFPCM, DMSO for S, Cl, C, N, H and SDD for In in solution (Figure 5.3).

In the gas phase the calculated energy difference is -3.53 kJ mol\(^{-1}\), indicating that the asymmetric structure 4c is more stable than the symmetric structure 4c'. Likewise,
when solvent effects were considered in DMSO, the symmetric structure was more energetically favourable when compared to the asymmetric, with an energy difference of \(-7.69\) kJ mol\(^{-1}\). This was the largest for any of the complexes in strong agreement with NMR spectroscopy and crystallographic data, both of which show a strong preference for the symmetric isomer.

### 5.3 Radiochemistry experiments

A major goal of this Chapter was to establish whether the indium complexes could be readily radiolabelled, as this would render them useful as new SPECT molecular imaging probes. These experiments were carried out at the Oxford Siemens Laboratory, University of Oxford under the supervision of Dr Phillip A. Waghorn and Prof Jon Dilworth.

![Figure 5.4](image)

Figure 5.4. Overlay of UV-HPLC trace (black) of aromatic zinc bis(thiosemicarbazonato) precursor with the radio-HPLC trace (blue) of aromatic \(^{111}\)InCl bis(thiosemicarbazonato) complex with compound 1b.

In a typical experiment, a 1 mg/mL stock solution of the complex was prepared in DMSO, from which 10 µL was added to 100 µL of ethanol and was further diluted with 100 µL of water. Subsequently 10 µL of InCl\(_3\) (ca. 10 MBq) was added to the solution and was heated at 60°C. After 15 minutes, an aliquot of 30 µL was removed from the solution and analysed using HPLC with a C18 column and a mobile phase of 0.1 % (v/v) TFA in acetonitrile/water. Figure 5.4 shows an overlay of the UV-HPLC trace of the zinc
precursor and the indium-111 complex for 1b. Data show that could be made rapidly and cleanly for these complexes by transmetallation despite the UV traces indicating that 1c contained a mixture of symmetric and asymmetric isomers as shown by the two slightly differing retention times (Figure 5.5).

![Figure 5.5. Overlay of UV-HPLC trace (black) of aromatic zinc bis(thiosemicarbazone) precursor with the radio-HPLC trace (blue) of aromatic 111InCl bis(thiosemicarbazone) complex with compound 1c.](image)

Due to the importance of lipophillicity and liver uptake the partition coefficient (log $P$) was calculated for these complexes with values of $1.19 \pm 0.09$, $1.07 \pm 0.03$, $0.94 \pm 0.13$ and $1.20 \pm 0.11$ for compounds 4a, 4b, 4c and 4d respectively. The log $P$ values were comparable to yet slightly lower than their analogous copper complexes, importantly they are significantly less lipophilic than Cu[ATSM], with a log $P$ of ca. 1.48, indicating a likely reduction of accumulation in the liver with respect to this well-known copper analogue.\(^{149}\)

### 5.2 Single crystal X-ray crystallography

Crystals were grown in THF:Hexane by a standard liquid or gas phase diffusion method for complexes 4a, 4b, 4c and 4d. X-ray structure determinations showed that, in agreement with NMR spectroscopy and DFT calculations, there is a strong preference for the symmetric isomer formation, and this was observed in the crystal structures of
all compounds studied here. As was observed with symmetric gallium compounds, each indium complex possessed a plane of symmetry across the backbone and symmetric binding to the metal, yet the terminal functionalities (Et, Ph and Allyl) are asymmetric (Figures 5.6 and 5.7).

Table 5.1: Selected bond lengths (Å) and bond angles (°) for compounds 4a-d, determined by X-ray diffraction

<table>
<thead>
<tr>
<th>Compound/ Molecular Parameters</th>
<th>4a (E,E)</th>
<th>4b (E,E)</th>
<th>4c (E,E)</th>
<th>4d (E,E)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-Cl</td>
<td>2.434(4)</td>
<td>2.4180(16)</td>
<td>2.3960(15)</td>
<td>2.4157(8)</td>
</tr>
<tr>
<td>M-N</td>
<td>2.260(13)</td>
<td>2.244(4)</td>
<td>2.281(4)</td>
<td>2.257(2)</td>
</tr>
<tr>
<td>M-S</td>
<td>2.305(14)</td>
<td>2.250(4)</td>
<td>2.278(4)</td>
<td>2.270(2)</td>
</tr>
<tr>
<td>C-C</td>
<td>2.474(5)</td>
<td>2.4602(14)</td>
<td>2.4638(14)</td>
<td>2.4784(7)</td>
</tr>
<tr>
<td>N-N</td>
<td>2.472(5)</td>
<td>2.4658(14)</td>
<td>2.4998(14)</td>
<td>2.4693(8)</td>
</tr>
<tr>
<td>C=N</td>
<td>1.492(2)</td>
<td>1.487(7)</td>
<td>1.478(7)</td>
<td>1.492(4)</td>
</tr>
<tr>
<td>N-N</td>
<td>1.349(18)</td>
<td>1.360(6)</td>
<td>1.349(6)</td>
<td>1.364(3)</td>
</tr>
<tr>
<td>S-M-S</td>
<td>123.19(15)</td>
<td>117.93(5)</td>
<td>120.54(5)</td>
<td>120.15(3)</td>
</tr>
<tr>
<td>S-M-N</td>
<td>76.8(4)</td>
<td>77.39(11)</td>
<td>75.46(11)</td>
<td>76.72(6)</td>
</tr>
<tr>
<td>N-M-N</td>
<td>74.2(4)</td>
<td>77.47(11)</td>
<td>76.44(11)</td>
<td>77.03(6)</td>
</tr>
<tr>
<td>S-M-Cl</td>
<td>72.2(4)</td>
<td>73.17(15)</td>
<td>71.55(15)</td>
<td>72.56(8)</td>
</tr>
<tr>
<td>N-M-Cl</td>
<td>109.5(2)</td>
<td>106.42(5)</td>
<td>106.72(5)</td>
<td>105.63(3)</td>
</tr>
<tr>
<td>N-M-Cl</td>
<td>108.2(2)</td>
<td>107.14(5)</td>
<td>106.41(5)</td>
<td>104.52(3)</td>
</tr>
</tbody>
</table>

A distorted square pyramidal around the metal centre was also notable and was likewise comparable to the gallium complexes with the Cl in the axial position with the base formed by the two nitrogens and two sulphurs. The bond distance of M-Cl is similar for 4a and 4b (2.434(4) Å and 2.4180(16) Å correspondingly) and 4b and 4d (2.4180(16) Å and 2.4157(8) Å respectively) (Table 5.1), but is smaller for 4c (2.3960(15) Å). Likewise the N-M-N angle was shorter for 4c (71.55(15)°) than for 4b (71.22(7)°).
(73.17(15)°) and 4d (72.56(8)°) (whereby the N-M-N of 4a (72.2(4)°) was similar to each of the respective indium complexes.) Moreover, the S-M-S angle of 4b (117.93(5)°) was shorter than those of 4c (120.54(5)°) and 4d (120.15(3)°), which were similar, whilst 4a (123.19(15)°) was notably larger than those of the other indium complexes of this family. Notably, the N-M-Cl angle appeared to increase the larger the functionality (91.0(4)° for methyl-substituted indium complex 4a and 102.2(1)° for phenyl-functionalised indium complex 4c) with the opposite observed for the S-M-Cl angle (e.g. 109.5(2)° in 4a and 106.72(5)° in 4c). The other bond lengths and angles of these indium compounds were comparable indicating that despite the functionality at the exocyclic nitrogen atoms having some effect on the rest of the structure, this effect is limited.

![Figure 5.6](image.png) Figure 5.6. Molecular structures of compounds 4a (above) and 4b (below), ellipsoids drawn at 30% probability, hydrogens omitted for clarity.

Since the phenyl-substituted complex crystal structures were acquired during this study, each possessing the symmetric conformation their comparison can shed light upon the effect of the metal centre on the structure and will be considered below (Table 5.2). For all metal bonds, M-X (where X is Cl or DMSO), M-N, M-S, the bond lengths
were longest for the indium complex, followed by the zinc and the gallium, with the copper complex possessing the shortest metal bonds. Interestingly, the C-C bond is similar for each metal complex, therefore it can be speculated that the difference in this bond length reported previously in the group between (E,Z) isomer of the zinc complex 1d and the (E,E) isomer of the copper complex may be due to crystal packing forces rather than an isomeric or metal-dependant effect.127

### Table 5.2

Selected bond lengths (Å) and bond angles (°) for compounds 1c-4c, determined by X-ray diffraction, where A is adduct atom.

<table>
<thead>
<tr>
<th>Compound/Molecular Parameters</th>
<th>1c (E,E) Isomer</th>
<th>2c (E,E) Isomer</th>
<th>3c (E,E) Isomer</th>
<th>4c (E,E) Isomer</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-Cl/M-O</td>
<td>*2.0642(12)</td>
<td>2.2096(14)</td>
<td>2.3960(15)</td>
<td></td>
</tr>
<tr>
<td>M-N</td>
<td>2.1092(14)</td>
<td>1.967(5)</td>
<td>2.069(4)</td>
<td>2.281(4)</td>
</tr>
<tr>
<td>M-S</td>
<td>2.1603(14)</td>
<td>1.976(5)</td>
<td>2.075(4)</td>
<td>2.278(4)</td>
</tr>
<tr>
<td>C-C</td>
<td>2.3330(5)</td>
<td>2.2508(17)</td>
<td>2.3130(16)</td>
<td>2.4638(14)</td>
</tr>
<tr>
<td>C=N</td>
<td>2.3691(5)</td>
<td>2.2598(18)</td>
<td>2.3171(15)</td>
<td>2.4998(14)</td>
</tr>
<tr>
<td>N-N</td>
<td>1.499(3)</td>
<td>1.481(8)</td>
<td>1.488(6)</td>
<td>1.478(7)</td>
</tr>
<tr>
<td>N-M</td>
<td>1.364(2)</td>
<td>1.382(6)</td>
<td>1.372(5)</td>
<td>1.349(6)</td>
</tr>
<tr>
<td>N-M-A</td>
<td>1.364(2)</td>
<td>1.384(6)</td>
<td>1.371(5)</td>
<td>1.359(6)</td>
</tr>
<tr>
<td>S-M-S</td>
<td>1.294(2)</td>
<td>1.286(7)</td>
<td>1.383(7)</td>
<td>1.297(7)</td>
</tr>
<tr>
<td>S-M-N</td>
<td>1.292(2)</td>
<td>1.293(7)</td>
<td>1.369(7)</td>
<td>1.292(7)</td>
</tr>
<tr>
<td>S-M-A</td>
<td>115.636(19)</td>
<td>110.99(7)</td>
<td>105.11(5)</td>
<td>120.54(5)</td>
</tr>
<tr>
<td>S-M-A</td>
<td>80.27(4)</td>
<td>83.64(16)</td>
<td>81.42(12)</td>
<td>75.46(11)</td>
</tr>
<tr>
<td>S-M-A</td>
<td>78.96(4)</td>
<td>83.72(15)</td>
<td>81.43(11)</td>
<td>76.44(11)</td>
</tr>
<tr>
<td>N-M-N</td>
<td>81.65(5)</td>
<td>81.6(2)</td>
<td>77.33(15)</td>
<td>71.55(15)</td>
</tr>
<tr>
<td>N-M-A</td>
<td>104.31(4)</td>
<td>n/a</td>
<td>108.56(6)</td>
<td>106.72(5)</td>
</tr>
<tr>
<td>N-M-A</td>
<td>102.01(4)</td>
<td>n/a</td>
<td>108.38(6)</td>
<td>106.41(5)</td>
</tr>
<tr>
<td>N-M-A</td>
<td>101.11(5)</td>
<td>n/a</td>
<td>99.7(1)</td>
<td>102.2(1)</td>
</tr>
<tr>
<td>N-M-A</td>
<td>93.77(5)</td>
<td>n/a</td>
<td>98.4(1)</td>
<td>100.1(1)</td>
</tr>
</tbody>
</table>

*For 1c this is representative of a DMSO adduct*

Interestingly, the C=N of the gallium complex is significantly longer than that of the other molecules, for which this bond is similar in length. Moreover, the S-M-A angle
was largest for gallium compound \(3c\) \((108.56(6))°\) and smallest for zinc complex \(1c\) \((104.31(4))°\), with the N-M-A largest for indium complex \(4c\) \((102.2(1))°\). The angles S-M-N and N-M-N are largest with the copper metal centre \((83.64(16))°\) and \(81.6(2)°\) respectively), followed by with a gallium atom \((81.42(12))°\) and \(77.33(15)°\) correspondingly), whereby for the indium complex these angles are the smallest \((75.46(11))°\) and \(71.55(15)°\) respectively). The opposite trend is true (with the gallium the smallest angle rather than the copper) for S-M-S angle indicating that whilst the sulphur atoms are closer in comparison to each other, the nitrogen atoms are further away from each other around the metal centre for the indium complexes, when compared to the copper and gallium complexes.

**Figure 5.7.** Crystal structures of \(4c\) (above) and \(4d\) (below), ellipsoids drawn at 30% probability, hydrogens omitted for clarity.

### 5.4 Spectroscopy

Fluorescence spectra were obtained in DMSO for initial scans between 200-800 nm, in order to obtain information about intrinsic fluorescence and \(\lambda_{em-max}\) using solutions of
100 μM. Ranges of absorption and emission, which are important with regards to cytotoxicity assays and fluorescence imaging, were also assessed using the 2D contours as a guideline (Figure 5.8). Relevant cytotoxicity assays use absorbance readings at 570 nm, therefore there should be minimal or no excitation at this wavelength, which was true for all of the indium complexes, with very low absorbance for each.

**Figure 5.8.** Excitation/emission map at 100 μM in DMSO of compound 4b (above) and 4c (below)

Complexes 4a, 4b and 4d displayed very similar absorption (ca. 250 – 570 nm) and emissions (ca. 515 – 685 nm), each with $\lambda_{\text{max}}$ of emission ca. 560 nm (Table 5.3). Each compound had a $\lambda_{\text{max}}$ of 400 nm, with 4c possessing a much broader range of excitation
and emission than the other functionalities, as well as a higher $\lambda_{\text{max}}$ of emission of 590 nm.

Table 5.3. Fluorescence quantum yield measurements of complexes 4a-4d.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$\lambda_{\text{ex-max}}$ / nm</th>
<th>Excitation range / nm</th>
<th>$\lambda_{\text{em-max}}$ / nm</th>
<th>Emission range / nm</th>
<th>Quantum Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>4a</td>
<td>400</td>
<td>250-570</td>
<td>556</td>
<td>520-680</td>
<td>0.31</td>
</tr>
<tr>
<td>4b</td>
<td>400</td>
<td>250-570</td>
<td>556</td>
<td>515-685</td>
<td>0.19</td>
</tr>
<tr>
<td>4c</td>
<td>400</td>
<td>250-610</td>
<td>590</td>
<td>540-740</td>
<td>0.15</td>
</tr>
<tr>
<td>4d</td>
<td>400</td>
<td>250-580</td>
<td>559</td>
<td>515-685</td>
<td>0.13</td>
</tr>
</tbody>
</table>

\[
\Phi_S = \Phi_R \cdot \left( \frac{D_S}{D_R} \right) \cdot \left( \frac{A_R}{A_S} \right) \cdot \left( \frac{I_R}{I_S} \right) \cdot \left( \frac{\eta_S}{\eta_R} \right)^2
\]

Quantum yields were calculated using the standard solution of [Ru(bipy)$_3$](PF$_6$)$_2$ in water as a reference and utilising the equation above. Where $\Phi$ = quantum yield, $D$ = integrated area under emission band, $S$ = sample, $A$ = absorbance of solution at excitation wavelength, $R$ = reference, $\eta$ = refractive index of solvent, $I$ = maximum intensity of excitation band [Ru(bipy)$_3$]PF$_6$ in water was used as a reference, with a quantum yield of 0.042. The quantum yields of the indium compounds higher than their respective gallium complexes and were sufficient to proceed to fluorescence imaging in biological cells.
5.5 Kinetic stability tests

In vitro assays were carried out to determine the stability of complexes for use as medical imaging probes. Demetallation could result in non-target tissue uptake and poor image contrast in vivo. Stability is therefore essential for the intended function of these complexes and a series of assays with excess of a numerous biologically relevant chemicals have been carried out up to 24 hours. These include Glutathione (GSH), L-cysteine, L-histidine, L-methionine, citric acid, ethylenediaminetetraacetic acid (EDTA), biomimetic eagle’s modified essential media (EMEM) (both with and without foetal calf serum) and biologically relevant pH buffers. Indeed, stability of these complexes in a range of pHs is significant due to the tendency of cancer cells to be slightly more acidic than non-cancerous cells.

UV-visible spectroscopy was utilised therefore to assess the kinetic stability of complexes and fluorescence to provide further insight whilst in the presence of biological media. Tests were carried out at room temperature at 100 µM in a 1:1 DMSO:assay agent solution to ensure sufficient solubility and at a concentration providing detectable fluorescence, with the assistance of Johan Stojanović, technician student from ESCOM, France.

![Fluorescence spectroscopy of compound 4a: FCS stability assay (100 µM FCS: DMSO, 1: 1).](image)

Figure 5.9. Fluorescence spectroscopy of compound 4a: FCS stability assay (100 µM FCS: DMSO, 1: 1).
Figure 5.10. Fluorescence spectroscopy of compound 4c: FCS stability assay (100 µM FCS: DMSO, 1:1).

Preliminary fluorescence studies focussing on EMEM containing 10% Foetal Calf Serum (FCS) demonstrated a general decreased intensity with time for all indium compounds, with the most significant reduction within the first hour, followed by equilibration after ca. 6h (Figures 5.9-5.10, Appendix Figures D.31-34, D.46). There was only a very limited a shift in the fluorescence maxima implying that no new species evolved. Spectra are almost identical for 4a, 4b and 4d, whereas 4c had a much lower initial fluorescence, but also a much lower rate of decline, indeed 4c displayed notable fluorescence even after 24 h. Due to the significantly greater fluorescence of the indium complexes in comparison with the free ligand, fluorescence intensity observed is indicative of complex stability (as was also observed with the gallium complexes). However, since the free ligand does possess some fluorescence, it is important to consider the UV-Visible spectra of complexes to confirm stability under these conditions.
Figure 5.11. Serum assays (99% FCS, 1% DMSO) by UV/Vis (below) for compound 4d, at 100 µM, monitored at time intervals up to 24h at room temperature and filtered using a 0.45 µm PTFE microfilter. (above) The structure of [GaCl(ATSM)] is also shown. This compound which was found not to be stable under comparable conditions.

In conjunction with a study comparing [In-Cl(ATSM)], which was found to form the corresponding to free ligand within 5 minutes in 99% FCS, 1% DMSO, with indium complex 4d tested under analogous conditions (Figure 5.11). Whilst changes in the UV/Vis spectra for the indium complex 4d were observed over an incubation of 24 h in serum, it is apparent that the product of the challenge has not completely converted to free ligand iid.
Table 5.4. Summary of estimated complex remaining from UV-visible data at 1:1 DMSO:biologically relevant agent at 15 minutes and 24 h where Citric Acid, EDTA, L-Cys, L-His, L-Met and GSH were in MilliQ water.

<table>
<thead>
<tr>
<th>Assay</th>
<th>4d, 100 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15 min&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>DMSO</td>
<td>86.6</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>94.8</td>
</tr>
<tr>
<td>PBS</td>
<td>66.8</td>
</tr>
<tr>
<td>5% FCS, MEM</td>
<td>77.9</td>
</tr>
<tr>
<td>SFM, MEM</td>
<td>81.4</td>
</tr>
<tr>
<td>Citric Acid</td>
<td>85.2</td>
</tr>
<tr>
<td>EDTA</td>
<td>94.2</td>
</tr>
<tr>
<td>L-Cys</td>
<td>94.3</td>
</tr>
<tr>
<td>L-His</td>
<td>80.8</td>
</tr>
<tr>
<td>L-Met</td>
<td>77.6</td>
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<tr>
<td>GSH</td>
<td>62.1</td>
</tr>
</tbody>
</table>

Following initial stability tests in pH buffers, in EMEM and in FCS a more in-depth study focussing on the indium allyl derivative as a representative example of this class of compounds. Indium complex 4d, however exhibited good stability in each media with no significant change in absorbance between 15 minutes and 24 hours room temperature incubation in DMSO and high percentage remaining complex after 24 h incubation in each of the above solvents (94.4 %, 61.5 %, 69.4 %, 76.9 % respectively).

This investigation utilised both UV-visible absorption (at 10 µM and at 100 µM) and fluorescence emission at 100 µM, with 1:1 DMSO:challanger to limit precipitation. This allowed a % stability to be estimated via UV-Visible spectroscopy at 15 minutes and 24 hours as calculated by (100 % - percentage converted to free ligand) (Table 5.4). The first assays carried out in DMSO, water, PBS, 5% FCS EMEM and SFM EMEM enabled complex stability to be understood in media likely to be encountered in a cell biology experiment.
Indium complex 4d, contrary to the gallium complex 3d exhibited good stability in each media with no significant change in absorbance between 15 minutes and 24 hours in DMSO and high percentage remaining complex after 24 h incubation in each of the aforementioned solvents (86.6, 94.4 %, 61.5 %, 69.4 %, 76.9 % respectively) (Figures 5.12-15, Appendix Figures D.31, D.46). The fluorescence study was carried out in parallel largely confirming the UV-visible spectroscopy data. There was no significant change in fluorescence intensity of the indium complex in DMSO over a period of 24 h.

As was observed for the gallium complex, the fluorescence intensity for the indium complex in PBS were significantly lower than in water, combined with a shift in
absorbance is indicative of some decomposition into bis-substituted free ligand (*Figure 5.13*). The absorbance of the indium complex remained yet reduced at 500 nm after 24 h, in contrast characteristic bands of the gallium complex (*Chapter 4.5*) were no longer present and displayed the form of the bis-substituted free ligand as investigated using UV-visible spectroscopy.

*Figure 5.13.* PBS assays (50% DMSO, 50% PBS) for compound 4d at 100 µM, by fluorescence spectroscopy (above) UV/Vis (below) monitored at time intervals up to 24h at room temperature.

This suggests that in the case of the indium complex a significant proportion of the complex has remains intact over the observation period. This was in contrast to the finding for the gallium complex, which appeared to have converted into the ligand within 15 minutes incubation under the conditions above. This may be assigned to the
higher affinity of the indium complex for soft donor ligands, such as a bis(thiosemicarbazone).

![Graphs showing fluorescence and absorption spectra](image)

**Figure 5.14.** EEMEM SFM assays (50% DMSO, 50% SFM) for compound 4d at 100 µM, by Fluorescence (above) UV/Vis (below) spectroscopies monitored at time intervals up to 24h at room temperature.

Interestingly, the zinc complex 1d displayed little change in fluorescence and maintained its characteristic absorption spectrum (*Chapter 3.7*), signifying better stability than that of the gallium and indium complex in PBS. The stability of the complex 4d was tested against Eagle’s Minimum Essential Medium Serum Free Medium (SFM), which complexes are often incubated in for cell imaging.
The fluorescence emission of each of the complex decreases over the time period studied at the $\lambda_{\text{max}}$. Compound 4d showed a very significant decrease in fluorescence over 24 h (to 17 % of the original), coupled with a small red shift from 551.5 nm to 556.5 nm. Since the free ligand formation would be accompanied by a blue shift (approaching $\lambda_{\text{max}}$ 531 nm) presumably the shift and intensity decrease are due to quenching, which may be caused by the interaction with the media, protonation or excimer formation, rather than significant decomposition to free ligand.
Moreover, these data suggest that it is likely that the complex remains intact under the conditions investigated hereby. When incubated in FCS EMEM, indium complex 4d displayed a significant reduction in fluorescence and an increase in absorbance at 468 nm, this could either indicate conversion to Compound iid (λ_{max} 412 nm) or interaction with protein molecules present in serum and quenching. It is apparent, however, that 4d did not fully convert into the corresponding free ligand iid, confirming stability of this complex up to 24 hours (Figure 5.15).

The complex 4d was therefore found to be intact for all five of the above media (DMSO, water, PBS, SFM and 5% FCS EMEM). It was thus appropriate to explore specific interactions with several specific biologically relevant agents in common use.
5.5.1 Assays with biologically relevant agents

Biologically relevant agents were chosen to provide a better insight into the stability of these complexes, as were introduced in (Chapter 3).\textsuperscript{145} Complexes were tested in presence of 2 equivalents of citric acid, L-methionine, Glutathione (GSH), L-cysteine, L-histidine, and EDTA. Indium complex 4d displayed very similar properties in citric acid, and sulphur containing L-Met and L-Cys in water in that there was little observable change in the spectral properties between 15 minutes and 24 h (Figure 5.16 and Appendix Figure D.34). The characteristic absorbance spectrum for Compound 4d was observable indicating very good stability of this complex up to 24 h in these solutions.

Figure 5.16. L-cysteine spectroscopic stability assays (50% DMSO, 50% water, 2 eq of L-cysteine) for compound 4d at 100 µM, by fluorescence spectroscopy (above) UV/Vis (below) spectra monitored at time intervals up to 24h at room temperature.
Figure 5.17. L-histidine challenge by spectroscopic assays (50% DMSO, 50% water, 2 eq of L-histidine) for compound 4d at 100 µM, by fluorescence spectroscopy (above) UV/Vis (below) monitored at time intervals up to 24h at room temperature.

The fluorescence spectroscopy assay involving the addition of N-donating L-His to solutions of Compound 4d resulted in a significant increase in fluorescence emission (505 nm). UV-Visible Spectroscopy showed that the mixture possessed spectral characteristics typical of 4d. The absorbance band decreased slightly, but remained notable (Figure 5.17). This therefore suggests that whilst some of the indium complex may have been converted to free ligand it appears that most of it remains chelated.
When GSH was added to compound 4d a notable increase in fluorescence emission and a slight increase in absorbance band (in the UV-Vis spectrum at both 10 µM and 100 µM concentrations) with the distinctive 4d spectrum were observable up to 24 h. This assay also suggests the compound stability under these conditions (Figure 5.18).

![Graph showing fluorescence and absorbance spectra](image)

**Figure 5.18.** Glutathione challenge by spectroscopic assays (50% DMSO, 50% water, 2 eq of Glutathione) for compound 4d at 100 µM, by fluorescence spectroscopy (above) UV/Vis (below) monitored at time intervals up to 24h at room temperature.

When compound 4d was assayed with 2 equivalents of EDTA, in aqueous media, fluorescence spectroscopies showed a notable decrease in fluorescence emission (12% of original) and blue shift of 64 nm. A decrease in absorbance band was observed in UV-Vis spectroscopy and the characteristic shape of 4d spectra was found. This
demonstrates some stability of this complex up to 24 h, indicative of the high stability of this complex in the presence of a strong chelator (*Figure 5.20*).

![Graph](image.png)

**Figure 5.19.** L-methionine spectroscopic assays (50% DMSO, 50% PBS, 2 eq of L-methionine) for compound 4d at 100 μM, by fluorescence spectroscopy (above) UV/Vis (below) spectra were monitored at time intervals up to 24h at room temperature.

Additionally, the kinetic stability of compound 4d was evaluated in aqueous solutions of PBS (phosphate buffered saline, which contains NaCl, KCl, Na\textsubscript{2}O\textsubscript{4}HP and KO\textsubscript{4}H\textsubscript{2}P). Unlike similar assays with L-Met or citric acid carried out without PBS (which all showed disappearance of most of the fluorescence emission intensity) an increased
absorbance at 460 nm and the decrease of the band at ca. 505 nm was observed agents tested, suggesting that complex had demetallated and therefore that PBS reduces the stability of these complexes (*Figure 5.19, Appendix Figure D.32-34*).

*Figure 5.20.* EDTA assays (50% DMSO, 50% water, 2 eq of EDTA) for compound 4d at 100 µM, by fluorescence spectroscopy (above) UV/Vis (below) monitored at time intervals up to 24h at room temperature.

Overall these tests provide strong evidence that 4d is stable in the presence of biologically relevant agents in water, whereby challenger assays did not reveal a specific stability based vulnerability.
5.5.2 pH buffer stability tests

The stability of complexes at biologically relevant pH is an important factor in this study. Cancer cells are slightly more acid than non-cancerous cells it is important that the molecular probes are stable at lower pH. All fluorescent scans were carried out with an excitation wavelength of 400 nm and at a concentration of 100 µM, with a 1:1 DMSO:buffer solution. An initial preliminary study was carried out using fluorescence spectroscopy for gallium and indium complexes. There is a clear trend in the behaviour of the indium complexes in these buffers. All displayed fluorescence between pH 2 and pH 9, indicating that compounds have good stability at normal physiological pH and reflecting their potential for protonation and deprotonation (Figure 5.21). Under more alkaline conditions the emission bands are slightly blueshifted, which indicates that a different species may be present, perhaps OH⁻ has interchanged with the Cl⁻.

Table 5.5. Summary of UV-visible data at 50: 50 DMSO: pH buffer at 15 minutes and 24 h, where NS = not stable.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Measured pH</th>
<th>% stability at 15 mins</th>
<th>% stability at 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 1.1</td>
<td>1.6</td>
<td>76.1</td>
<td>NS</td>
</tr>
<tr>
<td>pH 2</td>
<td>2.5</td>
<td>99.0</td>
<td>NS</td>
</tr>
<tr>
<td>pH 3</td>
<td>4.4</td>
<td>95.1</td>
<td>95.0</td>
</tr>
<tr>
<td>pH 5</td>
<td>6.9</td>
<td>93.3</td>
<td>93.0</td>
</tr>
<tr>
<td>pH 7</td>
<td>9.4</td>
<td>86.4</td>
<td>37.3</td>
</tr>
<tr>
<td>pH 9</td>
<td>9.35</td>
<td>83.9</td>
<td>81.4</td>
</tr>
<tr>
<td>pH 10</td>
<td>12.7</td>
<td>87.9</td>
<td>87.7</td>
</tr>
</tbody>
</table>

Following the above preliminary study allyl substituted compounds were selected as representative of the other functionality to assess the stability of newly synthesised gallium and indium complexes using UV-visible and fluorescence spectroscopy both at 15 minutes and 24 hours, with 1:1 DMSO:buffer solution. The pH of the samples was
measured and found to have no significant difference to the blank, however with a notable variance from that of the original pH solution in each case (Table 5.5).

Furthermore complex 4d (Figures 5.22-5.23 and Appendix Figure D.39-45) appeared most stable at either pH 2 or pH 3 after 15 minutes or less, which is also in agreement with data acquired where citric acid was a component of the buffer solutions. When incubated in pH 1.1 buffer the absorption spectrum of 4d maintained some of its original characteristic shape, suggesting some yet poor stability especially when comparing its fluorescence spectrum, which was almost identical to that of iid (proligand iid buffer study Appendix Figures D.50 and D.51). As was observed with
both 1d and 3d after 24 h fluorescence and absorption of 4d diminished signifying complete decomposition and instability of these complexes under the harsh conditions of a pH 1.1 buffer. Interestingly, despite complex 4d displaying very high stability by both fluorescence and UV-visible after 15 minutes, after 24 h absorbance has decreased and no longer possessed the characteristic 4d complex absorption spectrum in pH 2 buffer. It is most likely, however that some complex does remain since the fluorescence intensity is significantly greater than that of free ligand iid and the UV-visible spectrum of 4d after the 24 h incubation does not resemble that of the free ligand.

Incubations in pH 3.0 and pH 5.0 buffers demonstrated very strong stability, with almost identical spectra by both UV-visible and fluorescence spectroscopy after 15 minutes and 24 hours, each displaying the typical 4d complex absorption spectrum. When challenged with a pH 7.0 and pH 9.0 buffer highly comparable results were found in that good fluorescence and characteristic UV-visible spectra were observable after 15 minutes. This was followed by a blue shift in absorbance and a considerable decrease in fluorescence indicating that a large proportion of the complex has likely formed the free ligand Compound iid, yet with a significant proportion of the indium complex 4d remaining intact under the conditions tested.

Similar to observations made with regards to the zinc and gallium complexes, stability appeared poorer when incubated with a pH 10.0 buffer than with a pH 9.0 or pH 7.0 buffer, here with the blue shift observable after 15 minutes when compared to the characteristic shape of the absorption spectrum of 4d and a similar reduction in fluorescence after 24 h that was observed with the previously discussed pH 7.0 and pH 9.0 buffers. Interestingly, the UV-visible spectrum after 15 minutes was identical to that after 24 hours signifying that the loss of fluorescence intensity could be due to quenching as a result of solvent interaction rather than demetallation.
Figure 5.22. Buffer stability assays (50% DMSO, 50% water) for compound 4d at 100 µM by fluorescence spectroscopy (above) and UV/Vis (below) monitored at 15 minutes at room temperature.

Therefore the stability of this indium complex 4d, representative of the other indium complexes of this family, can be considered to be very broad with significant stability for all buffers in exception of pH 1.1 after 15 minutes, thus displaying fluorescence within a range of pH 2.5 and pH 12.7 for short-term incubations. Longer-term incubations demonstrated fluorescence between pH 2.5 and pH 6.9, with stability observable by UV-visible spectroscopy within the ranges of pH 4.4 and pH 12.7. This
is well within the requirements of biological experiments in strong support of \textit{in vitro} experiments carried out on the indium complexes.

Figure 5.23. Buffer stability assays (50% DMSO, 50% water) for compound 4d at 100 µM by fluorescence spectroscopy (left) and UV/Vis (right) monitored at 24 hours at room temperature.

In summary, all complexes tested possess good stability in biological media within 15 minutes incubation, with the indium complexes appearing kinetically stable after 24 h when assayed with 5% FCS, SFM, Citric Acid, EDTA, L-Cys, L-His, L-Met, GSH and pH 3.0 to pH 10.0 buffers. It was notable that the gallium complex was considerably less kinetically stable than the indium complex under the conditions tested, which could be accountable to the greater affinity of indium for soft donor ligands such as
bis(thiosemicarbazonates) resulting in lower lability in aqueous media. These assays are a good indicator of strong biological stability, indicating that experiments could proceed to testing in vitro.

5.6 Two-photon fluorescence lifetime study

The two-photon investigation was introduced in Chapter 2 and was performed at the Rutherford Appleton Laboratory under the supervision of Dr S Botchway. Indium complexes were incubated at 50 μM, 0.5% DMSO, for 20 minutes, unless stated otherwise in the text. The fluorescence lifetime components $\tau_1$, of the indium complexes in solution were ca. 0.45 ns for 4a, ca. 0.4 ns for 4b, ca. 0.2 ns for 4c and ca. 3.5 ns for 4d (Table 5.6). TCSPC using either 810 nm or 910 nm gave highly similar results under the same conditions.

| Table 5.6. Two-photon Time Correlated Single Photon Counting data of solutions in DMSO of indium bis(thiosemicarbazonato)complexes. |
|-----------------|-------|-------|-------|
| $\lambda_{ex}$ | 4a    | 4b    | 4c    | 4d    |
| 810 nm          | $\chi^2 = 1.06$ | $\chi^2 = 1.19$ | $\chi^2 = 1.24$ | $\chi^2 = 1.11$ |
|                  | 0.454 ns 100% | 0.454 ns 100% | 0.174 ns 54.4% | 0.458 ns 100% |
| 910 nm          | $\chi^2 = 1.42$ | $\chi^2 = 1.32$ | $\chi^2 = 1.42$ | $\chi^2 = 1.43$ |
|                  | 0.466 ns 100% | 0.453 ns 100% | 0.182 ns 58.9% | 0.442 ns 100% |
|                  | 0.375 ns 41.1% |               |               |                 |

| Table 5.7. Two-photon fluorescence lifetime imaging data in HeLa cells of indium bis(thiosemicarbazonato) complexes, $\lambda_{ex} = 810$ nm. |
|-----------------|-------|-------|-------|
| Compound        | 4b    | 4c    |
| $\chi^2$        | 1.33  | 1.26  |
| $\tau_1$ %      | 92    | 83    |
| $\tau_1$ / ns   | 0.262 | 0.278 |
| FWHM / ns       | 0.066 | 0.412 |
| $\tau_2$ %      | 8     | 17    |
| $\tau_2$ / ns   | 1.314 | 2.265 |
| FWHM / ns       | 0.915 | 2.065 |
There were small differences between the $\tau_1$ values of the indium complexes in cancer cells and in solution, but this can largely be attributable to the interaction with cellular components or biological media. As was the case with the gallium complexes when excited with 810 nm or 910 nm in vitro, the minor component was longer than expected according to the solution data (Appendix Table F.8). A long component of ca. 2 ns for 4b and 4c was observable as a minor component using $\lambda_{ex} = 810$ nm indicating that a small proportion of the complex may convert to free ligand within the cell during the time course of this experiment. This could mean in both cases the presence of autofluorescence or of ligand precursor, however for the purposes of these experiments is not significant since the longer minor component was proportionally quite small (between 8 and 17 %) and therefore most of the complex is stable under these conditions (Table 5.7). Additionally, the indium compounds were considerably more fluorescent than their corresponding proligand at 910 nm, therefore the better intensity obtained at lower concentrations for the complexes is also indicative of stability (Figure 5.24).

Fluorescence lifetime imaging was therefore used to provide evidence that the newly synthesised bis(thiosemicarbazonato) complexes have good stability in cells. The
combined use of UV-Visible spectroscopy, fluorescence spectroscopy and FLIM offer a powerful method of investigating small molecule stability in both solution and in cells.

5.7 Laser scanning confocal microscopy

Cells were cultured using standard protocols, analogous to earlier investigations on fluorescent thiosemicarbazones, as described in Chapter 4.5. Imaging conditions for complexes 4a-4d were standardised in an identical manner to the gallium complexes, with standard incubations of 0.5 % DMSO, 50 µM for 20 minutes. Cells were washed three times with PBS, which was replaced with serum free medium. As was observed with complexes 3a-3d, compounds 4a-4d caused significant changes in cell morphology after 60 minutes and therefore 20 minute incubation times were used, which showed little cytotoxicity.

Figure 5.25. Single-photon confocal microscopy images of 4c in MCF-7 cells, 20 minute incubation, 50 µM 0.5% DMSO at 37°C (a) DIC image, (b) excitation at 405 nm, emission 420-480 nm (c) excitation at 488 nm, emission >505 nm, (d) micrograph of cells after excitation at 543 nm and (e) is an overlay of (a), (b), (c) and (d) Scalebar: 20 µm
Initial experiments were carried out with the complex incubated alone, whereby each compound displayed significant fluorescence within cells when excited at 488 nm, with a long pass filter of 515 nm (Figure 5.26). With the exception of 4b, each complex showed sufficiently weak fluorescence when excited at 543 nm, using a long pass filter at 606 nm enabling experiments with red colocalisation dyes to proceed (Figure 5.27).

There was seldom any fluorescence in the blue channel when exciting at 405 nm at 37°C for all of the indium complexes an example of which is included (Figures 5.26, 5.27 and Appendix G).

Figure 5.26. Single-photon confocal microscopy images of 4b at 50 μM 0.5% DMSO in HeLa cells, 20 minute incubation, at 37°C (b) excitation at 488 nm (c) micrograph of cells after excitation at 543 nm and (d) is an overlay of fluorescence micrographs excitation at 405 nm and (a-b) and (e), (f) and (g) are the DIC image from excitation at 405, 488 and 543 nm, respectively. Scalebar: 20 μm.

At 4°C red-shifted fluorescence emission was observed an effect that was also displayed for the analogous gallium complex (Appendix Figure G.20). Interestingly, rapid phototoxicity was observed when irradiating with light (405 nm, 488 nm or 543 nm)
when HeLa cells were incubated with 4b (Figure 5.27), which occurred in the time taken to acquire the image. Such an intense photoactivity was not observed for any other complex of this class, with the ethyl-substituted complex therefore possessing interesting photosensitive properties.

Furthermore, Figures 5.27 e-g shows blebbing occurring within the cell, however no changes in localisation were observed as with complex 3b (Section 4.5). Uptake was observed at both 37°C and 4°C, which indicates that entry to cells can occur by passive diffusion alone. Interestingly, emission in the green channel was much weaker at 4°C (Appendix Figure G.25), with increased fluorescence in the blue channel (λ_{ex} = 405) and in the red channel (λ_{ex} = 543). However, complex 4c was irradiated for ca. 10 minutes with 488 nm and did not result in a significant change in cell morphology or size indicating that this compound was not phototoxic under the conditions tested, confirming the significance of functionality to this study (Appendix Figures G.26-27).

Figure 5.27. Single-photon Epi-fluorescence microscopy images of 4d in FEK-4 cells, excitation with light 400-500 nm, emission >505 nm 3 hour incubation, 100 μM, 1% DMSO at 37 °C (left) and at 4°C (right). Scalebar: 20 μm.
Figure 5.28. Single-photon confocal microscopy images with Hoechst stain of 4a at 50 μM 0.5% DMSO at 37°C, and in HeLa cells after 20 minutes. (a) Micrographs of cells after excitation at 488 nm. b) Overlays of respectively, of the corresponding DIC image and the micrograph after excitation at 405 nm. Scalebar: 20 μm

Figure 5.29. Single-photon confocal microscopy images with Hoechst stain and 4c at 50 μM 0.5% DMSO at 37°C, 6 hour incubation in PC-3 cells (c-d). (a) Micrograph of cells after excitation at 488 nm. b) Overlay of (a), of the corresponding DIC image and the micrograph after excitation at 405 nm, emission 420-480 nm (a), (b) and (c). Scalebar: 20 μm

Incubations of 4b and 4d in FEK-4 cells over 3 hours (with concentrations as high as 100 μM) followed by Epi-Fluorescence microscopy showed that compounds entered cells at both 4°C and 37°C. Fluorescence imaging seems to suggest that the compound
enters the nucleus under both conditions (*Figures 5.28*). Additionally cellular morphology was not visibly affected on the timescale of this experiment, with highly comparable data acquired for 4b and 4d. This contrasts with the results observed at the incubation of these compounds (50 µM) in HeLa and PC-3 cells, which showed morphological changes even after 60 minutes at either 4°C and 37°C.

This suggests that these compounds may be more cytotoxic towards cancer cell lines than non-cancer cells and are explored in Chapter 6. To obtain further confirmation of the nuclear uptake, cells were co-stained with Hoechst (nuclear stains from Invitrogen) as well as the compounds. Cells were incubated with 50 µM of the compound using 0.5% DMSO for 20 minutes, 60 minutes and up to 6 hours. Complexes 4a, 4b and 4d entered the nucleus of cancer cells within 60 minutes, with low levels of uptake after 20 minutes (*Figure 5.29 and Appendix Figures G.35-45*). Compound 4c, however did not co-localise with Hoechst after 6 hours confirming that the compound does not enter the nucleus (*Figure 5.30 and Appendix Figure G.45*). Intact cellular morphologies were observed after 20 minute incubations suggesting that uptake does not compromise the nuclear membrane as a result of complex toxicity.

### 5.7.1 Fluorescence images of the nucleus and chromosomes

Due to the observed nuclear uptake a preliminary investigation was carried out at the Rutherford Appleton Laboratory with Dr Yusuf Mohammed and Ms Ana Estandarte to assess whether the complexes bind to chromosomes. Non-cancerous lung cell line CCD37-LU was cultured in DMEM containing 10% foetal bovine serum (FBS) and 1% L-Glutamine at 37°C in a 5% CO2 incubator. Chromosomes were isolated via a Colcemid (Gibco BRL) treatment before cells were trypsinised and subsequently resuspended in pre-warmed KCl hypotonic solution (0.075M) at 37°C for 12 minutes. This was followed by fixing in a 3:1 methanol/acetic acid solution, which was added onto a glass microscope slide, to which 100 µL of Compound 4d (100 µM) was aliquoted and was washed with DMSO after 15 minutes.
Finally images were acquired using a Zeiss Axio Imager 2 microscope, which showed uptake of the complex in both the nucleus and in chromosomes. The entry to the nucleus was expected and is in agreement with the Hoechst study above and with apparent uniform binding to chromosomes (Figure 5.30). Further work could be carried out to assess if band patterns observed with this complex 4d is different for cancer and non-cancer cells, which if so could present diagnostic potential as a chromosomal fluorescent stain.151

As discussed above, 4c did not enter the nucleus and therefore behaves differently when compared to other indium compounds. It was subsequently was found to localise within the endoplasmic reticulum, which is involved in protein and lipid synthesis (Figure 5.31a). It can be hypothesised that the aromatic functionality enables different binding modes within the cell when compared to the other substituent groups. Moreover compounds 4a, 4c and 4d appeared to localise in the lysosome and the mitochondria (Figure 5.31 b-d).
In summary uptake of indium complexes was similar to that of the gallium complexes in that localisation was observed in the mitochondria and the lysosome, long-term experiments more than 20 minutes caused cytotoxicity and photoactivity was observed for the ethyl-substituted complex. Interestingly localisation did not change when irradiated with light. Another notable difference was that indium complexes entered the nucleus much more readily than other compounds of this type, with a further study
Chapter 5

5.8 In vitro hypoxia selectivity testing

An analogous study to the one carried out using the gallium complexes in EMT6 (murine breast carcinoma) and PC-3 cells, was carried out under the supervision of Prof Eric Aboagye (by Dr Israt S. Alam) at Hammersmith Hospital, Imperial College London. Compounds 4c and 4d were incubated in cells at 50 µM, 4% DMSO, with normoxic conditions of 20.7% O₂ and 5% CO₂ at 37°C, with hypoxic samples pre-incubated for 20 minutes at 1% O₂, 5% CO₂ at 37°C before complex addition. Following addition of the compound, cells were incubated for a further 20 minutes and subsequently washed three times with PBS before being returned to serum free media and imaged immediately.

Figure 5.32. Confocal micrographs of 4d in FEK-4 cells under normoxic (left) and hypoxic (right) conditions.

An initial confocal study was carried out within which significant differences in fluorescence were biologically relevant to determine (Figure 5.32). Therefore a flow cytometry study was carried out, which as was observed in the gallium complex, fluorescence was lower under hypoxic conditions than normoxic conditions. For the allyl-substituted indium complex, the fluorescence was lower by 25.4% (*P<0.01) in EMT6 cells and by 19.1% (*P<0.05) in PC-3 cells. Meanwhile the fluorescence
emission under hypoxic conditions for complex 4c seems lower by 22% (*P<0.05) in EMT6 cells and by 8% (*P<0.05) in PC-3 cells (Figure 5.33).

![Diagram of MFU values under hypoxia and normoxia for EMT6 and PC-3 cells](image)

**Figure 5.33.** Flow cytometry studies under normoxia and hypoxia of 4d in EMT6 (above) and in PC-3 cells (below).

The level of hypoxia selectivity is therefore similar for 4c and 4d, with the selectivity of 4d slightly greater than that of 4c. It is likely as was observed with the gallium complex that under hypoxia and the resultant reduction of the metal centre the complex demetallated causing a loss of fluorescence. A future study using radiochemical techniques could further confirm the hypoxic selectivity of these complexes.
5.9 Summary to Chapter 5

New indium bis(thiosemicarbazonato) complexes have been synthesised and characterised by NMR spectroscopy, ES-MS and X-ray crystallography. Data show a strong preference towards the symmetric isomer, with only this isomer assignable by NMR X-ray crystallography and DFT calculations are in agreement with this, with the optimised symmetric complex more stable than the asymmetric complex in the gas phase and in solution (a finding not observed for the respective zinc, copper or gallium complex). Furthermore complexes were cleanly and rapidly radiolabelled in addition to possessing sufficient fluorescence to continue the study with an in vitro cell uptake study. Complexes were found to possess excellent stability via tests in solution biomimetic media, biologically relevant agents and possessed stability over a broad pH range (pH 4.4 to pH 12.7 by UV-visible spectroscopy and pH 2.5 to pH 12.7 by fluorescence spectroscopy). Furthermore, two-photon fluorescence lifetime imaging microscopy confirmed the stability of these complexes in cancer cells. Additionally, indium compounds of this type localised in the mitochondria and lysosome, with the phenyl-substituted complex entering the endoplasmic reticulum.

Moreover, the indium complex with an ethyl functionality displayed phototoxicity, as did the corresponding gallium complex, yet without a change in localisation within the cell. Interestingly the indium complexes (with the exception of the phenyl-substituted complex) localised within the nucleus and were found to bind to chromosomes in a preliminary study. Furthermore hypoxia selectivity was observed for complexes 4c and 4d in EMT6 and PC-3 cells by way of a reduction of fluorescence monitored by flow cytometry. In summary, these complexes show potential in diagnostics either as hypoxic or chromosome-binding contrast agents, with the possibility of therapy via photoactivity.
Chapter 6. Cellular investigations of new bis(thiosemicarbazonato) complexes by cytotoxicity assays

In this chapter pharmacological assays were performed in order to understand the cellular toxicity of the newly synthesised compounds. Standard MTT and LDH assays were adapted and used hereby. Cell viability and cytotoxicity assays cause a colorimetric response, which allow a chosen biological characteristic or process to be monitored. An increase in absorbance can either be the result of living cells retaining normal functions and/or morphology (cell viability assay) or alternatively the result of cellular disruption (cytotoxicity assay).

Various assays monitor different functions and do not always give the same result. Therefore it is beneficial to carry out more than one. On one hand, the Lactate Dehydrogenase (LDH) assay is informative on cell membrane lysis over short periods of time, but instead indicates overall toxicity for longer periods of time, such as 24 h. Methyl Tetrazolium (MTT) assay on the contrary is indicative of the number of live cells and therefore the overall toxicity of a particular drug. Mitochondria of live cells convert MTT into formazan, which absorbs broadly around 500 nm; increased toxicity therefore results in low absorbance, with all MTT assays carried out at 570 nm. The mitochondria of any non-viable cell will not be functional; therefore this assay is not informative per se on action on the mitochondria.

6.1 Time series assays by MTT

A preliminary study was carried out in FEK-4, a non-cancer fibroblast cell line at 10 μM at 1 hour, 3 hours, 6 hours and 24 hours to assess the effect of the complexes and Cu[ATSM] on cells with time by MTT assay. The allyl substituted complexes were chosen as representatives of the other functionalities to allow the effect of the metal on cytotoxicity to be considered, whilst the indium complexes were selected for the purpose of assessing the influence of the substituent (Figure 6.1).
The analogous allyl compounds and Cu[ATSM] enabled an initial comparison between metal centre, the addition of the acenaphthenequinone backbone and allyl functionality to be investigated for the first time. Zinc and copper, metals of biological significance represented the acenaphthenequinone complexes of highest and lowest cytotoxicity respectively. The gallium and indium compounds displayed very similar, gradual toxicity, which indicates that the mechanism of action may be due to their polarity, since both bear a chloride counter-ion.

Figure 6.1. MTT assays at 10 μM in FEK-4 in SFM EMEM of allyl complexes (above), indium complexes (below).

In contrast 1d and Cu[ATSM] demonstrated more rapid activity upon cells each with ca. 60% viability after 3 hr, with 2d, 3d and 4d ca. 80% viability after incubation at this timepoint. The comparison between Cu[ATSM] and 2d implies that the addition of the acenaphthenequinone backbone significantly decreased toxicity, which is a desired
attribute for a potential diagnostic. This could perhaps be due to the greater stability and subsequently lower reactivity of 2d with respect to Cu[ATSM].

The absorbances of 4a, 4b and 4c were comparable, indicating that the effect of the functionality may be less significant than that of the metal centre. 4d is an exception, with somewhat higher cytotoxicity displayed here and may be due to the greater reactivity of this substituent compared to the relatively unreactive methyl, ethyl or phenyl groups.

### 6.2 MI$_{50}$ determinations by MTT

To explore this further and to obtain quantitative measures of the cytotoxicity of this family of compounds the MI$_{50}$, the concentration at which metabolism is reduced to 50%, were calculated using MTT assay for a 48 h incubation with the compound, 1% DMSO. The value corresponding to 50% cells was corrected for background absorbance, which corresponded to the point at which all cells of a sample were dead.

Previous studies in the group using trypan blue and an incubation time of 48 hours found that zinc complex 1d and copper complex 2d possessed LD$_{50}$ values of ca. 15 µM and 25 µM respectively in MCF-7 cells,$^{127}$ with zinc complexes 1a, 1b and 1c cytotoxicity estimations of 15 µM.$^{128}$ Similarly, 2b had LD$_{50}$ values of ca. 12.5 µM in IGROV (ovarian cancer) cells and ca. 50 µM in MCF-7 (breast cancer) cells.$^{123}$ This hence indicates that the zinc complexes may be marginally more cytotoxic than the copper compounds. (Since quantitative data from trypan blue is achieved by the counting of cells, of which the dye stains the membrane of intact cells, metabolism is not measured and therefore was referred to as LD$_{50}$, here defined as the concentration to kill 50% of cells.) Cis-Platin (CP), in comparison was estimated to possess LD$_{50}$ values of ca. 4.0 µM in IGROV cells and ca. 12.0 µM for MCF-7, meaning that whilst the zinc and copper complexes appeared to possess comparable cytotoxicity to CP, however were slightly less toxic.
6.2.1 Cell viability assays in HeLa cells

MTT assay was chosen as an alternative to the more time consuming and less sensitive trypan blue. Control experiments were used to determine a MI$_{50}$ of CP and was calculated to be 27 µM ± 5 µM under comparable conditions and in agreement with the literature$^{152}$, whilst Cu[ATSM] was found to be 0.55 µM ± 0.03 µM in HeLa cells, a cervical cancer cell line. By crystal violet method Cu[ATSM] had previously been estimated to have a value of ca. 18.6 µM in HeLa indicating a stark difference in nature of the cytotoxicity tests, whereby crystal violet stains DNA as opposed to MTT assay which is dependent on metabolism of mitochondria.$^{153}$ Considering Table 6.1, which includes the MI$_{50}$ data gathered on all compounds tested in HeLa, each with at least 3 repeat experiments allowed this family of molecules to be compared in terms of their cytotoxicity.

Table 6.1. MI$_{50}$ data of compounds in HeLa cells, where N/A = no significant cytotoxicity and nd = not done. †Compound iib did not reach 100% cell death, therefore this number represents the minimum MI$_{50}$ value. *

<table>
<thead>
<tr>
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<th>a</th>
<th>b</th>
<th>c</th>
<th>d</th>
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<tbody>
<tr>
<td>i</td>
<td>0.105 µM ± 0.006 µM</td>
<td>6.5 µM ± 0.7 µM</td>
<td>6.4 µM ± 1.8 µM</td>
<td>0.18 µM ± 0.04 µM</td>
</tr>
<tr>
<td>ii</td>
<td>N/A</td>
<td>28 µM ± 13 µM†</td>
<td>5.2 µM ± 0.8 µM</td>
<td>N/A</td>
</tr>
<tr>
<td>1</td>
<td>nd</td>
<td>3.0 µM ± 0.2 µM</td>
<td>3.0 µM ± 0.6 µM</td>
<td>nd</td>
</tr>
<tr>
<td>2</td>
<td>nd</td>
<td>17 µM ± 4 µM</td>
<td>19 µM ± 1µM</td>
<td>nd</td>
</tr>
<tr>
<td>3</td>
<td>0.49 µM ± 0.03 µM</td>
<td>1.6 µM ± 0.2 µM</td>
<td>3.6 µM ± 0.8 µM</td>
<td>0.91 µM ± 0.03 µM</td>
</tr>
<tr>
<td>4</td>
<td>0.37 µM ± 0.10 µM</td>
<td>1.0 µM ± 0.2 µM</td>
<td>0.38 µM ± 0.07 µM</td>
<td>0.44 µM ± 0.16 µM</td>
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* Some MI$_{50}$ values in HeLa were obtained with assistance of Haobo Ge
General patterns were observable that the indium complexes possessed greater cytotoxicity than the gallium complexes, which in turn were more active than the zinc complexes (Appendix Figures H.1-H.9). Of the complexes copper was the least cytotoxic, with comparable potency to CP. Proligands iib demonstrated low levels of cytotoxicity, with compound iia and iid showing little cytotoxicity in preliminary experiments, indicating that chelation improves the activity of bis(thiosemicarbazones). Much greater cytotoxicity, however, was expressed by iic at 5.2 µM ± 0.8 µM, yet this was less toxic than its corresponding complexes, with the exception of the comparatively lower activity of the copper compound 2c. Mono-substituted free ligands displayed the most variable properties with ia the most potent compound tested and ib with similar cytotoxicity to 2b in HeLa cells. Interestingly compounds 1b and 1c possess significantly lower MI50 values by MTT assay when compared to trypan blue studies. Trypan blue experiments are dependent on cell membrane integrity therefore if
it were that this family of complexes cause little membrane damage including after longer incubation times it could explain why the estimations differ, this is explored in section 6.3.

### 6.2.2 Non-cancerous cells assays by MTT – investigations in FEK-4 cells

To prepare cancer diagnosing or treating agents, action against non-cancer cells is important to explore. In FEK-4 cells these data were greatly contrasted by ia causing limited cell death compared with in HeLa, with a MI₅₀ of 9 µM ± 3 µM, with id also possessing a MI₅₀ of much higher than in HeLa cells (Appendix Figures H.10-12). The MI₅₀ of the gallium and indium complexes tested were comparable, yet possessing notably lower toxicity against the non-cancer cell line. This data therefore provides strong evidence that these compounds possess selectivity towards cancer over non-cancer. Exhaustive studies in numerous cell lines (both cancer and non-cancer) could confirm if this effect is general for cancer.

<table>
<thead>
<tr>
<th>Table 6.2. MI₅₀ data of compounds in FEK-4 cells</th>
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<tr>
<td>a</td>
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<tr>
<td>i</td>
</tr>
<tr>
<td>3</td>
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<td>4</td>
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### 6.3 In vitro investigation to provide insight into the complex mode of action

Bis(thiosemicarbazonato) complexes had previously been tested for cytotoxicity by Pascu et al.,¹²⁷,¹²⁸ and Ackerman et al.¹⁵³. Compounds generally showed low to medium toxicity, theorised to be mediated via initial the mitochondria followed by entry into the nucleus.¹²⁸ To allow pharmacological comparison of the new compounds presented in Chapters 2-4 with known complexes such as Cu[ATSM], Zn[ATSM] as well as 1d, 3d and 2b a study of cytotoxicity and cell uptake was carried out using lactate dehydrogenase (LDH) assays in HeLa (cervical cancer cells), MCF-7 (breast cancer cells) and FEK-4 (fibroblast, non-cancerous cells).
LDH assay indicates potential cell membrane action as a mechanism of toxicity when cells are incubated with the drug for short periods of time (up to ca. 6 h). LDH catalyses the conversion of lactate into pyruvate, which reacts with tetrazolium salt INT to produce formazan. High absorbance therefore indicates high LDH activity and a high amount of cell lysis. For longer periods of time (e.g. 24 hours) this assay is representative of overall toxicity rather than action by membrane lysis. All LDH assays were carried out at 570 nm.

As illustrated in Figure 6.3 (Dilworth et al. 2005) one compound can behave differently according to the cell line it is in. It was therefore important to investigate the effect of each compound in this study on a number of various cell types. Cu[ATSM] is currently under clinical trials as a PET hypoxia molecular imaging probe for cancer and has so far been tested mainly in cancer lines. Because of its therapeutic potential, however, it may be more important to test its cytotoxicity on non-cancerous cells. In this study FEK-4 cells, which are non-cancerous were used for comparative purposes of anti-cancer selectivity. HeLa (cervical cancer) and MCF-7 (breast cancer) were the cancerous cell lines chosen to investigate this compound class. Each dataset is a repeat of at least 3 experiments.
Figure 6.4. MTT assay (above) and LDH assay (below) of HeLa cells, where black is medium control, containing Foetal Calf Serum (FCS), dark grey is the serum free medium (SFM) control, light grey is the DMSO control (99:1, SFM:DMSO), orange is 3d, dark green is 1d, light green is Zn[ATSM], dark blue is 2b. Compounds were incubated in 99:1, SFM:DMSO at 10 μM concentration.

Both assays were in agreement that the (M = Zn and Cu) [ATSM] compounds showed greater overall toxicity and that the remaining compounds were of relatively equal toxicity in this cell line. Zn[ATSM] was the only compound with fast-acting cytotoxicity (Figure 6.4). 2b showed most of its toxicity within the first 3 hours, which implies that the complex has rapid action on HeLa cells.
Figure 6.5. MTT assay (above) and (below) LDH assay of MCF-7 cells, where black is medium control, containing FCS, dark grey is the SFM control, light grey is the DMSO control (99:1, SFM:DMSO), orange is 3d, dark green is 1d, light green is Zn[ATSM], dark blue is 2b. Compounds were incubated in 99:1, SFM:DMSO at 10 μM concentration.

LDH leakage was higher than for controls in the first 3 hours for the (M = Zn and Cu) [ATSM] complexes and 2b, indicating that they cause cell membrane lysis and possibly explaining why there was such a strong correlation between the MI₅₀ and the LD₅₀ values obtained for 2b. The 1d and 3d, however, did not appear to act on the cell membrane, with similar LDH leakage when compared to the SFM control after 24 hours.
This could clarify the discrepancy of lower cytotoxicity observed for the zinc complexes when tested using trypan blue, which relies on cell membrane damage to assess cytotoxicity.

![Graph of MTT and LDH assays](image)

**Figure 6.6.** MTT assay (above) and LDH assay (below) of FEK-4 cells, where black is medium control, containing FCS, dark grey is the SFM control, light grey is the DMSO control (99:1, SFM:DMSO), orange is 3d, dark green is 1d, light green is Zn[ATSM], dark blue is 2b. Compounds were incubated in 99:1, SFM:DMSO at 10 μM concentration.

All (M = Zn and Cu) [ATSM] and 1d and 3d displayed a significant toxicity from 3 hours onwards, where 3d and Zn[ATSM] showed faster acting toxicity, with maximum
effect during the first hour for Zn[ATSM] (*Figure 6.5*). 2b, in comparison showed little toxicity until 24 hours. The LDH assays indicated that some membrane activity was displayed by (M = Zn and Cu) [ATSM] compounds and 3d.

With exception of Zn[ATSM] all compounds displayed little toxicity until 3 hours. From 6 hours onwards, however, all compounds, excluding 2b showed high toxicity. The LDH assays indicate significant cell membrane lysis by the (M = Zn and Cu) [ATSM] complexes and 2b and slight lysis by both 1d and 3d compounds. After discussing these compounds in the respective cell lines it was next possible to consider them per compound.

### 6.3.1 Discussion of trends emerging from *in vitro* cytotoxicity investigations

#### 6.3.1.1 Compounds: 1d and 3d

1d and 3d displayed rapid moderate toxicity, showing significant difference from the controls at 3 h for FeK4 (non-cancer) and MCF-7 and at 6 h for HeLa and a gradual decrease in cell viability for all cell lines (*MTT assays, Figures 6.4-6.6a*). 1d showed significantly greater LDH leakage after 24h in MCF-7 and FEK-4 cells, rendering membrane activity very unlikely for this compound.

For 3d the LDH assays showed a considerable leakage in MCF-7 and slight leakage in FEK-4 at 6 h, but there was no significant difference between the leakage of controls in HeLa cells. It is therefore possible that for 3d cell membrane lysis is a mechanism of action in MCF-7, whereas it is more likely to be another mechanism, perhaps DNA damage for HeLa cells. This is a possibility due to the gradual nuclear uptake of 1d (previously carried out by the group) and 3d observed in this study by fluorescence microscopy (*Chapter 4.5*). Furthermore, a long-term LDH assay showed less LDH leakage for 1d and 3d than controls, which could be indicative of DNA repair by Poly-ADP ribose polymerase (PARP) (*Figure 6.7*).\(^{154}\)
Figure 6.7. long-term LDH assay in HeLa cells (above), where black is medium control, containing FCS, dark grey is the SFM control, light grey is the DMSO control (99:1, SFM:DMSO), orange is 3d, dark green is 1d, light green is Zn[ATSM], dark blue is 2b. Compounds were incubated in 99:1, SFM:DMSO at 10 μM concentration. A comparison between the pathways of PARP and LDH, indicating that DNA repair in cancer cells could be the cause of reduced absorbance in an LDH assay (below).

6.3.1.2 Known compound: Zn[ATSM]

Zn[ATSM] displayed rapid toxicity on all cell types, resulting in significantly less cell viability after only 1h incubation and was therefore the most toxic of the compounds tested. Microscopy by Dilworth et al. (Figure 6.3) showed nuclear uptake by MCF-7, PC-3 and IGROV (however most of the localisation for IGROV was in the lysosome), indicating that DNA damage is one possible mechanism of toxicity. Leakage was
observed after 3h in all cell lines tested pointing to cell membrane damage as a likely cytotoxicity mechanism (Figures 6.4-6.6b), which is supported by the work of Dilworth et al. in that fluorescence was observed in the plasma membrane of MCF-7 cells (Figure 6.3).\(^{62}\)

### 6.3.1.3 Known Compound: Cu[ATSM]

The known compound Cu[ATSM] displayed medium toxicity to the cancer cell lines tested and there was significantly less toxicity to non-cancerous FEK-4 cells. This is a promising result since \(^{64}\)Cu[ATSM] is currently in clinical trials as an *in vitro* PET molecular imaging probe, indicating that cytotoxicity is negligible at concentrations required for nuclear imaging. The data show that this compound is likely to cause cell membrane damage in FEK-4 and HeLa cells, and may also in MCF-7 cells, since LDH leakage was observed up to 6h incubation with the complex. Zn[ATSM] is used as a model for the cell uptake of Cu[ATSM], which is not fluorescent due to quenching by the Cu(II) (3d\(^9\)). Therefore in consideration that Zn[ATSM] enters the nucleus it is reasonable to expect Cu[ATSM] to do likewise indicating DNA damage as another possible mechanism.

### 6.3.1.4 Compound: 2b

Cell viability observed in MCF-7 and FEK-4 when incubated with 2b showed little variation with controls, making it the least toxic of the compounds tested in this study. The compound, however, showed medium toxicity towards cervical cancer cells, indicating that investigations with further cell lines are necessary to fully understand the cytotoxicity of 2b. The LDH study indicated significant cell leakage from 1h in HeLa and FEK-4 cells, which decreased with time for HeLa cells. This could indicate that the mechanism of cytotoxicity is by a non-linear mechanism of cell membrane lysis. The microscopy study indicated localisation in the cell membrane, which is suggestive of membrane lysis (Chapter 2.4).
6.4 Summary to Chapter 6

The cytotoxicity of this family of compounds have been investigated in three cell lines allowing comparisons to be made between metal centre, functionality, relative speed of and mode of action on cancerous and non-cancerous cells. It was possible to make general conclusions such as: (a) the metal complexes were more toxic than their bis-substituted free ligands, (b) copper complexes were the least cytotoxic of the metal compounds tested, (c) for all compounds tested so far more activity was observed against cancerous cells than non-cancerous cells indicating a possibility of selectivity.

Further experiments however, in numerous cell lines are needed to fully elucidate if this is a universal prospect for future cancer treatment and diagnosis. The phototoxic effect of these compounds discovered in previous Chapters could be explored as it was not investigated using cell viability or cytotoxicity assays. Whilst there was some exposure to light as the compounds were added to cells, all samples were incubated in absence of light.

The comprehensive localisation of these compounds in a number of organelles observed in previous Chapters indicate that numerous modes of action are plausible, many of which could be investigated further in another study. However the activity on the cell membrane observed in this project suggest that this is a plausible mode of action for the copper complexes, Cu[ATSM] and 2b, with the lack of membrane activity by the zinc complexes indicative of the different results obtained here by MTT assay and by trypan blue assays.
Chapter 7. Investigations towards the syntheses of a peptide-targeted bis(thiosemicarbazone) complex, spectroscopic and in vitro imaging

7.1 Introduction to Chapter 7

Receptors of many peptides are overexpressed in a large number of cancers and therefore form a means of targeting cancer. Furthermore peptides have an advantage in that they enable fast uptake into tissue of interest and more efficient body clearance when compared to antibodies. Imaging of tumours using peptides was first achieved in 1989 as Krenning et al. detected radiolabelled somatostatin using a gamma counter. Each peptide facilitates the targeting of its own unique selection of receptors and tumours, for example somatostatin analogues provide a means of targeting neuroendocrine tumours.

![Image of complex conjugated to bombesin analogue and intercalator](image)

**Figure 7.1.** A rhenium tricarbonyl complex conjugated to a bombesin analogue and an intercalator, (right), visualised in fixed PC-3 cells by fluorescence microscopy (left), where green represents the complex and blue the DAPI nuclear stain.

Furthermore bombesin analogues target numerous tumours notably prostate, breast, gastrointestinal and lung cancer, making it an attractive peptide as a component of a molecular imaging agent. Bombesin is a 14-amino acid peptide isolated from amphibian skin, is an analogue of the gastrin-releasing peptide found in mammals and possesses strong affinity for a G protein-coupled receptor known
Chapter 7

as GRPR.

The first *in vivo* investigation of bombesin analogues was carried out by Van de Wiele *et al.* showing targeting of breast and prostate cancer patients in 2000. More recently tricarbonyl rhenium and technetium complexes with acridine derivatives showed nuclear uptake *via* fluorescence and activity based studies respectively.\(^{44}\) Alberto *et al.* developed rhenium and technetium complexes comprising of a DNA intercalator for nuclear targeting, a biologically active molecule (here a bombesin analogue) and a linker, cleavable upon cell entry displaying uptake in both the nucleus and the cytoplasm (*Figure 7.1*).\(^{157,158}\)

In addition to bombesin-targeted copper thiosemicarbazonato complexes mentioned in the Introduction, copper and gallium bombesin-conjugates have been synthesised paving the way for further investigations with this peptide. For example, a bombesin analogue conjugated to NO2A radiolabelled with \(^{64}\)Cu allowed visualisation of PC-3 xenografts, with preliminary *in vitro* studies recently carried out on a new cyclam bombesin derivative.\(^{53,159}\) A DOTA-PEG\(_4\)-BN(7-14) bombesin analogue was labelled with \(^{67/68}\)Ga and \(^{177}\)Lu, showing good tumour uptake in PC-3 xenografted nude mice, where \(^{67/68}\)Ga can act as a diagnostic radionuclide and \(^{177}\)Lu as a therapeutic.\(^{99}\)

Conjugation of a bombesin analogue to the acenaphthenequinone bis(thiosemicarbazone) complex could result in the development of dual-modal fluorescent and PET or SPECT imaging probes with potential for simultaneous therapy due to their cytotoxicity. Furthermore, cell studies under normoxic conditions have shown that all compounds have toxicity towards non-cancerous as well as cancerous cells (*Chapter 6*). Therefore, it would be beneficial to add a targeting group such as bombesin onto the compounds for added cancer selectivity. For complexes that are found not to be hypoxia selective, this would provide a means of targeting cancer and thus the aim of this chapter was to synthesise an acenaphthenequinone bis(thiosemicarbazone) complex conjugated to bombesin. The first objective was to synthesise a stable and intrinsically fluorescent precursor, which *via* a linker could be attached to the peptide, followed by its spectroscopy and *in vitro* characterisation. Finally the goal of this Chapter was to investigate the
possibility to couple the complex to the peptide and analyse it by the same means as the precursor.

### 7.2 Attempted ligand synthesis

A free ligand mono(thiosemicarbazonato) ligand was attempted by my mixing 1 equivalent of thiocarbohydrazide with an equivalent of acenaphthenequinone in 150 mL of ethanol (Figure 7.2). Upon reflux 4 drops of glacial acetic acid were added and after 3 hours the suspension was filtered whilst hot yielding a yellow solid, which was analysed by mass spectrometry and NMR spectroscopy. Following this, the reaction was repeated with an excess of thiocarbohydrazide, in 30 mL of ethanol and with 4 drops of acetic acid as before. After three hours the reaction was filtered, producing a similarly coloured powder. Furthermore, from the filtrate crystals suitable for X-ray diffraction formed overnight (Figure 7.3).

![Figure 7.2. Synthesis attempt to make a free ligand suitable for peptide conjugation](image)

Figure 7.3. ORTEP representation of the free ligand produced by the reaction described above.

The NMR spectra were identical for the solid and the crystals from this reaction as well as the solid from the reaction using one equivalent of thiocarbohydrazide, indicating that the same product had been formed. The crystal structure (below) as well as a mass spectrum of 289.074 confirmed that the desired product of a
thiosemicarbazonato free ligand analogous to those of previous chapters as well as suitable for addition of a targeting group had not been formed and that another route should be pursued (the calculated value was 289.075).

7.3 Initial attempts via protecting group chemistries

7.3.1 Synthesis of the mono-substituted t-Boc protected amine thiosemicarbazide

A zinc precursor complex with t-Boc protected amine which could be de-protected under acidic conditions to a mono-substituted t-Boc protected amine thiosemicarbazide was therefore desirable. Prior to the synthesis of the complex DFT calculations were carried out, which confirmed the theoretical thermodynamic stability of the complex (Figure 7.4).

Figure 7.4. DFT optimisation of the symmetric isomer of a zinc bis(thiosemicarbazonato complex with a 7 amino acid bombesin analogue as one substituent and a t-Boc protected amine as the other in the gas phase by B3LYP 6-31G(d,p). Where grey = carbon, white = hydrogen, blue = nitrogen, red = oxygen, dark green = zinc.
Firstly 500 mg of thiocarbohydrazide was stirred in 5 mL DMSO for 20 minutes, followed by dropwise addition of an equivalent of Boc anhydride (1030 mg). After stirring at room temperature for 2 hours the reaction was quenched by adding 5 mL of water dropwise. The solution was frozen using dry ice and acetone, which lead to the precipitation of a white solid. Mass spectrometry and NMR spectroscopy showed that the resultant product was a mixture of a mono-substituted and a bis-substituted thiocarbohydrazide. TLC plates showed that both compounds formed long tails for a broad range of concentrations tested and therefore would not be readily isolated by a separation column. Purifications and extractions based upon solubility were also found to be unsuccessful and since the zinc complex synthesis described in Chapter 3 requires vast excess of thiosemicarbazide separation by semi-preparative HPLC was not a practical alternative.

![Reaction Scheme](image)

**Figure 7.5.** Synthesis of mono-substituted and bis-substituted thiocarbohydrazide

The reaction conditions were therefore modified. Firstly using more dilute conditions resulted in very little precipitation; secondly using four equivalents of thiocarbohydrazide yielded a mixture of thiocarbohydrazide as well as the other two aforementioned products. This was confirmed by mass spectrometry m/z values of 129.02 for thiocarbohydrazide, 229.07 for mono-substituted thiocarbohydrazide and 329.13 for bis-substituted thiocarbohydrazide.
7.3.2 Attempt to synthesise a Zinc(II) complex incorporated a t-Boc protected thiosemicarbazide

Using the assumption that the bis-substituted t-Boc protected thiocarbohydrazide would not react with diketone acenaphthenequinone and would possess significantly different solubility to the resultant zinc complex a synthesis was attempted using a mixture that did not include thiocarbohydrazide. An initial trial using a 50 mg mass of acenaphthenequinone was undertaken under analogous conditions as were used for the synthesis of the zinc bis(thiosemicarbazonato) complexes in 2 mL of glacial acetic acid. This method was not successful, producing a black solid, which was likely a polymer.

Next, these conditions were modified to be carried out in 50 mL of 1,4-dioxane with 1 mL of acetic acid, over molecular sieves (Figure 7.6). After 30 hours the reaction was filtered whilst hot, which gave a white solid corresponding to crushed sieves. The filtrate was allowed to cool to room temperature resulting in the precipitation of an orange solid, in which mass spectrometry confirmed the presence of the desired compound, whilst NMR spectroscopy showed that the product was impure. The temperature of the filtrate was reduced, which resulted in further precipitation, with the orange solid produced giving similar results as described above. Therefore, the filtrate was rotary evaporated and washed with 50 mL of diethyl ether providing the desired product that by NMR spectroscopy showed significantly better purity, which was confirmed by LC-MS, and gave 34% yield and was successful on only one occasion.

Figure 7.6. Synthesis of acenaphthenequinone t-Boc protected amine zinc bis(thiosemicarbazone (1e)
Figure 7.7. Chromatogram of 1e from HLPC, showing a single major peak at 2.4 minutes

The compound, characterised by LC-MS, which gave peaks for [M-H]⁻ and [2M-H]⁻ m/z at 619.09 and 1243.18 respectively with a single LC trace at 2.4 minutes and was investigated by ¹H NMR spectroscopy (Figure 7.7).

7.3.2.1 NMR spectroscopy study of t-Boc protected bis(thiosemicarbazonato) complex, 1e

Figure 7.8. Numbering of atoms of 1e.
Selected resonances from the $^1$H NMR spectroscopy of 1e in the region of $\delta$ 7.5-9.6 ppm. 

Due to exchange with deuterated solvent NH protons appear as broad singlets. The spectra show two sets of doublets and one triplet as would be expected from an aromatic backbone (Figures 7.8 and 7.9). This is indicative of a predominantly symmetric structure, however small peaks such as at 8.46 signify that there may be a small proportion of asymmetric isomers present, which would be consistent with previous studies by the group $^{31}$. A singlet would be expected of the tert-butyl groups and since this is not a clean signal and a greater integral than would be expected is observed, it may also indicate some asymmetric isomer presence.

7.3.3 Spectroscopic investigations in solution

An initial 200-800 nm 2D scan in DMSO at 100 $\mu$M was used in order to assess intrinsic fluorescence of 1e, the excitation wavelength of maximum emission and the range of excitation and emission (Figure 7.10). The compound has a broad excitation and emission of 260-660 nm and 480-755 nm respectively, with the excitation wavelength resulting in maximum emission at 510 nm at 572.5 nm. It may be possible that interchange with the dimeric form of the compound may reduce fluorescence.
emission. Compound 1e has been shown to be weakly fluorescent and therefore has potential for fluorescence imaging.

Figure 7.10. 2D fluorescence scan of the 1e in DMSO at 100 μM.

Quantum yield is the proportion of absorbed and emitted photons and has been estimated in DMSO at 100 μM as 0.007 by:

\[
\Phi_S = \Phi_R \cdot \left( \frac{D_S}{D_R} \right) \cdot \left( \frac{A_R}{A_S} \right) \cdot \left( \frac{I_R}{I_S} \right) \cdot \left( \frac{\eta_S}{\eta_R} \right)^2
\]

Where \( \Phi = \) quantum yield, \( D = \) integrated area under emission band, \( S = \) sample, \( A = \) absorbance of solution at excitation wavelength, \( R = \) reference, \( \eta = \) refractive index of solvent, \( I = \) maximum intensity of excitation band [Ru(bipy)\(_3\)]PF\(_6\)\(_2\) in water was used as a reference, with a quantum yield of 0.042. The quantum yield result confirms that the compound is weakly fluorescent in comparison with previously synthesised compounds.\(^4\)
7.3.5 Synthesis of t-Boc protected amine copper(II) bis(thiosemicarbazonato) complex

The analogous copper complex (2e) was synthesised by stirring with 2 equivalents of copper acetate with 1e in methanol for 72 h, following isolation of a black solid by rotary evaporation in 40% yield.

![Synthesis reaction diagram](image)

**Figure 7.11.** Synthesis of acenaphthenequinone t-Boc protected amine copper bis(thiosemicarbazone (2e)

![HPLC trace image](image)

**Figure 7.12.** HPLC trace of compound 2e.

7.3.6 Radiolabelling experiments

7.3.6.1 Radiolabelling with Copper-64
The complex, 1e was radiolabelled with $^{64}\text{Cu}$, using $^{64}\text{Cu(OAc)}_2$ as the starting material. After 2 h stirring at room temperature the desired $^{64}\text{Cu}$ complex had not been cleanly made; however after 24 h the radio-trace appeared quite clean (Figure 7.13), indicating that the complex can be radiolabelled cleanly despite it not being a rapid process. It is possible that some of these peaks represent the mono-deprotected, di-deprotected, a dimeric form of the compound and asymmetric isomers.

![Graph 1: 1e + Cu-64 after 2h](image1.png)

![Graph 2: 1e + Cu-64 after 20 h](image2.png)

*Figure 7.13.* Transmetallation of 1e to 2e with Cu-64 (a) after ca. 2 hours and (b) after ca. 24 hours
7.3.6.2 Radiolabelling with Indium-111

1e was further radiolabelled with indium-111 yielding two major peaks, one indicative of $^{111}$InCl$_3$ at 3.8 minutes and the other at 7.6 minutes could be due to the indium(III) tert-butyl carbamate (4e) substituted bis(thiosemicarbazone) complex (Figure 7.15). There was sufficient 1e for radiolabelling, however there was not enough to make 4e to enable spectroscopic characterisation. Peak-splitting observed in the Radio channel could be explained by formation of a mixture of symmetrical and unsymmetrical isomers, or could be explained by de-protection of the tert-butyl groups resulting in the formation of two species of similar size and polarity. NMR spectroscopy studies of 4e once it is synthesised in sufficient quantities would determine the proportion of the respective isomers. Unfortunately the reproducibility of the mono-substituted t-Boc protected thiocarbohydrazide synthesis was poor and other synthetic routes were investigated.
7.4 Synthesis of an asymmetric zinc complex precursor

As an alternative synthetic means of developing a peptide-targeted bis(thiosemicarbazonato) complex, an equivalent of mono-substituted thiosemicarbazone, i.e. ia-id, was added to three equivalents of zinc acetate. To this a vast excess of thiocarbohydrazide was added, with the mixture refluxed in acetic acid for 30 h. The suspension was filtered whilst hot and washed with 100 mL of diethyl ether giving a yellow solid for 1aN and 1bN, a dark red solid for 1cN and a brown solid for 1dN. Successful synthesis was confirmed by ES-MS, with m/z values of 420.00, 434.02, 482.02 and 446.02 for 1aN, 1bN, 1cN and 1dN respectively and by \(^1\)H-NMR spectroscopy (Figures 7.17-7.19).

![Reaction scheme](image)

**Figure 7.16.** Reaction scheme for amine-functionalised zinc bis(thiosemicarbazonato) complexes.

Interestingly, the \(^1\)H NMR spectroscopy or 1cN clearly shows the presence of one isomer, with H-1 present as one doublet representing 2 protons, which would be otherwise non-equivalent for the asymmetric isomer. The H-2 and H-2’ resonances were non-equivalent, but overlapping, indicating that the amine group does have a small effect on the symmetry of the complex. The exchange observed in previous chapters would in fact cause two isomers to be formed as below, which would display up to six resonances for H-2 and H-2’. Contrary to 1cN, 1bN appears to exist as more than one isomer.
Figure 7.17. Proposed isomeric exchange for amine-functionalised zinc complexes

![Diagram showing isomeric exchange for amine-functionalised zinc complexes.]

Figure 7.18. $^1$H-NMR spectroscopy of 1cN in $d_6$-DMSO, 300 MHz.

![Graph showing $^1$H-NMR spectroscopy of 1cN in $d_6$-DMSO, 300 MHz.]

Figure 7.19. $^1$H-NMR spectroscopy of 1bN in $d_6$-DMSO, 300 MHz.

![Graph showing $^1$H-NMR spectroscopy of 1bN in $d_6$-DMSO, 300 MHz.]

Furthermore in order to synthesise a gallium complex the asymmetric ethyl and amine-substituted 50 mg of zinc 1bN was heated into 2.5 mL of DMSO, to which an excess of gallium chloride was added and 30 mL of MeOH. This was refluxed under an
atmosphere of nitrogen for 6 hours and was filtered after cooling to room temperature. The solution was removed in vacuo and the resultant material washed with 10 mL of ethyl acetate, followed by two times 10 mL of diethyl ether. This yielded an orange solid of 45 mg, which was confirmed as \(3bN\) by m/z of 438.01 by mass spectrometry and \(^1\)H NMR, which displayed the presence of one isomer only (Figure 7.20).

![Figure 7.20](image)

**Figure 7.20.** \(^1\)H-NMR spectroscopy of \(3bN\) in \(d_6\)-DMSO, 300 MHz.

### 7.4.1 Fluorescence spectroscopy

Fluorescence spectra were obtained in DMSO for initial scans between 200-800 nm, in order to obtain information about intrinsic fluorescence and \(\lambda_{\text{em-max}}\) using solutions of 100 \(\mu\)M. Ranges of absorption and emission, which are important with regards to cytotoxicity assays and fluorescence imaging, were also assessed using the 2D contours as a guideline (Figures 7.21-7.22 and Table 7.1). Relevant cytotoxicity assays use absorbance readings at 570 nm, therefore there should be minimal or no excitation at this wavelength, which was true for each complex. Fluorescence emission corresponding to the amine-substituted zinc and gallium complexes was notably weaker than previously characterised symmetric zinc complexes and gallium complexes. Interestingly the \(\lambda_{\text{max}}\) of emission was blue shifted, yet with \(\lambda_{\text{max}}\) of excitation being brought to 470 nm. Furthermore, the excitation and emission maxima of gallium complex \(3bN\) (340 nm, 526 nm) were significantly lower than that of \(3b\) (500 nm, 556 nm), indicating a large Stoke’s shift for this complex. The excitation range of \(3bN\) was
lower than 3b; however the range of emission was broader. The amine group therefore has a considerable influence on the spectroscopic properties of these complexes.

Figure 7.21. 2D fluorescence scan in DMSO at 100 μM a) of 1d, b) of 1dN

Figure 7.22. Fluorescence mapping: 2D fluorescence scan in DMSO at 100 μM a) of 3b, b) of 3bN

Table 7.1. Fluorescence spectroscopy of zinc and gallium bis(thiosemicarbazonato) complexes

<table>
<thead>
<tr>
<th>Compound</th>
<th>λ&lt;sub&gt;ex-max&lt;/sub&gt; / nm</th>
<th>Excitation range / nm</th>
<th>λ&lt;sub&gt;em-max&lt;/sub&gt; / nm</th>
<th>Emission range / nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1c</td>
<td>440</td>
<td>250-590</td>
<td>581.5</td>
<td>535-723.5</td>
</tr>
<tr>
<td>1d</td>
<td>520</td>
<td>250-570</td>
<td>551</td>
<td>490-695</td>
</tr>
<tr>
<td>1cN</td>
<td>470</td>
<td>250-650</td>
<td>553</td>
<td>496-734</td>
</tr>
<tr>
<td>1dN</td>
<td>470</td>
<td>260-570</td>
<td>539</td>
<td>480-738</td>
</tr>
<tr>
<td>3b</td>
<td>500</td>
<td>250-570</td>
<td>556</td>
<td>500-685</td>
</tr>
<tr>
<td>3bN</td>
<td>340</td>
<td>250-530</td>
<td>526</td>
<td>459.5-684.5</td>
</tr>
</tbody>
</table>
7.4.2 In vitro cellular imaging

7.4.2.1 Laser scanning confocal microscopy and fluorescence lifetime imaging study

Cells were cultured using standard protocols, analogous with earlier investigations on fluorescent thiosemicarbazones and following the same method as described in previous chapters. As was observed above, fluorescence was weak, however emission significantly greater than autofluorescence was measured within the cell when incubated in cancer cells at 100 μM. In agreement with the study on symmetric bis(thiosemicarbazonato) complexes of this family of compounds, uptake appeared to be well dispersed within the cytoplasm of the cell. The uptake of the zinc bis(thiosemicarbazonato) complexes (1bN, 1cN and 1dN) with an amine functionality displayed comparable uptake to each other when comparing the different substituent complexes (Figure 7.23).

![Image of cellular imaging](image)

Figure 7.23. Two-photon fluorescence imaging of complex 1bN, 100 μM, 1% DMSO, λex = 910 nm, incubated in MCF-7 cells for 20 minutes. a) DIC micrograph. b) Micrograph of green channel with excitation at 488 nm. c) Overlay of a) and b). e) Fluorescence intensity profile. f) Fluorescence lifetime map (τm). g) The corresponding lifetime distribution plot in ns.
The TCSPC in DMSO at 10 mM of these zinc complexes were similar each with a major component of ca. 0.05 ns, with a minor component at ca. 0.2 ns. The minor component observed in solution closely resembled the major component acquired in cancer cells at both wavelengths for all cell lines that these compounds were tested in indicating stability and a conformational change in presence of cells. Furthermore, the asymmetric gallium complex, 3bN, with slightly stronger fluorescence was observable in HeLa and PC-3 cells at 50 μM, displayed similar uptake as was displayed by the analogous zinc complex (Figure 7.24). The fluorescence lifetime (TCSPC) of 3bN in DMSO, 10 mM possessed two components, which when excited by 810 nm and 910 nm gave comparable results of ca. 0.65 ns and 2.4 ns, each of almost equal weighting.

The same components for complex 3bN were observed in HeLa and PC-3 cells, with the shorter component of 0.465 ns, 0.266 ns FWHM and 0.442 ns, 0.274 respectively accounting for 86% of the fluorescence lifetime indicating a difference in molecular conformation in the cellular environment. In the same way, these data and the agreement in solution and in cells provide strong evidence that this complex remains intact in vitro. Interestingly this shows a large difference in fluorescence lifetime when changing the metal, which was much more significant when comparing the lifetimes of the symmetric bis(thiosemicarbazonato) complexes.

**Figure 7.24.** Single-photon confocal microscopy images of 3bN in PC-3 cells, 50 μM, 0.5% DMSO, 20 minutes, at 37°C (a) DIC image, (b) excitation at 488 nm, emission >505 nm, (c) is an overlay of (a) and (b) Scalebar: 20 μm.
7.4.3 *In vitro* cytotoxicity investigations

After demonstrating that these novel complexes enter cells it was possible to estimate their cytotoxicity, using the same method as described in Chapter 6 to obtain $MI_{50}$ values. These were determined in HeLa cells as $8 \mu M \pm 2 \mu M$ for $1aN$, $38 \mu M \pm 8 \mu M$ for $1bN$, $0.68 \mu M \pm 0.08 \mu M$ for $1cN$ and $16 \mu M \pm 1 \mu M$ for $1dN$. It is interesting and surprising that symmetric zinc complexes $1b$ and $1c$ possessed comparable cytotoxicity, yet the modification of functionality to include an amine group resulted in a decrease in cytotoxicity for $1bN$ and an increase in cytotoxicity for $1cN$. Furthermore $3bN$ did not cause 100% cell death indicating its low cytotoxicity. The $MI_{50}$ value was therefore determined to be of greater than $47 \mu M \pm 6 \mu M$, which is significantly less toxic than $3b$ ($1.6 \mu M \pm 0.2 \mu M$), since $3b$ and $3bN$ possess similar solubility. This indicates a notable difference in cellular activity followed by this molecular alteration, but also greater suitability as a diagnostic, which requires low cytotoxicity.

7.4.4 Derivatisation of a targeting peptide model

These complexes possessing an amine group were designed to allow a targeting group such as a peptide to be added and due to the suitability observed it was appropriate to continue and attempt to couple these complexes. A seven amino acid peptide, bombesin analogue was synthesised by Jennifer Williams and Fatima Merzoug via solid state synthesis and purified using semi-preparative HPLC by the author. Prior to peptide coupling it was necessary to derivatise the peptide with a linker, for which purpose pyruvic acid was utilised. An equivalent of pyruvic acid was added to coupling agents EDC (1-ethyl-3-(3’-dimethylaminopropyl) carbodiimide) and HOBt (hydroxybenzotriazole) on ice in DMF. After 45 minutes 5 mg, (1 equivalent) of bombesin was added followed by dropwise addition of DIPEA ($N,N’$-diisopropylethylamine) until pH 8 was reached. This was allowed to react for 2 hours and was subsequently extracted from DMF and purified using a solid phase extraction cartridge, with the resultant product confirmed by mass spectrometry and HPLC.
7.4.5 Peptide coupling to a zinc(II) complex

Following this 5 mg of derivatised bombesin analogue (4.55 μmols) was added to 2.0 mg (4.55 μmols) of zinc complex, compound 1bN or compound 1dN in 400 μL of DMSO, for the purpose of synthesising compound 1bB (ethyl derivative) or 1dB (allyl derivative) (Figure 7.25). To this mixture 10 mL of HPLC grade methanol was added followed by 1 drop of concentrated HCl (32%), resulting in an instant colour change from dark orange to yellow. After stirring at 30°C for ca. 16 hours, the solution became pale orange and was freeze-dried in vacuo. Reaction mixtures of complexes 1bB and 1dB were then purified using semi-preparative HPLC as below, $\lambda_{\text{abs}} = 400$ nm (Figures 7.32-7.33, Figures B.4-5). Preliminary imaging in three cancer cell lines has been carried out, demonstrating characteristics significantly different from other bis(thiosemicarbazone) complexes previously observed. Attempts have been made to ionise the new complex for analysis by mass spectrometry, but have so far been unsuccessful.

![Compound 1bN and Compound 1bB](image)

**Figure 7.25.** Molecular diagrams of zinc bis(thiosemicarbazone) complexes (left) the amine functionalised precursor (right) the bombesin analogue conjugated complex.
Figure 7.26. HPLC of compound 1bB reaction mixture ($\lambda_{abs} = 400$ nm), reference peaks to note under the same conditions – compound 1bN $R_t = 9.2$ minutes and derivatised bombesin analogue $R_t = 4.8$ minutes (HPLC method E).

Figure 7.27. HPLC of purified compound 1bB ($\lambda_{abs} = 400$ nm), HPLC method E.
Due to limited access to facilities, initial *in vitro* experiments were carried out on a reaction mixture of compound 1bB. This was analysed using HPLC and showed a single major peak that did not correspond to starting materials and was rotary evaporated and freeze-dried overnight (*Figure 7.28 and 7.29*).

**Figure 7.28.** HPLC traces of the derivatised bombesin (red), the zinc complex 1bN starting material (black) and the reaction mixture (blue) with the derivatised bombesin analogue (red), at 400 nm, HPLC method B.

**Figure 7.29.** HPLC trace of the reaction mixture at 400 nm, HPLC method B.
7.4.6 Initial solution study, cell uptake study, laser scanning confocal microscopy and two-photon fluorescence lifetime study

7.4.6.1 Solution and HeLa cell study

The fluorescence lifetime of the reaction mixture of complex \textit{1bB} in solution (10 mM in DMSO) was found to contain 3 components, the first at 0.030 ns (77.2%), the second at 0.670 ns (21.3%) and the third at 2.307 ns (1.5%), $\chi^2 = 1.28$. Complex \textit{1bN} in solution (10 mM in DMSO) was found to contain 2 components, the first at 0.045 ns (92.7%) and the second at 0.236 ns (7.3%), which displayed precipitation ($\chi^2 = 3.28$). The bombesin-conjugated complex reaction mixture, displayed uptake in all three cell lines tested HeLa (cervical cancer), PC-3 (prostate cancer) and MCF-7 (breast cancer) cells. Interestingly the characteristics and uptake demonstrated by the complex was very cell line dependent, which is unprecedented for this family of complexes. In HeLa, the conjugated complex \textit{1bB} was modelled to two component fluorescence lifetimes with $\chi^2 \approx 1.20$, $\tau_1 = 0.689$ ns, 0.406 ns FWHM, 80%; $\tau_2 = 3.333$ ns, 1.450 FWHM, 20%, which is similar to the lifetimes to the minor components observed in solution and indicates stability as well as interaction with cells or cellular media (Figure 7.30).

\textbf{Figure 7.30.} (a – c) Microscopy of complex \textit{1bB}, 100 \textmu M, 1\% DMSO, incubated in HeLa cells for 20 minutes where a) single-photon confocal overlay of DIC channel and with excitation at 488 nm Scalebar = 20 \textmu m, b) and c) two-photon fluorescence imaging where b) the fluorescence lifetime map of $\tau_m$ and c) is the corresponding lifetime distribution plot in ns of $\tau_m$. 

\textit{Note:} Scalebar = 20 \textmu m.
7.4.5.2 PC-3, prostate cancer cell line

The data obtained in PC-3 cells was particularly interesting in that the complex appeared to greatly damage the plasma membrane, almost instantly after addition of the complex, which was monitored by plasma membrane colocalisation with Alexa Fluor dye (Figure 7.32). This is an effect that had not previously been observed for this family of compounds. Further work could be to carry out lactate dehydrogenase (LDH) assays to test for cell membrane damage. The fluorescence lifetime was modelled to 2 components, which were $\chi^2 = 1.25$, $\tau_1 = 0.660$ ns, $0.329$ ns, 80% and $\tau_2 = 3.515$ ns, $2.570$ ns, 20%, again displaying highly comparable data to the minor components in solution as was observed in solution indicating that the complex is most likely stable and interacts with cells and/or cellular media (Figure 7.31).

![Figure 7.31](image-url) (a – c) Microscopy of complex 1bB, 100 μM, 1% DMSO, incubated in PC-3 cells for 20 minutes where a) single-photon confocal overlay of DIC channel and with excitation at 488 nm Scalebar = 20 μm, b) and c) two-photon fluorescence imaging where b) the fluorescence lifetime map of $\tau_m$ and c) is the corresponding lifetime distribution plot in ns of $\tau_m$. 

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Figure 7.32. PC-3 cells stained with Alexa fluor plasma membrane dye after addition of complex 1bB, final concentration within 5 minutes of addition at 100 µM, 1% DMSO, final concentration a) represents the DIC channel, (b) corresponds to excitation at 405 nm, (c) excitation at 488 nm and (d) are an overlay of the three channels were acquired addition. (Precipitation was observable since no cell washing occurred to enable the image to be taken as soon as the compound was added.) Scalebar: 20 µm.

7.4.6.3 MCF-7, breast cancer cell line

Complex 1bB reaction mixture primarily entered the plasma membrane of MCF-7, but slowly entered the cytoplasm, where it possessed a lower fluorescence lifetime indicated by the orange colour as opposed to the green observed in the membrane itself (Figure 7.32), displaying a very large contrast when compared to the uptake of complex 1bN in the same cell line under identical conditions (Figure 7.23). As was observed in HeLa and PC-3 cells the fluorescence lifetime possessed two components ($\chi^2 = 1.21$, $\tau_1 = 1.299$ ns, 0.523 ns FWHM 75%, $\tau_2 = 5.170$ ns, 2.007 ns FWHM, 25%) whereby the lifetimes were longer in comparison to those acquired in solution, which is attributable to the larger lifetime observed in the cell membrane of MCF-7 cells. Plasma membrane
uptake was confirmed by plasma membrane colocalisation with Alexa Fluor dye (Appendix Figures G.58-59).

Figure 7.33. (a – c) Microscopy of complex 1bB, 100 μM, 1% DMSO, incubated in MCF-7 cells for 20 minutes where a) single-photon confocal overlay of DIC channel and with excitation at 488 nm. Scalebar = 20 μm, b) and c) two-photon fluorescence imaging where b) the fluorescence lifetime map of $\tau_m$ and c) is the corresponding lifetime distribution plot in ns of $\tau_m$.

7.4.7 In vitro experiments performed on the purified complexes

Following purification, the complexes 1bB and 1dB were added to cells (Figure 7.34 and Appendix Figures G.61-64). Complex 1dB did not display good FLIM data in cells, which could be attributed to optimal emission with $\lambda_{ex} = 543$ nm (Appendix Figure G.62). Compound 1bB demonstrated presence of 3 components in the fluorescence lifetime, when excited by 810 nm and by 910 nm, whereby at 810 nm $\tau_1$ was 0.050 ns (83.6%), $\tau_2$ was 0.728 ns (11.0%) and $\tau_3$ was 3.508 ns (5.3%), $\chi^2 = 2.72$. At 910 nm the first component was 0.050 ns (corresponding to 74.0%), with a second component of 0.855 ns (22.9%) and third component of 7.232 ns (3.1%), $\chi^2 = 1.06$.

These values are different from those acquired from the reaction mixture, in that $\tau_1$ is slightly lower and $\tau_2$ is greater, which could be indicative of removal of impurities. The allyl-substituted complex possessed similar data to the ethyl functionalised-complex with slightly higher fluorescence lifetimes observed when exciting at 910 nm ($\chi^2 = 1.21$), for example $\tau_2$ at 810 nm was 0.832 ns (8.3%) and was 0.830 ns (13.1%) at 910 nm and $\tau_3$ was 4.422 ns (2.5%) at 810 nm and 9.302 ns (2.2%) at 910 nm. The major components where comparable at 0.048 ns (89.2%) and 0.049 ns (79.3%) respectively.
When incubated in cells complex 1bB possessed similar behaviour as before in that most of the uptake was observed in the cell membrane and the cytoplasm, giving comparable results as were seen before purification in HeLa and PC-3 cells (Figure 7.34). In MCF-7 distribution of the compound within the cell appeared similar to the other cell lines, therefore not giving an identical result as before, nevertheless minor components of the solution data closely match the fluorescence lifetimes observed in cells for the major component of 0.690 ns, 0.615 ns FWHM. Likewise in HeLa and PC-3 cell data were similar to the second component measured in solution whereby in HeLa at 810 nm the data were modelled to one component, which was 0.872 ns ± 0.490 ns FWHM, with the two components present when incubated in PC-3 at 810 nm were 0.889 ns ± 0.418 ns FWHM (first component) and 3.682 ns, 1.596 ns FWHM (second component). This is indicative of the likely stability of the complex and its interaction with cells/cellular media. Attempts were made to ionise the compound by MALDI-TOF mass spectrometry, which were unsuccessful. These experiments however, present interesting results and advance this research towards targeted small molecules for personalised medicine.

**Figure 7.34.** Compound 1bB, 1% 100 μM in PC-3 a) FLIM colour map where $\chi^2 = 1.27$, lifetime is 0.889 ns ± 0.418 ns FWHM, range of FLIM image is 0 to 5 ns, where $\lambda_{ex} = 810$ nm and b) is an overlay of DIC image and $\lambda_{ex} = 488$ nm, scalebar = 20 μm. $\tau_1 = 0.804$ ns, 0.720 ns FWHM 79%, $\tau_2 = 3.682$ ns, 1.596 ns FWHM, 19%.
7.5 Summary to Chapter 7

A number of approaches for the development of a targeted intrinsically fluorescent bis(thiosemicarbazonato) complex were attempted. Amine-functionalised zinc complexes and a gallium complex were synthesised and characterised displaying suitable properties as molecular imaging probes, showing uptake and stability within cells. A seven amino-acid analogue of bombesin was derivatised with pyruvic acid and a coupling reaction with zinc complexes was attempted. HPLC data were promising, however it could not confirm the presence of the desired peptide-conjugated complex.
Chapter 8. Conclusions and further work

The synthesis and characterisation of a number of intrinsically fluorescent acenaphthenequinone zinc(II), copper(II), gallium(III) and indium(III) bis(thiosemicarbazone) complexes was carried out. $^{64}$Cu, $^{68}$Ga and $^{111}$In were successfully used for clean and rapid radiolabelling by transmetallation from the zinc precursors. Biomimetic stability tests were performed using UV-Visible and fluorescence spectroscopy, which found the complexes to possess suitable properties for in vitro kinetic stability assays, cytotoxicity assays and cell uptake experiments.

The kinetic stability of the complexes was assayed with water, DMSO and PBS as well as Glutathione (GSH), L-cysteine, L-histidine, L-methionine, citric acid, ethylenediaminetetraacetic acid (EDTA) and biomimetic Eagle's modified essential media (EMEM) (both with and without foetal calf serum). These tests indicated excellent kinetic stability for the zinc and indium complexes, and appropriate stability for the gallium complexes for cellular experiments. Moreover, the complexes were tested with biologically relevant pH buffers and media, which indicated that the gallium complexes possessed sufficient stability between pH 2.5 and 7.35 within the timeframe of a cellular assay. The zinc and indium complexes, furthermore demonstrated kinetic stability within the range of pH 4.4 to 12.7 for up to 24 hours.

Subsequent fluorescence lifetime imaging experiments confirmed the stability of the bis(thiosemicarbazonato) complexes in solution and in biological cells. To the best of the author’s knowledge this was the first report in the literature of the use of this technique to assess the stability of a transition metal complex in biological cells.

As a consequence, a range of novel bis(thiosemicarbazonato) complexes, as well as previously synthesised compounds, were studied in vitro, all showing cell uptake and varying degrees of cytotoxicity. Colocalisation stains were used to evaluate the uptake in numerous organelles, including the nucleus, cell membrane, lysosome, mitochondria and endoplasmic reticulum. The gallium and indium complexes displayed nuclear uptake, while many of the complexes were seen to enter the lysosome and mitochondria.
Furthermore the phenyl-substituted derivatives localised mainly in the endoplasmic reticulum.

These findings provide some insight into possible mechanisms of action. Further studies would be required to confirm these hypotheses using biological assays. Interestingly, photoactivity was observed for some of the compounds. The ethyl-functionalised acenaphthenequinone gallium(III) bis(thiosemicarbazone) complex, in particular, displayed a significant change of uptake to form a punctuated appearance upon irradiation at 488 nm wavelength. Exclusively under these conditions cells were also observed to undergo blebbing, indicating cell death. This seems to indicate that these compounds could have potential for photodynamic therapy. A preliminary study was carried out to determine structural changes after irradiation using LEDs. NMR spectroscopy showed the presence of a new species, which, however, was not in sufficient proportions to allow assignment. Future work should investigate the structural changes initiated by light using high-powered lasers, in addition to by MI50 cytotoxicity experiments carried out under light irradiation.

The cytotoxicity of these complexes was evaluated with cells incubated in the dark using MTT and LDH assays. Results showed that a lower concentration was required to cause cell death in the cancerous cell line, HeLa, compared to the healthy cell line, FEK-4. Since some degree of toxicity was observed in non-cancerous cells, new zinc precursors and a gallium precursor featuring an amine group were synthesised to enable more selective targeting of cancer cells. Attempts to attach a targeting peptide via standard coupling reactions with a bombesin analogue were carried out. The product of which was purified by HPLC, with attempts to obtain a mass spectrum unsuccessful. Future work should explore different methods for coupling the precursor complexes to peptides and ionisation of the desired product. The crude and purified zinc complex reacted with a bombesin analogue were investigated for cell uptake. Each entered cells and possessed a comparable major fluorescence lifetime component with minor component solution data, which was different from the analogous amine-derivatised zinc complex. This therefore represents an advance towards attaching targeting these compounds to biologically active molecules for personalised medicine.
In vivo experiments in nude mice were carried out to assess the uptake of radiolabelled $^{64}\text{Cu}$ and $^{68}\text{Ga}$ and biodistribution of $^{64}\text{Cu}$ with a normoxic PC-3 Xenografted tumour (by collaborators in the Jason Lewis Group, MSKCC, NYC). These experiments showed very little uptake in the normoxic tumour combined with low non-target organ uptake compared to Cu[ATSM]. This may be seen as a promising result for an intended hypoxic selective probe. Furthermore, in vitro studies (carried out by collaborator Dr Israt S. Alam, Imperial College London) showed hypoxia selectivity in the case of gallium and indium complexes 3d, 4c and 4d. Future work should therefore include an investigation of hypoxic selectivity in nude mice to enable comparisons with the normoxic study. Moreover, the mechanism of hypoxic selectivity should be tested by cyclic voltammetry.

Finally, if some of the compounds synthesised during the course of this PhD are found to possess hypoxia selectivity in vivo, the major goal of developing and testing an intrinsically fluorescent, kinetically stable, hypoxic selective – and potentially therapeutic – molecular agent will have been achieved.
Chapter 9. Experimental

9.1 General Information

Mass spectrometry data was acquired on a Bruker Daltonics ESI-TOF and NMR spectra on a Bruker 300 Ultrashield™ (\(^\text{1}H\): 300 MHz, \(^\text{13}C\): 75.5 MHz). Mass spectrometry was carried out in HPLC-grade methanol or distilled water, in some cases with 1% DMSO. NMR spectroscopy solvents, acenaphthenquinone, Zinc acetate, copper acetate, gallium trichloride and indium trichloride were obtained commercially and used as received. Gallium trichloride and indium trichloride were weighed out in an MBraun glovebox at \(O_2, H_2O > 0.3\) ppm.

Continuous wave electron paramagnetic resonance (cwEPR) spectra at the X-band (9.450 GHz) were measured at 1mM in DMF at 60 K with a Bruker EMX spectrometer by Dr M. W. Jones, University of Oxford.

Elemental analyses were carried out by Mr Alan Carver, University of Bath, in parallel with A. Rosie Chhatwal (for compounds iia-iid and 2a-2d.)

Crystal structure determination by X-ray diffraction
Single crystal X-ray diffraction data were obtained by Dr Gabriele Kociok-Köhn, Dr Sofia I. Pascu or Dr Simon K. Brayshaw for compounds iiib-s, iid, L\(_\text{B}\), 1c, 2c, 3a, 3b, 3c, 4a, 4b, 4c and 4d. Crystals were selected using the oil drop technique, in perfluoropolyether oil and mounted at 150(2) K with an Oxford Cryostream \(N_2\) open-flow cooling device. Intensity data for 2c, 3a, 3c, 4a – 4d were collected on an Nonius Kappa CCD single crystal diffractometer using graphite monochromated Mo-K\(\alpha\) radiation (\(\lambda = 0.71073\) Å). Data were processed using the Nonius Software.\(^{160}\) For L\(_\text{B}\), 1c and 3b data were collected on an Oxford Diffraction Gemini single crystal diffractometer at 150 K. Data were processed using the Oxford Diffraction software package (CrysAlisPro Version 1.171.33.55). Data for iiib-s and iid were collected at Diamond using Synchrotron radiation (\(\lambda = 0.68890\) Å) on a CrystalLogic Kappa (3
Experimental

circle), Rigaku Saturn724 at 100K. Data were processed using the Rikagu software package (CrystalClear-SM Expert 2.0 r5).

For most structures a symmetry-related (multi-scan) absorption correction had been applied. Crystal parameters and details on data collection, solution and refinement for the complexes are provided in Tables A.1, A.2 and A.3.

The structures were solved by direct methods using the programmes SIR97\textsuperscript{161} or SHELXS-97\textsuperscript{162} followed by full-matrix least squares refinement on $F^2$ using SHELXL-97\textsuperscript{162} implemented in the WINGX-1.80 suite of programmes throughout. Additional programmes used for analysing data and graphically handling them included: SHELXle,\textsuperscript{163} PLATON,\textsuperscript{164} and ORTEP 3 for windows. Hydrogen atoms were placed onto calculated positions and isotropically refined using a riding model.

**HPLC characterisation was carried out by one of the following methods**

**HPLC method A** was carried out using a Symmetry® C-18 column (4.6 x 260 mm) with UV/visible detection measured at $\lambda_{\text{obs}} = 200$ nm, 300 nm, 400 nm, 450 nm, 500 nm, 600 nm, 700 nm and 800 nm. The gradient elution was 1.1 mL/minute, with 0.1% TFA MilliQ water as solvent A and 0.1% TFA MeCN as solvent B. Start 95% A reverse gradient until 5% A at 7.5 minutes, isocratic until 15 minutes, reverse gradient until 17.5 minutes 95% A, then hold to 18 minutes.

**HPLC method B** was carried out using a Symmetry® C-18 column (4.6 x 260 mm) with UV/visible detection measured at eight wavelengths from $\lambda_{\text{obs}} = 200$ nm, 220 nm, 280 nm, 300 nm, 400 nm, 450 nm, 500 nm, 600 nm, 700 nm and 800 nm. The gradient elution was 0.8 mL/minute, with 0.1% TFA MilliQ water as solvent A and 0.1% TFA MeCN as solvent B. Start 95% A reverse gradient until 5% A at 7.5 minutes, isocratic until 15 minutes, reverse gradient until 17.5 minutes 95% A, then hold to 18 minutes.

**HPLC method C** was carried out using a Acclaim® 120 C-18 column (4.6 x 150 mm) with UV/visible detection measured at up to four $\lambda_{\text{obs}} = 214$ nm, 220 nm, 254 nm, 280 nm, 300 nm and 400 nm. The gradient elution was 1.0 mL/minute, with 0.1% TFA MilliQ water as solvent A and 0.1% TFA MeCN as solvent B. Start 95% A reverse
Experimental

gradient until 5% A at 7.5 minutes, isocratic until 15 minutes, reverse gradient from 15.1 minutes 95% A, then hold to 18 minutes.

**HPLC method D** was carried out using a Waters C-18 column (4.6 x 250 mm) with UV/visible detection measured at up to four $\lambda_{obs} = 254$ nm. The gradient elution was 1.0 mL/minute, with 0.1% TFA MilliQ water as solvent A and 0.1% TFA MeCN as solvent B. Start 95% A reverse gradient until 5% A at 12 minutes, isocratic until 15 minutes, reverse gradient from 15.1 minutes 95% A, then hold until 21 minutes.

**HPLC method E** was carried out using a Acclaim® 120 C-18 column (4.6 x 150 mm) with UV/visible detection measured at up to four $\lambda_{obs} = 214$ nm, 220 nm, 254 nm, 280 nm, 300 nm and 400 nm. The gradient elution was 1.0 mL/minute, with 0.1% TFA MilliQ water as solvent A and 0.1% TFA MeCN as solvent B. Start 95% A reverse gradient until 5% A at 10 minutes, isocratic until 15 minutes, reverse gradient from 15.1 minutes 95% A, then hold to 18 minutes.

**HPLC method F** was carried out using a Waters C-18 column (4.6 x 250 mm) with UV/visible detection measured at $\lambda_{obs} = 254$ nm. The gradient elution was 1.0 mL/minute, with 0.1% TFA MilliQ water as solvent A and 0.1% TFA MeCN as solvent B. Start 95% A reverse gradient until 5% A at 10 minutes, isocratic until 12 minutes, reverse gradient until 95% A at 14 minutes, then hold to 15 minutes at 95% A.

**HPLC method G** was carried out using a Waters C-18 column (4.6 x 250 mm) with UV/visible detection measured at up to four $\lambda_{obs} = 214$ nm, 220 nm, 254 nm, 280 nm, 300 nm and 400 nm. The gradient elution was 1.0 mL/minute, with 0.1% TFA MilliQ water as solvent A and 0.1% TFA MeCN as solvent B. Start 95% A reverse gradient until 5% A at 15 minutes, isocratic until 22.5 minutes, reverse gradient from 22.6 minutes 95% A, then hold to 25.5 minutes.

**HPLC method H** was carried out using an Eclipse C-18 column (2 x 50 mm) with UV/visible detection measured at up to four $\lambda_{obs} = 214$ nm, 220 nm, 254 nm, 280 nm, 300 nm and 400 nm. The gradient elution was 1.0 mL/minute, with 0.1% TFA MilliQ
water as solvent A and 0.1% TFA MeOH as solvent B. Start 95 % A reverse gradient until 5% A at 8 minutes, hold until 10 minutes.

**HPLC method I** was carried out using a Phenomenex Ultracarb C-18 column (4.6 x 150 mm) with UV/visible detection measured at up to four $\lambda_{obs} = 254$ nm. The gradient elution was 1.0 mL/minute, with 0.1% TFA MilliQ water as solvent A and 0.1% TFA MeOH as solvent B. Start 95 % A reverse gradient until 5% A at 12 minutes, hold until 15 minutes.

**Semi-Preparative HPLC** was carried out using a Phenomenex® Gemini C-18 column (10 x 250 mm) with UV/visible detection measured at up to four $\lambda_{obs} = 214$ nm, 220 nm, 254 nm, 280 nm, 300 nm and 400 nm. The gradient elution was 1.0 mL/minute, with 0.1% TFA MilliQ water as solvent A and 0.1% TFA MeCN as solvent B. Start 95 % A reverse gradient until 5% A at 15 minutes, isocratic until 22.5 minutes, reverse gradient from 22.6 minutes 95% A, then hold to 25.5 minutes.

**General Radiochemistry Procedures**

Copper and indium experiments were carried out at either University of Oxford or University of Cambridge under the supervision of Prof Jonathan R. Dilworth or Dr Franklin I. Aigbirhio respectively. The zinc complex was prepared as either 1 mg/mL or 2 mg/mL in DMSO. For copper $^{64}$Cu$^{2+}$ was extracted from the nickel target and purified from $^{64}$Ni$^{2+}$ using an ion exchange column. An aqueous $^{64}$CuCl$_2$ solution was acquired in 0.1 mol.dm$^{-3}$ HCl, which was diluted with 0.2 mL $^{64}$CuCl$_2$ in 0.1 mol.dm$^{-3}$ Na(CH$_3$CO$_2$)$_2$. 200 μL of < 10 MBq $^{64}$Cu(CH$_3$CO$_2$)$_2$ was added to 100 μL of 1 mg/mL zinc complex in DMSO and 400 μL water in a 2 mL reaction vial. After stirring at room temperature 25 μL of this solution was entered into the HPLC, which was combined with both a UV tracer detecting at 254 nm and NI scintillation crystal detection with approximately a 10s delay. For indium 50 μL of the zinc complex in DMSO at a concentration of 1 mg/mL was added to 150 μL of MeOH and 2.5 μL $^{111}$InCl$_3$, < 10 MBq per experiment. The solutions were stirred at 60 °C and entered into the HPLC after ca. 1h. Gallium experiments were carried out by Mr Frazer Twyman, Hammersmith Hospital, Imperial College London, whereby 10 mL of 0.1M HCl was...
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used to elute ca. 6mCi of $^{68}$Ga$^{3+}$ from the generator and was subsequently trapped on a 30 mg/mL Strata X-C cartridge. This was eluted with 700 μL of 0.02M HCL / 98% acetone and dried for 15 minutes under a stream of nitrogen at 110°C. Next, 25 μL of 2 mg/mL zinc complex in DMSO and 2 mL of HPLC-grade ethanol. The solution was heated for 30 minutes at 90 °C. HPLC methods F, G, H and I were used in all cases.

Log $P$ calculations

$^{64}$CuEt/$^{111}$In (ca. 0.5 MBq) in 0.5 mL de-ionized water was added to 0.5 mL of octanol in an Eppendorf tube. The tube was vigorously vortexed for 5 min and centrifuged at 5000×g for 5 min. Aliquots (50 μL) of both aqueous and octanol layers were collected in triplicate and the associated activity counted using a gamma counter (Triathler, Hidex). Log $P$ values were then calculated and compared with that of Cu[ATSM] standard, using the formula:

$$\log P = \log(\text{counts(octanol)})/\text{counts(H}_2\text{O})$$

Animal Xenograft Models

All experiments were carried out following an Institutional Animal Care and Use Committee-approved protocol, Memorial Sloan-Kettering Cancer Center, New York by Dr Brian Zeglis. Athymic mice aging six to eight weeks were obtained from Taconic Farms Incorporated (Hudson, NY). The animals were given ca. 1 week prior to treatment to acclimatise in ventilated cages and were provided with food and water ad libitum. Xenografts were induced on the right shoulder by subcutaneous injection of 100 μL of 3 x 10$^6$ PC-3 cells.

Small-animal PET imaging

Small-animal PET imaging was performed at MSKCC, New York, on a micro-PET R4 rodent scanner (Concorde Microsystems) by Dr Brian Zeglis. Mice were injected with complex radiolabelled with Cu-64 or Ga-68 of 10-15 MBq and anesthetised by inhalation of 2% isoflurane (Baxter Healthcare, Deerfield, IL)/oxygen gas mixture. The animal was positioned on the scanner bed with anesthesia maintained by a 1% isoflurane/gas mixture. Measurements for each mouse were recorded using static scans
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at time points between 30 minutes and 24 hours. Each scan occurred for between 10 and 45 minutes and contained at least 20 million coincident events. The energy window used was 350-700 keV, with a coincidence timing window of 6 ns. Data were arranged into 2D histograms by Fourier rebinning, and transverse images were reconstructed by filtered back-projection into a 128 x 128 x 63 (0.72 x 0.72 x 1.3 mm$^3$) matrix. Images were normalised to correct for non-uniformity of response of the PET, dead-time count losses, positron branching ratio, and physical decay to the time of injection but no attenuation, scatter, or partial-volume averaging correction was applied. The counting rates of the reconstructed images were converted to activity concentrations (percentage of injected dose [%ID] per gram of tissue) using a calibration factor based on the image of a mouse-sized water-equivalent phantom containing either Cu-64 or Ga-68. ASIPro VM™ software (Concorde Microsystems) was used to analyse the data.

**Biodistribution study**

Biodistribution experiments were carried out at MSKCC, New York, by Dr Brian Zeglis, with mice bearing PC-3 tumour grafts as described above and randomised prior to the study. Prior to intravenous tail injection with Cu-64 radiolabelled 2b and 2c (0.5 – 1.0 MBq) the mice were gently warmed with a heat lamp for 5 minutes. Mice were euthanised by CO$_2$ gas asphyxiation at 1h, 2h, 6h, 12h and 24h, after which organs (including the tumour) were removed, rinsed with water, dried in air, weighed and counted using a Cu-64 calibrated gamma counter. Count data were converted to activity concentrations (percentage of injected dose [%ID] per gram of tissue).

**UV-visible spectra** were obtained using a Perkin-Elmer Spectrometer, Lamda 650 in DMSO and processed using UV Winlab 3 software. The orientation of the 1.00 cm quartz cuvette was the same for each experiment for consistency.

**Fluorescence spectra** were measured in a 1.00 cm quartz cuvette using a Perkin-Elmer LS55 luminescence spectrophotometer. A scan from 200-800nm with increments of 10nm was initially carried out to discover excitation wavelength of maximum emission ($\lambda_{ex-max}$) and wavelength of maximum emission ($\lambda_{em-max}$). Quantum yields were estimated by reference to [Ru(bipy)$_3$](PF$_6$)$_2$ in water ($\Phi_R = 0.042$ in water at 420 nm) using the equation:
\[ \Phi_S = \Phi_R \cdot \frac{D_S}{D_R} \cdot \frac{A_R}{A_S} \cdot \frac{I_R}{I_S} \cdot \left( \frac{\eta_S}{\eta_R} \right)^2 \]

\( \Phi \) is the quantum yield, \( D \) is the integrated area of the emission band, \( A \) is the absorption of the solution at the excitation wavelength, \( I \) is the maximum intensity of the excitation band and \( \eta \) is the refractive index of the solvent. \( S \) is the sample and \( R \) is the reference [Ru(bipy)_3](PF_6)_2.

**Fluorescence pH and biomimetic media studies**

Buffers from pH 1.1 to pH 10 were made using a Fisher brand Hydrus 600 pH meter in order to investigate the stability of compounds by fluorescence spectroscopy. The following buffer systems were used, following the procedure described in “Buffers: A guide for the preparation and use of buffers in biological systems”; Chandra Mohan, 1997. \(^{165}\)

**pH Buffer systems**

1.1 KCl/HCl
2.0 KCl/HCl
3.0 Citric acid/Sodium citrate dehydrate OR Glycine / HCl
5.0 Citric acid/Sodium citrate dehydrate OR Acetic Acid / Sodium acetate
7.0 Citric acid/Dibasic Sodium phosphate OR Sodium phosphate Monobasic / Sodium phosphate Dibasic
9.0 Glycine/Sodium Hydroxide
10.0 Sodium Carbonate Anhydrous/Sodium Hydrogen Carbonate

A 4 mL solution of twice the final concentration was added to 4 mL of the buffer system or biomimetic media and then scanned for fluorescence between 200-800 nm with an excitation wavelength of \( \lambda_{\text{max}} \). If precipitation was observed samples were filtered using a Millex\textsuperscript{TM} 0.22 \( \mu \)m filter prior to scanning. Eagle’s Minimum Essential Medium containing 0.5% penicillin/streptomycin and 1% glutamine was used to assess the stability of the compounds in biomimetic media and also in relevance to the cell studies. Two equivalents of biologically relevant agents glutathione (GSH), L-cysteine, L-histidine, L-methionine or citric acid or chelator ethylenediaminetetraacetic acid (EDTA) were used.
Cell culturing and cell plate preparation.

Cells were cultured at 37 °C in a humidified atmosphere in air and diluted once confluence had been reached. Culture occurred in Eagle’s Minimum Essential Medium (EMEM) for HeLa (cervical cancer cells) and FEK-4 (epithelial fibroblast cells), Dulbecco’s Modified Eagle’s Medium (DMEM) for MCF-7, RPMI 1640 for PC3 (prostate cancer cells) and Weymouth’s medium for EMT6 (murine breast carcinoma). The media contained foetal calf serum (FCS) (10% for HeLa, PC-3, EMT6 and MCF-7 and 15% for FEK-4), 0.5% penicillin/streptomycin (10,000 IU mL⁻¹/10,000 mg mL⁻¹) and 200 mM L-Glutamine (5 mL). All steps were performed in absence of phenol red. Surplus supernatant containing dead cell matter and excess protein was aspirated. The live adherent cells were then washed with 2 x 10 mL aliquots of phosphate buffer saline (PBS) solution to remove any remaining media containing FCS, which inactivates trypsin. Cells were resuspended in solution by incubation in 3 mL of trypsin–PBS solution (0.25% trypsin) for 5 min at 37 °C. After trypsinisation, 5 mL of medium containing 10% serum was added to inactivate the trypsin and the solution was centrifuged for 5 min (1000 rpm, 25 ºC) to remove any remaining dead cell matter. The supernatant liquid was aspirated and 5 mL of cell medium (10% or 15% FCS) was added to the cell matter left behind. Cells were counted using a haemocytometer and then seeded as appropriate.

MTT assays. Cells cultured as above were plated (5 x 10⁴ cells mL⁻¹) and left for 48 h to adhere fully. All steps were carried out in the absence of phenol red. (a) For MI₅₀ estimations by MTT assays, cells were incubated with each compound tested for 48 h at 37 °C. Concentrations used were 1 nM, 100 nM, 1 µM, 10 µM, 50 µM, 100 µM and 250 µM (1% DMSO, 99% Eagle’s Modified Essential Medium containing FCS at standard concentration of the cell line). Subsequently, cells were washed three times with PBS and 100 mL of MTT was added (0.5 mg mL⁻¹, 10% PBS: SFM) followed by a 2 h incubation. Following aspiration, 100 mL of DMSO was added and 96 well plates were read at an ELISA plate reader, Molecular Devices Versa Max (BN02877). Data were obtained from five consistent results and MI₅₀ was calculated using Origin 8 as half the height of the fitted curve for each compound and for each individual experiment. Due to background absorbance, 100% cell death would not correspond to zero absorbance, therefore the height of the curve was calculated as the highest absorbance of the fit plot.
minus the minimum absorbance of the curve, at which point death of all cells has been achieved. Where 100% cell death had not been attained, in the case of compounds iiib and 3bN, the MI$_{50}$ was calculated using the same method in that the minimum absorbance of the fitted curve was subtracted. This value therefore indicates the minimum MI$_{50}$ for this compound and is stated in the text/figure legend. The mean MI$_{50}$ was calculated from the average of the five MI$_{50}$ values obtained from five consistent experiments. The error reported was the standard error of the mean and shown as ± S.E.M.

(b) Time lapse studies by MTT assays were carried out in HeLa, MCF-7 and FEK-4 cells by an analogous method to that described at (a) above, but under serum free EMEM conditions aiming to link directly cytotoxicity tests to fluorescence imaging experiments. Concentrations used were 10 µM and 50 µM in 1% DMSO, 99% Eagle’s Modified Essential Medium. Conclusions were reached following observations of cells’ viability within 1 h incubation (37 °C) interval.

LDH assay
The medium containing serum was aspirated and the compounds were added in serum free medium (SFM). After drug treatment, the supernatant was removed and added to a separate 96 well plate. To the remaining cells 100 µL SFM containing SDS was added. A preliminary study showed that the optimum percentage of SDS for FEK-4 and MCF-7 cells was 0.015% and for HeLa cells 0.1 %. These were mixed by pipette and 20 µL of LDH reaction kit, Roche Scientific ® (1:40 catalyst:dye) was added to each well. After 20 min the 96 well plates were read by Molecular Devices Versa Max (BN02877) at 570 nm. % LDH leakage was calculated by supernatant OD divided by (supernatant OD + cell OD).

Fluorescence microscopy. At Bath fluorescence microscopy experiments were carried out on a Nikon eclipse TE2000 epi-fluorescence or a Zeiss LSM510META microscope, whereas at the Rutherford Appleton Laboratory a modified Nikon TE2000-U microscope was used. For all fluorescence microscopy experiments, cells were cultured as above and plated in glass-bottomed dishes as $1.5 \times 10^5$ cells per dish (ca. 60% coverage) and incubated for 12 h for HeLa, EMT-6 and FEK-4, 48 h for MCF-7 and 72 h for PC-3 cells. All steps were carried out in the absence of phenol red. This timing
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was found to be minimum necessary for the two different cell lines to adhere to the glass bottomed Petri dishes used for imaging. Prior to compound addition, cells were washed 3 times with PBS, before adding serum free medium (2 mL). Subsequently, a small volume of medium was removed (10 mL) and compound in DMSO was added to obtain a final volume of 1 mL and the desired concentration. The final concentration of compounds on the cell plate was 50 mM in medium, containing 0.5% DMSO or 1% DMSO, depending on the compound solubility. After 20 min or 1 h incubation with the compound cells were washed 3 times with PBS and fresh serum free medium was added (1 mL) and images were recorded immediately.

Co-localisation studies. Cells were cultured using standard protocols, as described above, in RPMI-1640 (PC-3). Prior to addition of any commercial co-localisation dye, cells were washed 5 times with PBS. Protocols adapted from Invitrogen were used throughout.

(a) Hoechst A stock solution of 100 mg mL\(^{-1}\) Hoechst 33342 (Invitrogen) was prepared in sterile MilliQ water. To 10 µL of the stock solution, a volume of 990 µL was added giving a final concentration of 1 mg mL\(^{-1}\). After cells were incubated with this solution for 30 min a further 5 washings with PBS were carried out, before the addition of 1 mL of SFM. Subsequently, a small volume of serum free medium (SFM) was removed (e.g. 10 µL) and compound in DMSO was added in equal volume to what was removed, to obtain a final volume of 1 mL and the desired concentration (50 mM in cell medium containing 0.5% DMSO). After 20 min or 1 h incubation with the compound cells were washed 3 times with PBS and fresh serum free medium was added (1 mL) and images were recorded immediately.

(b) Mitotracker A stock solution of 20 µM Mitotracker® Red FM (Invitrogen) was prepared in DMSO. To 10 µL of stock solution, 990 µL was added giving a final concentration of 200 nM. After cells were incubated with this solution for 30 min a further 5 washings with PBS were carried out, before the addition of 1 mL of SFM. Subsequently, a small volume of SFM was removed (e.g. 10 µL) and compound in DMSO was added in equal volume to what was removed, to obtain a final volume of 2mL and the desired concentration (50 mM in cell medium containing 0.5% DMSO).
After 20 min or 1 h incubation with the compound cells were washed 3 times with PBS and fresh serum free medium was added (1 mL) and images were recorded immediately.

(c) Lysotracker A stock solution of 20 μM Lysotracker® Red DND-99 (Invitrogen) was prepared in DMSO. To 10 μL of stock solution, 990 μL was added giving a final concentration of 200 nM. After cells were incubated with this solution for 60 min a further 3 washings with PBS were carried out, before the addition of 1 mL of SFM. Subsequently, a small volume of SFM was removed (e.g. 10 μL) and compound in DMSO was added in equal volume to that which was removed to obtain a final volume of 1 mL and the desired concentration (50 mM in cell medium containing 0.5% DMSO). After 20 min or 1 h incubation with the compound cells were washed 3 times with PBS and fresh serum free medium was added (1 mL) and images were recorded immediately.

(d) ER tracker
A stock solution of 100 μM ER-tracker® Red (Invitrogen) was prepared in DMSO. To 10 μL of stock solution, 990 μL was added giving a final concentration of 100 μM. After cells were incubated with this solution for 20 min a further 3 washings with HBSS were carried out, before the addition of 1 mL of SFM. Subsequently, a small volume of SFM was removed (e.g. 10 μL) and compound in DMSO was added in equal volume to that which was removed to obtain a final volume of 1 mL and the desired concentration (e.g. 50 mM in cell medium containing 0.5% DMSO). After 20 min or 1 h incubation with the compound cells were washed 3 times with PBS and fresh serum free medium was added (1 mL) and images were recorded immediately.

(e) Nile Red
A stock solution of 10 μg/mL Nile Red (Invitrogen) was prepared in DMSO. To 10 μL of stock solution, 990 μL was added giving a final concentration of 100 ng/mL. After cells were incubated with this solution for 10 min a further 3 washings with HBSS were carried out, before the addition of 1 mL of SFM. Subsequently, a small volume of SFM was removed (e.g. 10 μL) and compound in DMSO was added in equal volume to that which was removed to obtain a final volume of 1 mL and the desired concentration (e.g. 50 mM in cell medium containing 0.5% DMSO). After 20 min or 1 h incubation with
the compound cells were washed 3 times with PBS and fresh serum free medium was added (1 mL) and images were recorded immediately.

(f) *Alexa Fluor*

A stock solution of 50 µg/mL Alexa fluor® 350 conjugate (*Invitrogen*) was prepared in HBSS (Hank’s Buffered Salt Solution). To 10 µL of stock solution, 990 µL was added giving a final concentration of 100 µM. After cells were incubated with this solution for 10 min a further 3 washings with HBSS were carried out, before the addition of 1 mL of SFM. Subsequently, a small volume of SFM was removed (e.g. 10 µL) and compound in DMSO was added in equal volume to that which was removed to obtain a final volume of 1 mL and the desired concentration (e.g. 50 mM in cell medium containing 0.5% DMSO). After 20 min or 1 h incubation with the compound cells were washed 3 times with PBS and fresh serum free medium was added (1 mL) and images were recorded immediately.

**Fixed cell imaging**

Cells were plated in a Petri dish containing a glass cover slip. Before fluorescence imaging, the serum containing medium was replaced with SFM. After the drug treatment cells were fixed by inverting the coverslip placing it onto 50 µL of paraformaldehyde-PBS (4% paraformaldehyde). After 15 min, the coverslip was mounted to a slide using Vectashield®, hard set mounting medium with DAPI (Vector Laboratories, Inc.)

**Two-photon excitation experiments** were performed at the Rutherford Appleton Laboratory following the methodology described in Botchway *et al.* 2008\(^{137}\) and in our recent publication (Pascu *et al.* 2011\(^{138}\)) with assistance of Dr S Botchway, Dr P Burgos, Mr A. Mckenzie and Mr A. Henman. An optical parametric oscillator was pumped by a mode locked Mira titanium sapphire laser (Coherent Lasers Ltd), generating 180 fs pulses at 75 MHz and emitting light at a wavelength of 580-630nm nm. The laser was pumped by a solid state continuous wave 532 nm laser (Verdi V18, Coherent Laser Ltd), with the oscillator fundamental output of 915 ± 2 nm or 810 ± 2 nm. The laser beam was focused to a diffraction limited spot through a water immersion ultraviolet
corrected objective (Nikon VC x60, NA1.2) and specimens illuminated at the microscope stage of a modified Nikon TE2000-U with UV transmitting optics.

The focused laser spot was raster scanned using an XY galvanometer (GSI Lumonics). Fluorescence emission was collected without de-scanning, bypassing the scanning system and passed through a coloured glass (BG39) filter. The scan was operated in normal mode and line, frame and pixel clock signals were generated and synchronised with an external fast microchannel plate photomultiplier tube used as the detector (R3809-U, Hamamatsu, Japan). These were linked via a Time-Correlated Single Photon Counting (TCSPC) PC module SPC830. Lifetime calculations were obtained using SPCImage analysis software (Becker and Hickl, Germany) or Edinburgh Instruments F900 TCSPC analysis software. Preliminary single-photon FLIM investigations were conducted using the Becker and Hickl DCS120 system with a 40 ps 473 nm diode laser.

**Fluorescence imaging of the nucleus and chromosomes**

Due to the observed nuclear uptake a preliminary investigation was carried out at the Rutherford Appleton Laboratory with Dr Yusuf Mohammed and Ms Ana Estandarte to assess whether the complexes bind to chromosomes. Non-cancerous lung cell line CCD37-LU was cultured in DMEM containing 10% foetal bovine serum (FBS) and 1% L-Glutamine at 37°C in a 5% CO₂ incubator. Chromosomes were isolated via a Colcemid (Gibco BRL) treatment before cells were trypsinised and subsequently resuspended in pre-warmed KCl hypotonic solution (0.075M) at 37°C for 12 minutes. This was followed by fixing in a 3:1 methanol/acetic acid solution, which was added onto a glass microscope slide, to which 100 µL of Compound 4d (100 µM) was aliquoted and was washed with DMSO after 15 minutes. Finally images were acquired using a Zeiss Axio Imager 2 microscope, which showed uptake of the complex in both the nucleus and in chromosomes.

**In vitro hypoxia testing study**

Cells were cultured as described above with the final concentration on the cell plate 50 μM, containing 4% DMSO for 20 minutes with the hypoxia investigation carried out by Dr Israt S. Alam, Imperial College London. Normoxic conditions were 20.7% O₂ and 5% CO₂ at 37 °C, with hypoxic samples pre-incubated for 20 minutes at 1% O₂, 5% CO₂ at
37 °C before complex addition. Cells were washed 3 times with PBS, which was replaced with 2mL of serum free media, with images recorded immediately.

Flow cytometry studies
Cells were seeded as $3 \times 10^5$ cells per well in a 6 well plate and incubated for ca. 12 h. The cells were subsequently washed twice with PBS, with the media replaced with serum free medium and compound of 50 μM 4%, DMSO final concentration. This was incubated for 20 minutes at 20.7% O$_2$ and 5% CO$_2$ at 37°C for the normoxic sample. Hypoxic conditions were obtained by pre-incubating the cells for 20 minutes at 1% O$_2$ and 5% CO$_2$ at 37°C, followed by incubation of a further 20 minutes under the same conditions with the compound. Following this cells were washed three times with PBS, trypsinised and centrifuged at 600g for three minutes. The precipitate was washed with PBS, resuspended in 1 mL of serum free medium, kept on ice and analysed in an LSRII cytometer (BD Biosciences, Rockville, MD USA), with 10 000 cells counted per event. Each experiment was carried out at least three times, with data analysis performed using FlowJo software (TreeStar, USA).

Radioactive cell uptake investigation
Cells were seeded as $3 \times 10^5$ cells per well in a 6 well plate and incubated for ca. 12 h. The cell medium was aspirated and replaced with serum free medium containing the Ga-68 radiolabelled complex (following the radiochemical procedure above). This was incubated at 20.7% O$_2$ and 5% CO$_2$ at 37°C for the normoxic sample. Hypoxic conditions were obtained by pre-incubating the cells for 20 minutes at 1% O$_2$ and 5% CO$_2$ at 37°C, followed by incubation under the same conditions with the compound for the time course of the study. Cell plates were put on ice, washed 3 times with ice-cold PBS and lysed using 0.2 mL RIPA buffer for 10 minutes (Thermo Fisher Scientific Inc., Rockford, IL, USA). PBS (0.5 mL) was added to each well and cell lysates were transferred to counting tubes, with measurements of decay-corrected radioactivity performed using a gamma counter (Cobra II Auto-Gamma counter, Packard Biosciences Co, Pangbourne, UK). Aliquots were snap-frozen and subsequently protein determination was carried out using a BCA 96-well plate assay (Thermo Fisher Scientific Inc., Rockford, IL, USA). Decay corrected counts were corrected to protein concentration, with data presented as percent of total radioactivity per mg of protein.
Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM), unless stated in the text. Student's t test (Prism v5.0 software for windows, GraphPad Software, San Diego, CA, USA) was used to assess the significance of comparison between two data sets. If $P \leq 0.05$ the differences between groups were regarded as significant.
9.2 Syntheses

Mono(substituted) 4-methyl-3-thiosemicarbazone acenaphthenequinone (ia)

For Compound ia, acenaphthenequinone (0.50 g, 2.74 mmol) and 4-methyl-3-thiosemicarbazide (0.29 g, 2.80 mmol) were added in absolute ethanol (15 mL) and refluxed for 2 hours. 10 drops of conc. HCl were added upon reflux. The solid was isolated by filtration whilst hot, resuspended in hot methanol (10 mL) and stirred for 15 minutes before filtering and washing with further methanol. The resultant yellow solid (0.60 g, 2.25 mmol, 82%), compound ia, was dried in vacuo.

$^1$H NMR (300 MHz, $d_6$--DMSO, 25 °C): $\delta$ 12.65 (s, 1H, N-NH), 9.38 (m, 1H, NHMe), 8.38 (d, 1H, H-3, $J = 8.3$ Hz), 8.14 (d, 1H, H-3', $J = 8.3$ Hz), 8.10 (d, 1H, H-1, $J = 7.1$ Hz), 7.98 (d, 1H, H-1', $J = 6.9$ Hz), 7.88 (overlapping t, 2H, H-2), 7.85 (overlapping t, 2H, H-2'), 3.13 (d, 3H, (CH$_3$)). $^{13}$C NMR (75.5 MHz, $d_6$--DMSO, 25°C): $\delta$ 188.6, 178.0, 139.1, 137.2, 132.9, 130.5, 130.1, 130.0, 129.0, 128.7, 127.2, 122.6, 118.3, 31.5. Mass spectrum ESI-MS calcd for C$_{14}$H$_{12}$N$_3$OS$^+$ [M + Na]$^+$ 292.0515, found m/z = 292.0494.

Mono(substituted) 4-ethyl-3-thiosemicarbazone acenaphthenequinone (ib)

For Compound ib, acenaphthenequinone (0.50 g, 2.74 mmol) and 4-ethyl-3-thiosemicarbazide (0.36 g, 3.00 mmol) were added in absolute ethanol (15 mL) and refluxed for 2 hours. 10 drops of conc. HCl were added upon reflux. The solid was isolated by filtration whilst hot, resuspended in hot methanol (10mL) and stirred for 15 minutes before filtering and washing with further methanol. The resultant yellow solid (0.66 g, 2.33 mmol, 85%), compound ib, was dried in vacuo.
Experimental

$^1$H NMR (300 MHz, $d_6$-DMSO, 25°C): $\delta$ 12.60 (s, 1H, N-NH), 9.42 (t, 1H, NHEt, $J = 5.9$ Hz), 8.38 (d, 1H, H-3, $J = 8.2$ Hz), 8.14 (d, 1H, H-3’, $J = 8.3$ Hz), 8.10 (d, 1H, H-1, $J = 7.0$ Hz), 8.01 (d, 1H, H-1’, $J = 6.9$ Hz), 7.88 (t+t, 2H, H-2 and H-2’), 3.68 (m, 2H, (CH$_2$)CH$_3$), 1.23 (t, 3H, CH$_2$CH$_3$, $J = 7.2$ Hz).

$^{13}$C NMR (75.5 MHz, $d_6$-DMSO, 25°C): $\delta$ 188.5, 176.9, 139.1, 137.2, 132.8, 130.5, 130.1, 129.9, 128.9, 128.6, 127.1, 122.5, 118.3, 39.1, 14.1. Mass spectrum ESI-MS calcd for C$_{15}$H$_{14}$N$_3$OS$^+$ [M + Na]$^+$ 306.0677, found 306.0667.

Mono(substituted) 4-phenyl-3-thiosemicarbazone acenaphthenequinone (ic)

For Compound ic, acenaphthenequinone (0.5136 g, 2.81 mmol) was added to 1.1 equivalents of 4-phenyl-3-thiosemicarbazide (0.5116 g, 3.05 mmol) were added in absolute ethanol (15mL) and refluxed for 2 hours. 10 drops of conc. HCl were added upon reflux. The solid was isolated by filtration whilst hot, resuspended in hot methanol (10mL) and stirred for 15 minutes before filtering and washing with further methanol. The resultant light orange solid (0.800 g, 2.41 mmol, 85%), compound ic, was dried in vacuo.

$^1$H-NMR (300 MHz, $d_6$-DMSO, 25°C): $\delta$ 12.79 (s, 1H, N-NH), 10.93 (s, 1H, NHPh), 8.36 (d, 1H, H-3, $J = 8.1$ Hz), 8.13 (d, 1H, H-3’, $J = 8.3$ Hz), 8.10 (d, 1H, H-1, $J = 6.9$ Hz), 8.08 (d, 1H, H-1’, $J = 7.0$ Hz), 7.86 (overlapping t, 1H, H-2), 7.82 (overlapping t, 1H, H-2’), 7.65 (obscured d, 2H, o-H), 7.45 (t, 2H, m-H, $J = 7.6$ Hz, 8.3 Hz), 7.29 (m, 1H, o-H). $^{13}$C-NMR (75.5 MHz, $d_6$-DMSO, 25°C): $\delta$ 188.6, 176.6, 139.4, 138.5, 137.5, 132.8, 130.4, 129.99, 129.93, 128.9, 128.6, 128.4, 127.2, 125.8, 122.5, 120.9, 118.8. Mass spectrum ESI-MS calcd for C$_{19}$H$_{14}$N$_3$OS$^+$ [M+H]$^+$ 332.0858, found 332.0861. Elem. Anal.: Found: C; 68.10 %, H; 3.96 %, N; 13.10 %, Calc.: C; 68.86 %, H; 3.95 %, N; 12.68 %.
Mono(substituted) 4-allyl-3-thiosemicarbazone acenaphthenequinone (id)

For Compound id, acenaphthenequinone (0.500 g, 2.745 mmol) and 4-allyl-3-thiosemicarbazide (0.393 g, 3.293 mmol) were added in absolute ethanol (15mL) and refluxed for 2 hours. 10 drops of conc. HCl were added upon reflux. The solid was isolated by filtration whilst hot, resuspended in hot methanol (10mL) and stirred for 15 minutes before filtering and washing with further methanol. The resultant yellow solid (0.595 g, 1.723 mmol, 63%), compound id, was dried in vacuo.

$^1$H NMR ($d_6$-DMSO, 25 °C): $\delta$ 12.61 (s, 1H, N-NH'), 9.57 (t, 1H, NHAllyl, $J = 6.0$ Hz), 8.34 (d, 1H, H-3, $J = 8.2$ Hz), 8.11 (d, 1H, H-3', $J = 8.3$ Hz), 8.06 (d, 1H, H-1', $J = 7.0$ Hz), 7.96 (d, 1H, H-1, $J = 6.9$ Hz), 7.85 (overlapping t, 1H, H-2 or H-2'), 7.81 (overlapping t, 1H, H-2 or H-2'), 5.95 (m, 1H, -CH$_2$CHCH$_2$), 5.27 + 5.21 (q, 1H, $H_{trans}$, $J = 17.3$ Hz), 5.19 + 5.15 (q, 1H, $H_{cis}$, $J = 10.3$ Hz), 4.30 (ddt, 2H, -CH$_2$CHCH$_2$).

$^{13}$C NMR ($d_6$-DMSO, 25 °C): $\delta$ 188.5, 177.5, 139.1, 137.3, 134.0, 132.8, 130.4, 130.0, 129.9, 128.9, 128.6, 127.1, 122.5, 118.4, 116.3, 46.5. Mass spectrum ESI-MS calcd for C$_{16}$H$_{13}$N$_3$NaOS$^+$ 318.0677 [M+Na]$^+$, found 318.0665. Elem. Anal.: Found: C; 65.09 %, H; 4.43 %, N; 14.21 %, Calc.: C; 65.06 %, H; 4.44 %, N; 14.23 %.

Bis(substituted) 4-methyl-3-thiosemicarbazone acenaphthenequinone (iia)

Acenaphthenequinone (0.25g, 0.137 mmol) and methyl thiosemicarbazide (0.43 g, 0.411 mmol) were suspended in 40mL ethanol and refluxed for 4 hours. 10 drops of conc. HCl were added upon reflux. The solid was isolated by filtration whilst hot, resuspended in hot methanol (10 mL) and stirred for 15 minutes before filtering and
washing with further methanol. The resultant yellow solid (266.6 mg, 54 %), compound iia, was dried in vacuo.

\[^1\text{H} \text{NMR} \] (300 MHz, \textit{d}_6\text{-DMSO}, 25\,^\circ\text{C}) \, \delta \, 12.60 \, (s, \, 1\text{H}, \, \text{N-NH}'), \, 11.29 \, (s, \, 1\text{H}, \, \text{N-NH}), \, 9.15 \, (m, \, 1\text{H}, \, \text{NH'}\text{CH}_3), \, 8.86 \, (m, \, 1\text{H}, \, \text{NH}CH_3), \, 8.20 \, (d \, 1\text{H}, \, H-1', \, J = 7.3 \text{ Hz}), \, 8.13 \, (d, \, 1\text{H}, \, H-3', \, J = 8.3 \text{ Hz}), \, 8.07 \, (d, \, 1\text{H}, \, H-1', \, J = 7.0 \text{ Hz}), \, 8.03 \, (d, \, 1\text{H}, \, H-3, \, J = 8.3 \text{ Hz}), \, 7.79 \, (m, \, 2\text{H}, \, H-2 \text{ and } H-2'), \, 3.11 \, (m, \, 6\text{H}, \, overlapping \text{CH}_3 \text{ groups}).

\[^{13}\text{C} \text{NMR} \] (75.5 MHz, \textit{d}_6\text{-DMSO}, 25\,^\circ\text{C}) \, \delta \, 178.42, \, 138, \, 136.45, \, 133.23, \, 130.25, \, 129.20, \, 128.20, \, 128.18, \, 127.03, \, 125.02, \, 31.79, \, 31.6. \textbf{Mass spectrum} \text{ ESI-MS calcd for } C_{16}H_{15}N_6S_2^- \, [M-H]^- \, 355.0800, \, \text{found} \, 355.08\, 04.

\textbf{Elem. Anal.:} \, \text{Found C; 53.7 \%, H, 4.51 \%, N; 23.4 \%, Calc.: C; 53.91 \%, H; 4.52 \%, N; 23.58 \%}.

**Bis(substituted) 4-ethyl-3-thiosemicarbazone acenaphthenequinone (iib)**

\[
\begin{align*}
\text{Acenaphthenequinone (0.25 g, 0.137 mmol) and 4-ethyl-3-thiosemicarbazide (0.48 g, 0.411 mmol) were suspended in 40 mL ethanol and refluxed. 10 drops of conc. HCl were added upon reflux. The solid was isolated by filtration whilst hot, resuspended in hot methanol (10 mL) and stirred for 15 minutes before filtering and washing with further methanol. The resultant yellow solid (446.7 mg, 85%), compound iib, was dried in vacuo.}

\[^1\text{H} \text{NMR} \] (300 MHz, \textit{d}_6\text{-DMSO}, 25\,^\circ\text{C}) \, 12.55 \, (s, \, 1\text{H}, \, \text{N-NH}'), \, 11.21 \, (s, \, 1\text{H}, \, \text{N-NH}), \, 9.19 \, (t, \, 1\text{H}, \, \text{NH}\text{Et}, \, J = 6.0 \text{ Hz}), \, 8.80 \, (t, \, 1\text{H}, \, \text{NHEt}, \, J = 5.5 \text{ Hz}), \, 8.19 \, (d, \, 1\text{H}, \, H-1, \, J = 7.2 \text{ Hz}), \, 8.13 \, (d, \, 1\text{H}, \, H-3', \, J = 8.3 \text{ Hz}), \, 8.09 \, (d, \, 1\text{H}, \, H-1', \, J = 7.0 \text{ Hz}), \, 8.03 \, (d, \, 1\text{H}, \, H-3, \, J = 8.3 \text{ Hz}), \, 7.79 \, (\text{overlapping t, } 2\text{H}, \, H-2 \text{ and } H-2'), \, 3.66 \, (m, \, 4\text{H}, \, \text{CH}_2\text{CH}_3), \, 1.24 \, (m, \, 6\text{H}, \, \text{CH}_2\text{CH}_3). \, \textbf{Mass spectrum} \text{ ESI-MS calcd for } C_{18}H_{19}N_6S_2^- \, [M-H]^- \, 383.1113, \, \text{found} \, 383.1155.

\textbf{Elem. Anal.:} \, \text{Found C; 56.1 \%, H, 5.23 \%, N; 21.8 \%. Calc.: C; 56.22 \%, H; 5.24 \%, N; 21.86 \%.}
Experimental

Bis(substituted) 4-phenyl-3-thiosemicarbazone acenaphthenequinone (iic)

Acenaphthenequinone (0.25g, 0.137mmol) and 4-phenyl-3thiosemicarbazide (0.68g, 0.411mmol) were suspended in 40mL ethanol and refluxed 4 hours. 10 drops of conc. HCl were added upon reflux. The solid was isolated by filtration whilst hot, resuspended in hot methanol (10 mL) and stirred for 15 minutes before filtering and washing with further methanol. The resultant yellow solid (549 mg, 83%), compound iic, was dried in vacuo.

$^1$H NMR: (300 MHz, $d_6$-DMSO, 25°C) $\delta$ 12.95 (s, 1H, N-N($H')$), 11.54 (s, 1H, N-NH), 10.74 (s, 1H, NHPh or N-NH'), 10.67 (s, 1H, NHPh or N-NH'), 8.30 (overlapping d, 2H, $H$-$I$ and $H$-$I'$, $J = 7.6$ Hz and 7.4 Hz), 8.18 (d, 1H, $H$-3 or $H$-3', $J = 8.4$ Hz), 8.07 (d, 1H, $H$-3 or $H$-3', $J = 8.3$ Hz), 7.84 (m, 4H, $H$-2 and $H$-2', o-$H'$), 7.68 (d, 2H, o-$H'$, $J = 8.1$ Hz), 7.43 (m, 4H, m-$H$ and m-$H'$), 7.25 (overlapping t, 2H, p-$H$ and p-$H'$). $^{13}$C NMR: (75.5 MHz, $d_6$-DMSO, 25°C) $\delta$ 178.41, 176.55, 138.89, 138.81, 132.70, 130.0, 129.14, 128.34, 127.76, 127.00, 125.76, 124.30. Mass spectrum ESI-MS calcd for C$_{26}$H$_{19}$N$_6$S$_2$ $^{-}$[M-H]$^{-}$ 479.1118, found 479.1194. Elem. Anal.: Found C; 64.75 %, H, 4.19 %, N; 7.55 %. Calc.: C; 64.98 %, H, 4.19 %, N; 17.49 %.
Experimental

**Bis(substituted) 4-allyl-3-thiosemicarbazone acenaphthenequinone (iid)**

![Diagram]

Acenaphthenequinone (0.25g, 0.137mmol) and 4- allyl 3-thiosemicarbazide (0.54 g, 0.411 mmol) were suspended in 40mL ethanol and were refluxed. 10 drops of conc. HCl were added upon reflux. The solid was isolated by filtration whilst hot, resuspended in hot methanol (10 mL) and stirred for 15 minutes before filtering and washing with further methanol. The resultant yellow solid (415 mg, 74%), compound iid, was dried in vacuo.

**1H NMR:** (300 MHz, $d_6$-DMSO, 25ºC) δ 12.63 (s,1H, N-NH'), 11.27 (s,1H, N-NH), 9.32 (t, 1H, NHAllyl, $J = 6.0$ Hz), 9.02 (t, 1H, NH'Allyl, $J = 5.7$ Hz), 8.21 (d, 1H, $H-1$ or $H-1'$, $J = 7.2$ Hz), 8.11 (overlapping d, 2H, $H-3$ and $H-3'$), 8.01 (d, 1H, $H-1$ or $H-1'$, $J = 7.1$ Hz), 7.80 (overlapping t, 1H, $H-2$ or $H-2'$), 7.78 (overlapping t, 1H, $H-2$ or $H-2'$), 5.95 (m, 2H, CH$_2$CHCH$_2$) 5.35 + 529 (ddt, 2H, $H_{trans}$, $J = 17.4$ Hz), 5.25 + 5.18 (ddt, 2H, $H_{trans}$, $J = 17.4$ Hz), 5.17 + 5.14 (m, 2H, $H_{cis}$, $J = 10.6$ Hz), 4.31 (m, 4H, CH$_2$CHCH$_2$).  

**13C NMR:** (75.5 MHz, $d_6$-DMSO, 25ºC) δ 182.52, 178.14, 138.23, 136.45, 134.86, 134.40, 133.21, 130.32, 129.28, 128.88, 128.55, 128.18, 127.09, 124.93, 116.83, 116.28, 47.07, 46.52. **Mass spectrum** ESI-MS calcd for C$_{20}$H$_{19}$N$_6$S$_2$ [M-H]$^-$ 407.1113, found 407.1106. **Elem. Anal.**: Found C; 58.9 %, H; 4.94 %, N; 20.5 %. Calc.: C; 58.80 %, H, 4.93 %, N; 20.57 %.

**Zinc(II) bis(4-methyl-3-thiosemicarbazone) acenaphthenequinone (1a)**

![Diagram]

Zinc complex 1a was prepared by suspending 1 equivalent of acenaphthenequinone (502 mg, 2.76 mmols) and 3 equivalents of zinc acetate (1.82 g, 8.29 mmols) in acetic acid at 60 ºC. Vast excess 4-methyl-3-thiosemicarbazide (2.95 g, 28.04 mmols) was
added, the temperature increased to 120 °C and refluxed for 30h. Isolation was achieved by hot filtration, followed by washing with 100 mL diethyl ether, yielding an orange powder of 601 mg, 1.43 mmols, 52%. Comparison of $^1$H NMR spectra of these products with literature data, as well as mass spectroscopy, confirmed the desired structures.

$^1$H NMR ($d_6$-DMSO, 25 °C): δ 8.19 (d, 2H, $H-1$, $J = 6.8$ Hz), 7.99 (d, 2H, $H-3$, $J = 8.3$ Hz), 7.85 (m, 2H, N-H), 7.76 (apparent t, 2H, $H-2$, $J = 7.9$ and 7.4 Hz), 3.04 (d, 6H, $CH_3$, $J = 3.9$Hz), 1.91 (3H, $CH_3$, acetic acid) $^{13}$C NMR (300 MHz, $d_6$–DMSO, 25°C): δ 179.2, 140.0, 138.1, 130.6, 128.6, 128.2, 126.9, 123.1, 29.4. Mass spectrum ESI-MS calcd for $C_{16}H_{15}N_6S_2Zn^+$ [M + H]$^+$ 419.0086, found 419.0101. HPLC (Method H): $R_t = 2.0$ mins.

**Zinc(II) bis(4-ethyl-3-thiosemicarbazone) acenaphthenequinone (1b)**

Zinc complex 1b was prepared by suspending 1 equivalent of acenaphthenequinone (498 mg, 2.73 mmols) and 3 equivalents of zinc acetate (1.763 g, 8.03 mmols) in acetic acid at 60 °C. Vast excess of 4-ethyl-3-thiosemicarbazide (3.229 g, 27.09 mmols) was added, the temperature increased to 120 °C and was refluxed for 30h. Isolation was achieved by hot filtration, followed by washing with 100 mL diethyl ether, yielding an orange powder of 603 mg, 1.35 mmols, 49%. Comparison of $^1$H NMR spectra of these products with literature data, as well as mass spectroscopy, confirmed the desired structures.

$^1$H NMR ($d_6$-DMSO, 25 °C): δ 8.17 (d, 2H, $H-1$, $J = 7.0$ Hz), 7.98 (d, 2H, $H-3$, $J = 8.1$ Hz), 7.91 (m, 2H, NHEt), 7.75 (t, 2H, $H-2$, $J = 7.7$ Hz), 3.57 (q, 4H, $CH_2CH_3$, $J = 3.4$ Hz and 3.6 Hz), 1.23 (t, 6H, $CH_2CH_3$, $J = 7.2$ Hz).$^{13}$C NMR (300 MHz, $d_6$–DMSO, 25°C ): δ 178.8, 140.0, 138.3, 130.9, 129.0, 128.6, 127.2, 123.1, 37.5, 14.8. Mass spectrum ESI-MS calcd for $C_{18}H_{19}N_6S_2Zn^+$ [M + H]$^+$ 447.0399, found 447.0396.
Zinc(II) bis(4-phenyl-3-thiosemicarbazone) acenaphthenequinone (1c)

Zinc complex 1c was prepared by suspending 1 equivalent of acenaphthenequinone (0.504 g, 2.77 mmols) and 3 equivalents of zinc acetate (1.81 g, 8.25 mmols) in acetic acid at 60 °C. 4-phenyl-3-thiosemicarbazide was added, the temperature increased to 120 °C and was refluxed for 30h. Isolation was achieved by hot filtration, followed by washing with 100 mL diethyl ether, yielding a red powder of 1.020 g, 1.88 mmols, 68%. Comparison of 1H NMR spectra of these products with literature data, as well as mass spectroscopy, confirmed the desired structures.

1H NMR (d6-DMSO, 25 °C): δ 10.03 (s, 2H NHPh), 8.25 (d, 2H, H-1, J = 7.0 Hz), 8.10 (d, 2H, H-3, J = 8.2 Hz), 7.93 (d, 4H, α-H, J = 7.7 Hz), 7.85 (t, 2H, H-2, J = 7.1 Hz), 7.42 (t, 4H, m-H and m-H', J = 7.9 Hz), 7.08 (t, 2H, p-H and p-H', J = 7.3Hz). 13C NMR (300 MHz, d6-DMSO, 25 °C): δ 177.2, 142.3, 140.5, 138.6, 130.7, 128.6, 128.3, 128.0, 127.5, 123.5, 122.7, 120.9. Mass spectrum ESI-MS calcd for C26H19ZnN6S2+ [M + H]+ 543.0399, found 543.0348.

Zinc(II) bis(4-allyl-3-thiosemicarbazone) acenaphthenequinone (1d)

Zinc complex 1d was prepared by suspending 1 equivalent of acenaphthenequinone (0.502 mg, 2.76 mmols) and 3 equivalents of zinc acetate (1.815 g, 8.27 mmols) in acetic acid at 60 °C. Vast excess of 4-allyl-3-thiosemicarbazide was added the temperature increased to 120 °C and refluxed for 30h. Isolation was achieved by hot filtration, followed by washing with 100 mL diethyl ether, yielding an orange powder of 62%. Comparison of 1H NMR spectra of these products with literature data, as well as mass spectroscopy, confirmed the desired structures.
Experimental

$^1$H NMR ($d_6$-DMSO, 25 °C): $\delta$ 8.18 (d, 2H, $H$-1, $J = 6.8$ Hz), 7.95 (t, 2H, NH), 7.89 (d, 2H, $H$-3, $J = 8.1$ Hz), 7.68 (apparent t, 2H, $H$-2, $J = 8.1$ Hz and 7.2 Hz), 6.00 (m, 2H, -CH$_2$CH$_2$CH$_2$), 5.27 (dd, 2H, $H_{trans}$), 5.15 (dd, 2H, $H_{cis}$), 4.18 (bm, 4H, -CH$_2$CH$_2$)

$^{13}$C NMR (300 MHz, $d_6$-DMSO, 25 °C): $\delta$ 175.3, 155.4, 146.3, 135.3, 130.5, 129.9, 128.4, 126.9, 122.9, 117.1, 47.9. Mass spectrum ESI-MS calcd for C$_{20}$H$_{19}$N$_6$S$_2$Zn$^+$ [M + H]$^+$ 471.0396, found 471.0404.

Zinc(II) bis(t-Boc-protected amine thiosemicarbazone) acenaphthenequinone (1e)

Acenaphthenequinone (50 mg, 0.274 mmols) and zinc acetate (183 mg, 0.833 mmols) in 2:98 acetic acid:dioxane were added in the presence of activated molecular sieves was heated to 60°C. A mixture of Monoboc thiosemicarbazide (tert-butyl-2-hydrazinecarbonothioyl)hydrazinecarboxylate) and the di-substituted-boc, was added in excess to a suspension and the temperature raised to 110 °C. This was refluxed for 30 h and by filtrated whilst hot. The filtrate was dried in vacuo, washed with 50 mL diethyl ether and dried once more in vacuo. The product was obtained as an orange solid (58 mg, 0.093 mmols, 34%). Elemental analysis indicated that this complex could not be isolated in high purity.

$^1$H NMR ($d_6$-DMSO, 25 °C): $\delta$ 9.39 (s, 2H, NH), 8.92 (s, 2H, NH), 8.30 (d, 2H, $H$-1, $J = 7.1$ Hz), 8.06 (d, 2H, $H$-3, $J = 8.2$ Hz), 7.76 (t, 2H, $H$-2, $J = 7.6$ Hz), 3.50 (d, 1H, dioxane), 1.91 (s, 1H, acetic acid), 1.48 (s, 18H, t-Boc). Mass spectrum ESI-MS calcd for C$_{24}$H$_{27}$N$_8$O$_4$S$_2$Zn$^-$ [M-H] 619.0913, found 619.0893. Elem. Anal.: Found C; 35.3 %, H; 4.42 %, N; 18.05 %. Calc. C; 46.34 %; H; 4.54 %; N; 18.01 %. HPLC (Method H): $R_t = 2.4$ mins.
Experimental

Copper(II) bis(4-methyl-3-thiosemicarbazone) acenaphthenequinone (2a)

[Chemical structure image]

Synthesised from the proligand: 1 equivalent compound iia (0.1g, 0.028 mmol) and 3 equivalents copper acetate (0.152g, 0.0842mmol) were stirred at rt in 50 mL methanol for 24 hours. This was filtered and washed with water and diethyl ether yielding a brown solid 82.9 mg, 70.5%. Synthesis via zinc complex: 1a (50 mg, 0.119 mmols) was added to two equivalents of copper acetate (49.2 mg, 0.247 mmols) in methanol and stirred at room temperature for 24h, filtered and washed with diethyl ether. This yielded 36 mg (0.086 mmols, 72%) of brown solid. Mass spectrum ESI-MS calcd for C_{16}H_{15}CuN_{6}S_{2}^{+} [M + H]^+, 418.0900, found 418.0092. **Elem. Anal.:** Found: C; 45.6 %, H; 3.85 %, N; 18.8 %. Calc.: C; 45.97 %, H; 3.38 %, N; 20.11 %. **HPLC (Method A):** R_t = 11.3 mins, (Method H): R_t = 8.1 mins.

Copper(II) bis(4-ethyl-3-thiosemicarbazone) acenaphthenequinone (2b)

[Chemical structure image]

Synthesis from proligand: 1 equivalent bis ethyl thiosemicarbazone ligand (0.15g, 0.390 mmol) and 3 equivalents copper acetate (0.254g, 1.10 mmol) were stirred at rt in 50 mL methanol for 24 hours. This was filtered and washed with water and diethyl ether giving a brown solid, yielding 67.8 mg, 0.152 mmols, 39%. Synthesis from the zinc complex: 1b (50 mg, 0.112 m mols) was added to two equivalents of copper acetate (44.5 mg, 0.223 mmol) in methanol. This was stirred at rt in 50 mL methanol for 24 hours. It was filtered and washed with water and diethyl ether yielding a brown solid of 26.6 mg, 0.060 mmols, 53%. Mass spectrum ESI-MS calcd for C_{18}H_{19}CuN_{6}S_{2}^{+} [M + H]^+ 446.0403, found 446.0400. **Elem. Anal.:** Found: C; 47.9 %, H; 4.05 %, N; 17.60 %. Calc.: C; 47.73 %, H; 4.64%, N; 17.58%. **HPLC (Method H):** R_t = 9.3 mins.
Copper(II) bis(4-phenyl-3-thiosemicarbazone) acenaphthenequinone (2c)

Synthesis from zinc bis phenyl thiosemicarbazone: The zinc complex 1c, (101 mg, 0.186 mmols) added to two equivalents of copper acetate (77 mg, 0.386 mmols) in methanol and stirred at room temperature for 24h. The product (67 mg, 0.124 mmols, 66%) was isolated by filtration and washing with water and diethyl ether.

**Mass spectrum** ESI-MS calcd for C_{26}H_{17}CuN_{6}S_{2}^{-} [M-H]^{-} 540.0258, found 540.0237.

**Elem. Anal.:** Found: C; 55.8 %, H, 3.50 %; N; 14.60 %. Calc. (+MeOH): C; 56.48 %, H, 3.86 %, N; 14.64 %. **HPLC** (Method D): R_t = 14.9 mins.

Copper(II) bis(4-allyl-3-thiosemicarbazone) acenaphthenequinone (2d)

Synthesis from the ligand: 1 equivalent bis allyl thiosemicarbazone (0.2g, 0.049 mmol) and 3 equivalents copper acetate (0.32g, 0.147 mmol) were stirred at rt in 50 mL methanol for 24 hours. This was filtered and washed with water and diethyl ether yielding a brown solid of 95mg, 41%. Synthesis from zinc complex: 1d, (52 mg, 0.110 mmols) added to two equivalents of copper acetate (44 mg, 0.223 mmols) in methanol and stirred at room temperature for 24h. The product (33 mg, 0.070 mmols, 64%) was isolated by filtration and washing with water and diethyl ether.

**Mass spectrum** ESI-MS calcd for C_{20}H_{17}N_{6}S_{2}Cu^{-} [M-H]^{-} 468.0258, found 468.0260. **Elemental Analysis:** Found: C; 49.85 %, H, 3.82 %, N; 17.0 %. Calc.: C; 51.10 %, H, 3.86 %, N; 17.88 %. **HPLC** (Method H): R_t = 9.4 mins.
Copper(II) bis(t-Boc-protected amine thiosemicarbazone) acenaphthenequinone (2e)

The zinc complex 1e, (20 mg, 0.032 mmols) was added to two equivalents of copper acetate in methanol and stirred at room temperature for 24h. The product was isolated by rotary evaporation yielding 8 mg of very dark brown solid (0.013 mmols, 40%).

Mass spectrum ESI-MS calcd for $C_{24}H_{27}CuN_8O_4S_2^-\ [M-H]^- 618.0898$, found 618.0761.

HPLC (Method H): $R_t = 8.5$ mins.

Gallium(III) chloride bis(4-methyl-3-thiosemicarbazone) acenaphthenequinone (3a)

A suspension of zinc bis(4-methyl-3-thiosemicarbazide) acenaphthenequinone (0.101 g, 0. 242 mmol) in MeOH (50 mL) was added to GaCl$_3$ (0.193 g, 1.11 mmol). The resulting suspension was heated at reflux for 6 h. The red solid that precipitated on cooling to room temperature was then isolated by filtration, washed with Et$_2$O and dried under vacuum (0.0395 g, 0.086 mmol, 36%). The (E,E) and (E,Z) isomers were found to be present in the integral ratio of 57:43 (favouring the (E,E) isomer) in $d_6$-DMSO by $^1$H NMR analysis.

(E,E) isomer (57% relative abundance) $^1$H NMR ($d_6$-DMSO, 25 °C): $\delta$ 8.81 (broad s, 2H, NH), 8.29 (d, 2H, H-1, $J = 6.8$ Hz), 8.19 (d, 2H, H-3, $J = 8.3$ Hz), 7.88 (t, 2H, H-2, $J = 4.5$ Hz), 3.11 (d, 6H, CH$_3$). (E,Z) isomer (43% relative abundance) $^1$H NMR ($d_6$-DMSO, 25 °C): $\delta$ 9.53 (apparent d, 1H, NH, $J = 4.1$ Hz), 8.90 (apparent d, 1H, N(H'), $J = 4.5$ Hz), 8.58 (d, 1H, H-1 or H-1', $J = 7.0$ Hz ), 8.16 (obscured d, 1H, H-1 or H-1'), 8.07 (d, 1H, H-3 or H-3', $J = 8.2$Hz), 7.94 (d, 1H, H-3 or H-3', $J = 6.9$ Hz), 7.84 (t, 1H,
Experimental

$H-2$ or $H-2'$, $J = 7.7\text{Hz}$), 7.80 (t, 1H, $H-2$ or $H-2'$, $J = 7.6\text{Hz}$), 3.16 (d, 3H, $CH_3$ or $CH_3'$, $J = 4.4\text{Hz}$), 3.12 (observed d, 3H, $CH_3$ or $CH_3'$). $^{13}$C NMR ($d_6$-DMSO, 25 °C): $\delta$ 178.7, 174.7, 170.7, 147.5, 142.4, 138.9, 138.8, 136.0, 132.3, 130.4, 129.7, 129.4, 129.1, 129.04, 128.95, 128.90, 127.4, 126.2, 124.6, 118.4, 33.2, 30.2 29.4, 27.1. Mass spectrum ESI-MS calcd for $C_{16}H_{13}ClGaN_6S_2 - [M-H]$ 458.9587, found 458.9605. Elemental analysis: Calc.: C; 41.81 %, H; 3.07 %, N; 18.28 %, Found: C; 41.8 %, H; 3.04 %, N; 18.0 %.

Gallium(III) chloride bis(4-ethyl-3-thiosemicarbazone) acenaphthenequinone (3b)

A suspension of zinc bis(4-ethyl-3-thiosemicarbazide) acenaphthenequinone (0.216 g, 0.482 mmol) in MeOH (50 mL) was added to GaCl$_3$ (0.420 g, 1.478 mmol). The resulting suspension was heated at reflux for 6 h. The red solid that precipitated on cooling to room temperature was then isolated by filtration, washed with Et$_2$O and dried under vacuum (0.146 g, 0.299 mmol, 62%).

The $(E,E)$ and $(E,Z)$ isomers were found to be present in the integral ratio of approximately 1:1 in $d_6$-DMSO by $^1$H NMR analysis. $(E,Z)$ isomer (51%). $^1$H NMR ($d_6$-DMSO, 25 °C): $\delta$ 9.58 (t, 1H, NH), 8.87 (t, 1H, NH'), 8.50 (d, 1H, $H-1$, $J = 7.0\text{Hz}$), 8.11 (d, 1H, $H-3'$, $J = 8.3\text{Hz}$), 8.03 (d, 1H, $H-3$, $J = 8.1\text{Hz}$), 7.93 (d, 1H, $H-1'$, $J = 6.9\text{Hz}$), 7.83 (apparent t, 1H, $H-2$), 7.76 (apparent t, 1H, $H-2'$), 3.61 + 3.56 (m, 4H, $CH_2$), 1.26 + 1.25 (two t, 6H, $CH_3$, $J = 7.2\text{Hz}$). $(E,E)$ isomer (49%): $\delta$ 8.79 (broad t, 2H, NH), 8.16 (d, 2H, $H-1$, $J = 7.0\text{Hz}$), 8.09 (d, 2H, $H-3$, $J = 8.3\text{Hz}$), 7.75 (apparent t, 2H, $H-2$), 3.59 (m, 4H, $CH_2$), 1.25 (t, 6H, $CH_3$, $J = 7.2\text{Hz}$). $^{13}$C NMR ($d_6$-DMSO, 25 °C): $\delta$ 177.56, 173.20, 169.93, 138.72, 138.50, 135.84, 132.28, 130.25, 129.65, 129.31, 128.92, 128.47, 127.00, 126.83, 126.06, 124.23, 118.39, 114.13, 41.87, 38.34, 37.57, 14.60, 14.07, 13.67. Mass spectrum ESI-MS calcd for $C_{18}H_{17}ClGaN_6S_2 [M-H]^-$ 484.9900, found 484.9907.
Gallium(III) chloride bis(4-phenyl-3-thiosemicarbazone) acenaphthenequinone (3c)

A suspension of zinc bis(4-phenyl-3-thiosemicarbazide) acenaphthenequinone (0.100 g, 0.184 mmol) in MeOH (50 mL) was added to GaCl₃ (0.1567 g, 0.551 mmol). The resulting suspension was heated at reflux for 6 h. The red solid that precipitated on cooling to room temperature was then isolated by filtration, washed with Et₂O and dried under vacuum (72.4 mg, 0.124 mmols, 67%). The (E,E) and (E,Z) isomers were found to be present in the integral ratio of approximately 1:1 in d₆-DMSO by ¹H NMR analysis. (E,Z):(E,E) 27:73%. NMR data of the (E,Z) isomer was not assigned due to major overlapping with (E,E) peaks and poorly defined signals. (E,E) isomer (73%). ¹H NMR (d₆-DMSO, 25 °C): δ 10.81 (s, 2H, NH), 8.28 (two overlapping d, 4H, H-3 + H-I), 7.97 (apparent t, 2H, H-2), 7.87 (d, 4H, o-H, J = 8.0 Hz), 7.50 (apparent t, 4H, m-H), 7.20 (t, 2H, p-H, J = 7.3 Hz). ¹³C NMR (75.5 MHz, d₆-DMSO, 25 °C): weak due to poor solubility

Mass spectrum ESI-MS calcd for [C₂₆H₁₇ClGaN₆S₂]⁺ [M-H]⁺ 580.9900, found 580.9915.

Gallium(III) chloride bis(4-allyl-3-thiosemicarbazone) acenaphthenequinone (3d)

A suspension of zinc bis(4-allyl-3-thiosemicarbazide) acenaphthenequinone (0.050 g, 0.106 mmol) in MeOH (50 mL) was added to GaCl₃ (0.284 g, 1.635 mmol). The resulting suspension was heated at reflux for 6 h. After allowing cooling a red solid was then isolated by filtration, washed with Et₂O and dried under vacuum (0.029 g, 0.057 mmol, 54%). (E,Z) isomer (62% relative abundance) ¹H NMR (300 MHz, d₆-DMSO, 25 °C): δ 9.78 (m, 1H, NH), 9.08 (m, 1H, NH'), 8.52 (d, 1H, H-1, J = 7.2 Hz), 8.12 (overlapping d, 1H, H-1), 8.05 (d, 1H, H-3, J = 8.3 Hz), 7.94 (d, 1H, H-1, J = 7.0 Hz),
Experimental

7.79 (overlapping t, 1H, H-2 or H-2), 7.77 (overlapping t, 1H, H-2 or H-2), 5.87–6.09 (m, 2H, CH2CH2), 5.30 (two overlapping dd, 2H, \( H_{\text{trans}} \)), 5.17 (two overlapping dd, 2H \( H_{\text{cis}} \)), 4.23 (m, 2H, CH2CH2), 4.17 (m, 2H, CH2CH2). \( (E,E) \) isomer (38% relative abundance) \( ^1H \) NMR (300 MHz, \( d_6-\text{DMSO} \), 25 °C): \( \delta \) 9.02 NH, 8.21 (d, \( H-3, J = 7.2 \text{ Hz} \)), 8.15 \( H-1 \), 7.84 \( H-2 \), 5.87-6.09 (m, CH2CH2), 5.28 (dd, \( H_{\text{trans}} \)), 5.21 (dd, \( H_{\text{cis}} \)), 4.23 (m, 2H, CH2CH2). \( ^{13}C \) NMR (\( d_6-\text{DMSO} \), 25 °C): \( \delta \) 178.5, 170.3, 147.6, 142.6, 138.8, 135.9, 134.2, 133.7, 133.3, 132.2, 129.7, 129.6, 129.5, 128.9, 128.7, 128.5, 127.1, 127.0, 125.9, 124.4, 118.5, 117.3, 116.6, 116.5, 48.9, 45.7, 45.0. Mass spectrum ESI-MS calcd for \( C_{20}H_{17}ClGaNS_2 \) [M - H]+ 508.9900, found 508.9911. Elem. Anal.: Found C; 45.7%, H; 3.45 %, N; 15.6 %. Calc. (for 3·MeOH): C; 46.39 %, H; 4.08 %, N; 15.46 %.

**Indium(III) chloride bis(4-methyl-3-thiosemicarbazone) acenaphthenequinone (4a)**

A suspension of zinc bis(4-methyl-3-thiosemicarbazide) acenaphthenequinone (0.155 g, 0.370 mmol) in MeOH (50 mL) was added to InCl3 (0.365 g, 1.652 mmol). The resulting suspension was heated at reflux for 6 h. The red solid that precipitated on cooling to room temperature was then isolated by filtration, washed with Et2O and dried under vacuum (0.116 g, 0.230 mmol, 62%).

\( ^1H \) NMR (\( d_6-\text{DMSO} \), 25 °C): \( \delta \) 8.42 (d, 2H, \( H-1, J = 6.6 \text{ Hz} \)), 8.09 (d, 2H, \( H-3, J = 8.1 \text{ Hz} \)), 7.98 (m, 2H, \( NH \)), 7.75 (apparent t, 2H, \( H-2, J = 7.4 \text{ Hz and } 7.9 \text{ Hz} \)), 3.11 (d, 6H, \( CH_3 \), \( J = 4.7 \text{ Hz} \)). \( ^{13}C \) NMR (\( d_6-\text{DMSO} \), 25 °C): \( \delta \) 176.2, 139.9, 137.2, 130.3, 128.7, 128.6, 127.3, 124.2, 29.2. Mass spectrum ESI-MS calcd for \( C_{16}H_{13}ClInN_6S_2 \) [M - H]+ 502.9370, found 502.9375. Elem. Anal.: Calc.: C; 38.07 %, H; 2.80 %, N; 16.65 %, Found C; 37.65 %, H; 2.77 %, N; 16.2 %.
**Experimental**

**Indium(III) chloride bis(4-ethyl-3-thiosemicarbazone) acenaphthenequinone (4b)**

A suspension of zinc bis(4-ethyl-3-thiosemicarbazide) acenaphthenequinone (0.155 g, 0.370 mmol) in MeOH (50 mL) was added to InCl$_3$ (0.365 g, 1.652 mmol). The resulting suspension was heated at reflux for 6 h. The red solid that precipitated on cooling to room temperature was then isolated by filtration, washed with Et$_2$O and dried under vacuum (0.116 g, 0.230 mmol, 62%).

$^1$H NMR (d$_8$-THF, 25 °C): $\delta$ 8.29 (d, 2H, $H-1$, $J = 6.9$ Hz), 7.92 (d, 2H, $H-3$, $J = 8.3$ Hz), 7.68 (m, 4H, NH + H-2), 3.72 (m, 4H, CH$_2$), 1.34 (t, 6H, CH$_3$, $J = 7.3$ Hz).

$^{13}$C NMR (d$_8$-THF, 25 °C): $\delta$ 175.3, 142.4, 139.7, 131.8, 129.3, 129.2, 128.9, 125.5, 39.1, 14.7. **Mass spectrum** ESI-MS calcd for C$_{18}$H$_{17}$ClInN$_6$S$_2$ $[M-H]^{-}$ 530.9683, found 530.9679. **Elem. Anal.**: Found: C; 38.80 %, H; 3.42 %, N; 14.60 %. Calc. (for 1·MeOH): C; 40.40 %, H; 3.93 %, N; 14.88 %.

**Indium(III) chloride bis(4-phenyl-3-thiosemicarbazone) acenaphthenequinone (4c)**

A suspension of zinc bis(4-phenyl-3-thiosemicarbazide) acenaphthenequinone (0.100 g, 0.1838 mmol) in MeOH (50 mL) was added to InCl$_3$ (0.365 g, 1.652 mmol). The resulting suspension was heated at reflux for 6 h. 4c was isolated from the filtrate, which was dried under vacuum and subsequently washed with water and diethyl ether (49.9 mg, 0.079 mmol, 43%).

$^1$H NMR (d$_8$-THF, 25 °C): $\delta$ 9.60 (s,2H,NHPh), 8.40 (d, 2H, $H-3$, $J = 7.0$ Hz), 8.04 (d, 2H, $H-1$, $J = 8.2$ Hz), 7.85 (d, 4H, $\alpha$-H, $J = 7.6$ Hz), 7.73 (apparent t, 2H, $H-2$,), 7.42 (t, 4H, $m$-H, $J = 7.9$ Hz), 7.13 (t, 2H, $p$-H, $J = 7.4$ Hz). $^{13}$C NMR (d$_8$-THF, 25 °C): $\delta$ 174.2, 144.5, 141.0, 136.9, 132.0, 129.8, 129.6, 129.5, 128.7, 125.4, 124.7, 123.0. **Mass
**Experimental**

**spectra** ESI-MS calcd for C_{26}H_{17}ClInN_{6}S_{2} [M-H]^- 626.9683, found 626.9716. **Elem. Anal.**: Found C; 45.90 %, H; 3.27 %, N; 12.10 %. Calc. (for 2·H_{2}O): C; 46.97 %, H; 3.34 %, N; 12.64 %

**Indium(III) chloride bis(4-allyl-3-thiosemicarbazone) acenaphthenequinone (4d)**

A suspension of zinc bis(4-allyl-3-thiosemicarbazide) acenaphthenequinone (0.100 g, 0.212 mmol) in MeOH (50 mL) was added to InCl_{3} (0.209 g, 0.946 mmol). The resulting suspension was heated at reflux for 6 h. The red solid that precipitated on cooling to room temperature was then isolated by filtration, washed with Et_{2}O and dried under vacuum (0.031 g, 0.056 mmol, 27%). **^1H NMR** (300 MHz, d_{6}-DMSO, 25 °C): δ 8.35 (br s, 2H, H-1), 8.18 (s, 2H, NH), 8.04 (d, 2H, H-3, J = 8.2 Hz), 7.79 (t, 2H, H-2, J = 7.6 Hz), 6.01 (m, 2H, CH_{2}CHCH_{2}), 5.29 (dd, 2H, H_{trans}, J = 17.1 Hz), 5.14 (dd, 2H, H_{cis}, J = 10.3 Hz), 4.19 (s, 4H, CH_{2}CHCH_{2}). **^13C NMR** (75.5 MHz, d_{6}-DMSO, 25 °C): δ 176.0, 140.1, 137.2, 135.3, 130.3, 128.7, 127.4, 124.0, 115.9, 44.7. **Mass spectrum** ESI-MS calcd for C_{20}H_{17}ClInN_{6}S_{2} [M+H]^+ 554.9683, found 554.9683 **Elem. Anal.**: Found C; 42.40 %, H; 3.24 %, N; 14.8 %. Calc. (for 1·MeOH): C; 42.83 %, H; 3.77 %, N; 14.27 %

**Bis-acenaphthenequinone bridged thiosemicarbazonato proligand (L_{B})**

Excess of thiocarbohydrazide (1.43 g, 13.47 mmol) with an equivalent of acenaphthenequinone (0.5 g, 2.74 mmols) in 30 mL of ethanol (Figure 7.2). Upon reflux four drops of glacial acetic acid were added and after 3 hours the suspension was filtered whilst hot yielding a yellow solid (247.9 mg, 0.86 mmols). Crystals precipitated (372.9 mg, 1.29 mmols) from the filtrate and possessed identical spectral properties as the solid.
Experimental

$^1$H NMR (300 MHz, $d_6$-DMSO, 25 °C): $\delta$ 7.88 (overlapping d, 2H, $H-3$, $J = 8.1$ Hz), 7.73 (overlapping d, 2H, $H-1$, $J = 6.9$ Hz), 7.63 (overlapping t, 2H, $H-2$), 6.98 (s, 2H, OH), 4.61 (s, 4H, NH$_2$). $^{13}$C NMR (75.5 MHz, $d_6$-DMSO, 25 °C): $\delta$ 180.2, 139.2, 134.7, 130.4, 128.1, 125.2, 121.1, 95.5. **Mass spectrum** ESI-MS calcd for $C_{13}H_{13}N_4O_2S^+$ 289.0754, found 289.0741.

**Zinc(II) 4-methyl-3-thiosemicarbazono-thiocarbohydrazide acenaphthenequinone (1aN)**

Zinc complex 1aN was prepared by suspending 1 equivalent of compound 1a (0.1044g, 0.38 mmol) and 3 equivalents of zinc acetate (0.2486, 1.13 mmol) in 10 mL of acetic acid at 60 °C. 3 equivalents of thiocarbohydrazide (0.1196g, 1.12 mmol) was added, the temperature increased to 120 °C and refluxed for 30h. Isolation was achieved by hot filtration, followed by washing with 5 mL of THF and 100 mL diethyl ether, yielding yellow powder of 0.1215 g, 0.28 mmol, 74%.

$^1$H NMR (300 MHz, $d_6$-DMSO, 25 °C): All peaks: $\delta$ 9.96 (very broad, 1.3H, NH, (E,E)), 8.95 (broad, 0.3H, NH, (E,Z)), 8.77 (broad, 0.3H, NH, (E,Z)), 8.15-8.75 (m, 2H, Ar-$H-1$), 7.80-8.05 (m, 2H, Ar-$H-1$), 6.71 (broad, 0.46H, NH$_2$, (E,Z)), 6.60 (broad, 1.04H, NH$_2$, (E,E)), 3.01-3.14 (m, 3H, CH$_3$). $^{13}$C NMR (75.5 MHz, $d_6$-DMSO, 25 °C): $\delta$ 178.08, 175.59, 145.85, 135.09, 130.59, 129.51, 128.42, 127.81, 127.36, 127.11, 126.39, 120.17, 30.32, 22.64. **Mass spectrum** ESI-MS calcd for $C_{15}H_{12}N_7S_2Zn^+$ [M+H]$^+$ 420.0043, found 420.0066; $C_{15}H_{12}N_7S_2Zn^-$ [M-H]$^- 417.9887$, found 417.9911. **HPLC** (Method B): $R_t = 9.8$ mins.
Zinc(II) 4-ethyl-3-thiosemicarbazone-thiocarbohydrazide acenaphthenequinone (1bN)

A suspension of compound ib (250.6 mg, 0.885 mmols) was added to three equivalents of zinc acetate (587.3 mg, 2.68 mmols) was heated to 60°C in 10 mL of acetic acid. Three equivalents of thiocarbohydrazide (285.6 mg, 2.69 mmols) were added and the temperature increased to 120°C. This was refluxed for 28 h and then filtered whilst hot, washed with 5 mL of THF, 100 mL of diethyl ether, and dried under vacuum, yielding a yellow powder of 221.6 mg, 0.510 mmols, 58 %.

$^1$H NMR: (300 MHz, $d_6$-$\text{DMSO}$, 25 °C) All peaks: δ 9.87 (1H, very broad peak, NH $[E,E]$), 8.98 (0.39 H, NH, $E,Z$), 8.75 (0.36 H, NH’, $E,Z$), 8.15-8.63 (m, 2H, Ar-$H_{1/3}$), 7.68 (overlapping t, 2H, $H-2$ and $H-2’$), 6.64 (0.7H, NH$_2’$), 6.54 (1H, NH$_2$), 3.60 (t, 2H, $CH_2$), 1.28 (t, 3H, $CH_3$). $^{13}$C NMR (75.5 MHz, $d_6$-$\text{DMSO}$, 25 °C): δ 177.48, 175.67, 135.01, 130.67, 129.52, 128.35, 127.35, 125.76, 120.12, 37.96, 22.76, 14.18. Mass spectrum ESI-MS calcd for C$_{16}$H$_{16}$N$_7$S$_2$Zn $[M + H]^+$ 434.0200, found 434.0211.

HPLC (Method B): $R_t = 10.5$ mins, (Method E): $R_t = 9.2$ mins.

Zinc(II) 4-phenyl-3-thiosemicarbazone-thiocarbohydrazide acenaphthenequinone (1cN)

A suspension of compound ic (0.1025 g, 0.31 mmol) was added to three equivalents of zinc acetate (0.2048 g, 0.93 mmols) using was heated to 60°C in 10 mL of acetic acid. Three equivalents of (0.0998g, 0.94 mmol) thiocarbohydrazide were added and the temperature increased to 120°C. This was refluxed for 28 h and then filtered whilst hot,
washed with 5 mL of THF, 100 mL of diethyl ether, and dried under vacuum, yielding a dark red powder of 0.0982 g, 0.20 mmol, 65%.

\(^1\text{H-NMR}\) (300 MHz, \(d_6\)-DMSO, 25 °C): 8.67 (broad d, 1H, \(H-3\) or \(H-3'\)), 8.07 (broad, 1H, \(H-3\) or \(H-3'\)), 7.95 1H (d, H1 or H1', \(J = 8.29\) Hz) and 7.89 1H (d, \(H-1\) or \(H-1'\)), 7.71 (d, \(2H, o-H\)), 7.62 (d, 1H, \(H-2\) or \(H-2'\)), 7.51 (t, 1H, \(H-2\) or \(H-2'\)), 7.40 (t, 2H, \(m-H\)), 7.16 (t, 1H, \(p-H\)), 6.55 (s, 2H, NH\(_2\)).

\(^{13}\text{C NMR}\) (75.5 MHz, \(d_6\)-DMSO, 25 °C): 140.15, 135.02, 130.30, 128.54, 128.01, 125.74, 123.65, 122.70, 22.49. Mass spectrum ESI-MS calcd for \(^\text{C}_{20}\text{H}_{16}\text{N}_7\text{S}_2\text{Zn}^+\) [M+H]⁺ 482.0200, found 482.0200.

**Zinc(II) 4-allyl-3-thiosemicarbazone-thiocarbohydrazide acenaphthenequinone (1dN)**

A suspension of compound id (0.1034g, 0.35 mmol) was added to three equivalents of zinc acetate (0.2228g, 1.01 mmol) using was heated to 60°C in 10 mL of acetic acid. Three equivalents of thiocarbohydrazide (0.1091, 1.02 mmol) were added and the temperature increased to 120°C. This was refluxed for 28 h and then filtered whilst hot, washed with 5 mL of THF, 100 mL of diethyl ether, and dried under vacuum, yielding a light brown powder of 0.0787g, 0.17 mmol, 48%.

\((E,E)\) isomer, 67 % \(^1\text{H NMR}\) (300 MHz, \(d_6\)-DMSO, 25 °C): 8.92 (s, 1H, NH), 8.99 (s, 1H, NH'), 8.60 (d, 1H, \(H-1\)), 8.38 (broad, 1H, \(H-1'\)), 7.98 (d+d, 2H, \(H-3\), \(J = 7.9\) Hz), 7.68 (d+d, 2H, \(H-2\)), 6.59 (d, 2H, NH\(_2\)), 6.01 (m, 1H, CH\(_2\)CHCH\(_2\)), 5.28 (dd, 1H, \(H_{trans}\)), 5.14 (dd, 1H, \(H_{cis}\)), 4.18 (bm, 2H, CH\(_2\)CHCH\(_2\)). \((E,Z)\) isomer contained many overlapping peaks and was not assignable. \(^{13}\text{C NMR}\) (75.5 MHz, \(d_6\)-DMSO, 25 °C): (Weak data due to poor solubility) \(\delta\) 129.54, 115.75, 128.27, 127.26, 125.92, 115.75, 45.38, 22.61. Mass spectrum ESI-MS calcd for \(^\text{C}_{17}\text{H}_{16}\text{N}_7\text{S}_2\text{Zn}^+\) [M+H]⁺ 446.0200, found 446.0192. HPLC (Method B): \(R_s = 10.9\) mins, (Method E): \(R_s = 9.3\) mins.
Gallium(III) 4-ethyl-3-thiosemicarbazone-thiocarbohydrazide acenaphthenequinone (3bN)

50 mg, 0.115 mmols of zinc 1bN was solubilised in 2.5 mL of DMSO, to which an excess of gallium chloride was added and 30 mL of MeOH. This was refluxed under an atmosphere of nitrogen for 6 hours and was filtered after cooling to room temperature. The solution was removed \textit{in vacuo} and the resultant material washed with 10 mL of ethyl acetate, followed by two times 10 mL of diethyl ether. This yielded an orange solid of 45 mg, 0.095 mmols, 82.6\% \((E,E)\) isomer. $^1$H NMR (300 MHz, $d_6$-DMSO, 25 °C): $\delta$ 10.25 (s, 1H, NH), 8.60 (t, 1H, NH, $J = 6.41$ Hz), 8.15 (d, 1H, H-3, $J = 8.3$ Hz), 7.98 (m, 1H, H-3', $J = 8.2$ Hz), 7.85 (m, 2H, H-1/1') 7.75 (m, 2H, H-2/2'), 7.61 (s, 2H, NH$_2$), 4.12 (broad, 2H, CH$_2$), 1.30 (t, 3H, CH$_3$, $J = 7.3$ Hz). $^{13}$C NMR (75.5 MHz, $d_6$-DMSO, 25 °C): $\delta$ 129.81, 129.38, 128.27, 126.96, 125.78, 117.81, 37.96, 13.88. Mass spectrum ESI-MS calcd for C$_{20}$H$_{16}$N$_7$S$_2$GaCl $^-$ 471.9696, found 471.9729. 

HPLC (Method D): $R_t = 7.8$ mins.
Experimental

Bombesin analogue

Fmoc-protected rink amide resin (1g, 0.5 mmols) was swelled in a peptide vessel for 20 min with DCM. This was subsequently deprotected using a solution of 20% piperidine in DMF, with deprotection confirmed by Kaiser-test. For the first step, three equivalents of the amino-acid (Fmoc-Leu-OH, 530 mg, 1.5 mmols) of HOBt (203 mg, 1.5 mmols) and of base N,N'-Diisopropylcarbodiimide (235 µL, 1.5 mmols) in DMF was added to the resin. This was to react for 90 minutes, followed by deprotection using 20% piperidine in DMF as confirmed by the Kaiser-test. Subsequent steps used three equivalents of amino acids, 1.9 equivalents of HOBt (360 mg) and 6 equivalents of N,N'-Diisopropylcarbodiimide and were followed by deprotection as described above. The amino acids were Fmoc-His(Trt)-OH (619.7 mg), Fmoc-Gly-OH (446 mg), Fmoc-Val-OH (509.1 mg), Fmoc-Ala-OH (467 mg), Fmoc-Trp(Boc)-OH (790 mg), Fmoc-Gln(Trt)-OH (916.05 mg), respectively. Finally the peptide was t-boc deprotected by stirring for three hours with a cocktail of 95% TFA, 2.5% TIS and 2.5% H2O, with resin beads filtered and the solution collected. This was freeze-dried in vacuo and purified using semi-preparative HPLC yielding 60 mg of peptide.

Mass spectrum ESI-MS calcd for C_{38}H_{57}N_{12}O_{8}^+ 807.4417, found 807.4403. HPLC (Method E): R_t = 5.4 mins.
**Derivatised bombesin analogue**

An equivalent of pyruvic acid (0.4 mg, 4.55 µmols) was added to coupling agents EDC (1-ethyl-3-(3’-dimethylaminopropyl) carbodiimide) (0.9 mg, 5.80 µmols) and HOBT (hydroxybenzotriazole) (0.86 mg, 6.37 µmols) on ice in DMF, which was achieved using 10x stock solutions in DMF. After 45 minutes 5 mg, (1 equivalent, 4.55 µmols) of bombesin was added followed by dropwise addition of DIPEA (N,N’-diisopropylethylamine) until pH 8 was reached. This was allowed to react for 2 hours and was subsequently extracted from DMF and purified using a solid phase extraction cartridge. **Mass spectrum** ESI-MS calcd for C_{41}H_{57}N_{12}O_{10}^– 877.4321, found 877.4377. **HPLC** (Method E): R_t = 4.8 mins.

**Ethyl-bombesin derivatised zinc complex, 1bB**

Following this 5 mg of derivatised bombesin analogue (4.55 µmols) was added to 2.0 mg (4.55 µmols) of zinc complex, compound 1bN in 400 µL of DMSO, for the purpose of synthesising compound 1bB. To this mixture 10 mL of HPLC grade methanol was added followed by 1 drop of concentrated HCl (32%), resulting in an instant colour change from dark orange to yellow. After stirring at 30°C for ca. 16 hours, the solution became pale orange and was freeze-dried in vacuo.
Allyl-bombesin derivatised zinc complex, 1dB

Following this 5 mg of derivatised bombesin analogue (4.55 µmols) was added to 2.0 mg (4.55 µmols) of zinc complex, compound 1dN in 400 µL of DMSO, for the purpose of synthesising compound 1dB. To this mixture 10 mL of HPLC grade methanol was added followed by 1 drop of concentrated HCl (32%), resulting in an instant colour change from dark orange to yellow. After stirring at 30°C for ca. 16 hours, the solution became pale orange and was freeze-dried in vacuo.
10. References


10. References


Appendix A.  

X-ray crystallography data

<table>
<thead>
<tr>
<th></th>
<th>Sulfur-bridged dimer, iib₈₋₈</th>
<th>iid</th>
<th>L₈</th>
<th>1c</th>
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<td>Empirical formula</td>
<td>C₃₆H₃₈N₁₂S₄</td>
<td>C₂₀H₂₀N₅S₂</td>
<td>C₁₅H₁₂N₄O₂S</td>
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<td>17266 / 8480 /</td>
<td>22724 / 7566 /</td>
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<td>R₁⁺, wR₂ [I &gt; 2σ(I)]⁶</td>
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<td>R₁⁺, wR₂ (all data)⁶</td>
<td>0.0993, 0.2568</td>
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**iib₈₋₈** Potential Hydrogen atoms on N₅ and N₈. However, attaching these to the parent atoms changed the R value from 9.93 to a higher 9.97 %. Initially all N-H hydrogen atoms were refined freely but a couple of them had too low temperature factors. For N₅H₅ and N₆H₆ however the temperature factor shot up to unrealistic numbers.

**L₈**. The asymmetric unit contains two independent molecules of the proligand. All N-H and O-H hydrogen atoms have been located in the difference Fourier map and refined freely.
1c. The asymmetric unit contains two independent molecules of the DMSO and one DMSO adduct
Table A.2. X-ray crystallography data of 2c, 3a, 3b and 3c.

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<th>Empirical formula</th>
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2c. The asymmetric unit contains two independent molecules of THF

3a. The asymmetric unit contains two isomers of the gallium complex, 3a and one independent molecule of DMSO.

3c. The asymmetric unit contains two independent molecules of the gallium complex, 3c and four independent molecules of THF.
Table A.3. X-ray crystallography data of 4a, 4b, 4c and 4d.

<table>
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<th>Empirical formula</th>
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<td>2</td>
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<td>3.08 to 25.09</td>
<td>3.55 to 27.48</td>
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<td>0.1119</td>
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<td>0.0408, 0.0870</td>
<td>0.0497, 0.1132</td>
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<td>0.0755, 0.1271</td>
<td>0.0551, 0.0846</td>
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**4a.** The asymmetric unit contains two independent molecules of the indium complex, 4a. This was a very small, weak diffracting crystal. Hence the high \(R\)-(int) and high \(R\) factor.

4 non H atoms had to be refined isotropically.

**4b.** Hydrogen atoms attached to N have been located in the difference Fourier map and freely refine with idealised bond lengths.

**4c.** The asymmetric unit contains one molecule of THF.

**4d.** The asymmetrical unit consists of one In compound, 4d, one half molecule of THF which lies on a twofold axis and half a molecule of 1,4-Dioxane. The half molecule of 1,4-Dioxane is disordered over two sites with 25% occupancy. The O of the Dioxane are refined anisotropically and the C atoms of this solvent are refine
isotropically. There is hydrogen bonding between N-H and the THF and Cl...H-N of the neighbouring molecule.
Appendix CIF Contents

All CIFs are stored on the accompanying DVD

1. Crystallographic Information File for iib, sulphur-bridged ethyl-substituted bis(thiosemicarbazone) proligand dimer.
2. Crystallographic Information File for iid, allyl-substituted bis(thiosemicarbazone) proligand
3. Crystallographic Information File for L, bridgethiosemicarbazone proligand
4. Crystallographic Information File for 1e, zinc phenyl-substituted bis(thiosemicarbazone) complex
5. Crystallographic Information File for 2c, copper phenyl-substituted bis(thiosemicarbazone) complex
6. Crystallographic Information File for 3a, gallium methyl-substituted bis(thiosemicarbazone) complex
7. Crystallographic Information File for 3b, gallium ethyl-substituted bis(thiosemicarbazone) complex
8. Crystallographic Information File for 3c, gallium phenyl-substituted bis(thiosemicarbazone) complex
9. Crystallographic Information File for 4a, indium methyl-substituted bis(thiosemicarbazone) complex
10. Crystallographic Information File for 4b, indium ethyl-substituted bis(thiosemicarbazone) complex
11. Crystallographic Information File for 4c, indium phenyl-substituted bis(thiosemicarbazone) complex
12. Crystallographic Information File for 4d, indium allyl-substituted bis(thiosemicarbazone) complex
Appendix B. HPLC traces

HPLC traces for compounds without elemental analysis and/or X-ray crystal structures, and where solubility in DMSO was very poor, HPLC data is shown to represent complex purity.

**Figure B.1.** HPLC traces of: bombesin analogue (left), of derivatised bombesin analogue (right), method E, $\lambda_{obs} = 280$ nm.

**Figure B.2.** HPLC traces of: complex 1aN (left), of complex 1bN bombesin analogue (right), method B, $\lambda_{obs} = 400$ nm.

**Figure B.3.** HPLC traces of: complex 1dN (left), method B, $\lambda_{obs} = 400$ nm, complex 3bN (right), method D, $\lambda_{obs} = 400$ nm.
Figure B.4. HPLC of compound 1dB reaction mixture, $\lambda_{\text{obs}} = 400$ nm, reference peaks to note under the same conditions – compound 3 $R_t = 9.3$ minutes and derivatised BBN $R_t = 4.8$ minutes (HPLC method E).

Figure B.5. HPLC of purified compound from 1dB reaction mixture, $\lambda_{\text{obs}} = 400$ nm (HPLC method E).
Appendix B.

![Graph](image1)

**Figure B.6.** Overlay of UV-HPLC trace (black) of aromatic zinc bis(thiosemicarbazone) precursor a) 1a (R = methyl) and b) 1c (R = phenyl) with the radio-HPLC trace (blue) of aromatic $^{68}$GaCl bis(thiosemicarbazone) complex.

![Graph](image2)

**Figure B.7.** Overlay of UV-HPLC trace (black) of aromatic zinc bis(thiosemicarbazone) precursor a) 1d (R = allyl) and aromatic free ligand bis(thiosemicarbazone) precursor b) iiia (R = methyl) with the radio-HPLC trace (blue) of aromatic $^{68}$GaCl bis(thiosemicarbazone) complex.

![Graph](image3)

**Figure B.8.** Overlay of UV-HPLC trace (black) of aromatic free ligand bis(thiosemicarbazone) precursor iid (R = methyl) with the radio-HPLC trace (blue) of aromatic $^{68}$GaCl bis(thiosemicarbazone) complex.
Figure B.9. Overlay of UV-HPLC trace (black) of aromatic zinc bis(thiosemicarbazone) precursor with the radio-HPLC trace (blue) of aromatic $^{111}$InCl bis(thiosemicarbazone) complex with a) 1a and b) 1d.
Appendix C.  Fluorescence spectroscopy

Figure C.1. 2D representation of the fluorescence of compound iia.

Figure C.2. 100 μM in DMSO where left represents a 2D plot of complexes a) 3a and b) 3d

Figure C.3. 100 μM in DMSO where left represents a 2D plot of complexes a) 4c and b) 4d

^2 Spectrum 3d was acquired with Brett Kennedy
Appendix D. Stability Assays using UV-visible and Fluorescence Spectroscopy

Figure D.1. PBS challenges (50% DMSO, 50% PBS) for compound 1d at 100 µM, by fluorescence spectroscopy (left) UV/Vis (right) monitored at time intervals up to 24h at room temperature.

Figure D.2. EMEM SFM challenges (50% DMSO, 50% SFM) for compound 1d at 100 µM, by fluorescence spectroscopy (left) UV/Vis (right) monitored at time intervals up to 24h at room temperature.

Figure D.3. FCS assay (50% DMSO, 45% EMEM, 5% FCS) for compound 1d at 100 µM, by fluorescence spectroscopy (left) UV/Vis (right) monitored at time intervals up to 24h at room temperature.
Figure D.4. Citric acid challenges (50% DMSO, 50% water, 2 eq of citric acid) for compound 1d at 100 µM, by fluorescence spectroscopy (left) UV/Vis (right) monitored at time intervals up to 24h at room temperature.

Figure D.5. L-methionine challenges (50% DMSO, 50% water, 2 eq of L-methionine) for compound 1d at 100 µM, by fluorescence spectroscopy (left) UV/Vis (right) monitored at time intervals up to 24h at room temperature.

Figure D.6. L-methionine challenges (50% DMSO, 50% PBS, 2 eq of L-methionine) for compound 1d at 100 µM, by fluorescence spectroscopy (left) UV/Vis (right) monitored at time intervals up to 24h at room temperature.
Figure D.7. L-cysteine challenges (50% DMSO, 50% water, 2 eq of L-cysteine) for compound 1d at 100 µM, by fluorescence spectroscopy (left) UV/Vis (right) monitored at time intervals up to 24h at room temperature.

Figure D.8. L-histidine challenges (50% DMSO, 50% water, 2 eq of L-histidine) for compound 1d at 100 µM, by fluorescence spectroscopy (left) UV/Vis (right) monitored at time intervals up to 24h at room temperature.

Figure D.9. Glutathione challenges (50% DMSO, 50% water, 2 eq of Glutathione) for compound 1d at 100 µM, by fluorescence spectroscopy (left) UV/Vis (right) monitored at time intervals up to 24h at room temperature.
Figure D.10. All Buffer challenges (50% DMSO, 50% water) for compound 1d at 100 µM by fluorescence spectroscopy (left) and UV/Vis (right) monitored at 24 hours at room temperature.

Figure D.11. Citric acid challenges (50% DMSO, 50% water, 2 eq of citric acid) for compound 3d at 100 µM, by fluorescence spectroscopy (left) UV/Vis (right) monitored at time intervals up to 24h at room temperature.

Figure D.12. L-methionine challenges (50% DMSO, 50% water, 2 eq of L-methionine) for compound 3d at 100 µM, by fluorescence spectroscopy (left) UV/Vis (right) monitored at time intervals up to 24h at room temperature.
Figure D.13. L-methionine challenges (50% DMSO, 50% PBS, 2 eq of L-methionine) for compound 3d at 100 µM, by fluorescence spectroscopy (left) UV/Vis (right) monitored at time intervals up to 24h at room temperature.

Figure D.14. L-cysteine challenges (50% DMSO, 50% water, 2 eq of L-cysteine) for compound 3d at 100 µM, by fluorescence spectroscopy (left) UV/Vis (right) monitored at time intervals up to 24h at room temperature.

Figure D.15. L-histidine challenges (50% DMSO, 50% water, 2 eq of L-histidine) for compound 3d at 100 µM, by fluorescence spectroscopy (left) UV/Vis (right) monitored at time intervals up to 24h at room temperature.
Figure D.16. Fluorescence spectroscopy buffer assay (a) 3a pH study Arbitrary Units pH 2 >> pH 5 > pH 1.1 > pH 10 ≥ pH 9 > pH 7 ≥ pH 9.4 > pH 3. 0-40 and fluorescence intensity of complex with buffer. 3d pH study where pH 2 > pH 1.1 > pH 3 ≥ pH 9 > pH 10 > pH 5 ≥ pH 7 ≥ pH 8. *3d pH study was carried out with Brett Kennedy.

Figure D.17. (a) Citric acid challenges (50% DMSO, 50% water, 2 eq of citric acid) (b) EDTA challenges (50% DMSO, 50% water, 2 eq of EDTA) for compound 3d at 10 µM by UV/Vis monitored at time intervals up to 24h at room temperature.

Figure D.18. (a) Glutathione challenges (50% DMSO, 50% water, 2 eq of Glutathione) (b) L-cysteine challenges (50% DMSO, 50% water, 2 eq of L-cysteine) for compound 3d at 10 µM by UV/Vis monitored at time intervals up to 24h at room temperature.
Figure D.19. L-Histidine challenges (50% DMSO, 50% water, 2 eq of L-Histidine) for compound 3d at 10 µM by UV/Vis monitored at time intervals up to 24h at room temperature.

Figure D.20. Buffer challenges (50% DMSO, 50% water, pH 1.1 buffer, blank measured pH 1.6) for compound 3d at 100 µM by fluorescence spectroscopy (left) and UV/Vis (right) monitored at time intervals up to 24h at room temperature.

Figure D.21. Buffer challenges (50% DMSO, 50% water, pH 2.0 buffer, blank measured pH 2.5) for compound 3d at 100 µM by fluorescence spectroscopy (left) and UV/Vis (right) monitored at time intervals up to 24h at room temperature.
Figure D.22. Buffer challenges (50% DMSO, 50% water, pH 3.0 buffer, blank measured pH 4.4) for compound 3d at 100 µM by fluorescence spectroscopy (left) and UV/Vis (right) monitored at time intervals up to 24h at room temperature.

Figure D.23. Buffer challenges (50% DMSO, 50% water, pH 5.0 buffer, blank measured pH 6.9) for compound 3d at 100 µM by fluorescence spectroscopy (left) and UV/Vis (right) monitored at time intervals up to 24h at room temperature.

Figure D.24. Buffer challenges (50% DMSO, 50% water, pH 7.0 buffer, blank measured pH 9.4) for compound 3d at 100 µM by fluorescence spectroscopy (left) and UV/Vis (right) monitored at time intervals up to 24h at room temperature.
Figure D.25. Buffer challenges (50% DMSO, 50% water, pH 9.0 buffer, blank measured pH 9.35) for compound 3d at 100 µM by fluorescence spectroscopy (left) and UV/Vis (right) monitored at time intervals up to 24h at room temperature.

Figure D.26. Buffer challenges (50% DMSO, 50% water, pH 10.0 buffer, blank measured pH 12.7) for compound 3d at 100 µM by fluorescence spectroscopy (left) and UV/Vis (right) monitored at time intervals up to 24h at room temperature.

Figure D.27. All buffer challenges (50% DMSO, 50% water) for compound 3d at 100 µM by fluorescence spectroscopy (left) and UV/Vis (right) monitored at 15 minutes at room temperature.
Figure D.28. DMSO challenges for compound 3d at 100 µM, by fluorescence spectroscopy (left) UV/Vis (right) monitored at time intervals up to 24h at room temperature.

Figure D.29. Water challenges (50% DMSO, 50% water) for compound 3d at 100 µM, by fluorescence spectroscopy (left) UV/Vis (right) monitored at time intervals up to 24h at room temperature.

Figure D.30. 3b FCS assay
Figure D.31. DMSO challenges for compound 4d at 100 µM, by fluorescence spectroscopy (left) UV/Vis (right) monitored at time intervals up to 24h at room temperature.

Figure D.32. Citric acid challenges (50% DMSO, 50% water, 2 eq of citric acid) for compound 4d at 100 µM, by fluorescence spectroscopy (left) UV/Vis (right) monitored at time intervals up to 24h at room temperature.

Figure D.33. Citric acid challenges (50% DMSO, 50% PBS, 2 eq of citric acid) for compound 4d at 100 µM, by fluorescence spectroscopy (left) UV/Vis (right) monitored at time intervals up to 24h at room temperature.
Appendix D

Figure D.34. L-methionine challenges (50% DMSO, 50% water, 2 eq of L-methionine) for compound 4d at 100 µM, by fluorescence spectroscopy (left) UV/Vis (right) monitored at time intervals up to 24h at room temperature.

Figure D.35. (a) Citric acid challenges (50% DMSO, 50% water, 2 eq of citric acid) (b) EDTA challenges (50% DMSO, 50% water, 2 eq of EDTA) for compound 4d at 10 µM by UV/Vis monitored at time intervals up to 24h at room temperature.

Figure D.36. (a) Glutathione challenges (50% DMSO, 50% water, 2 eq of Glutathione) (b) L-cysteine challenges (50% DMSO, 50% water, 2 eq of L-cysteine) for compound 3d at 10 µM by UV/Vis monitored at time intervals up to 24h at room temperature.
Figure D.37. L-Histidine challenges (50% DMSO, 50% water, 2 eq of L-Histidine) for compound 4d at 10 µM by UV/Vis monitored at time intervals up to 24h at room temperature.

Figure D.38. Fluorescence spectroscopy buffer assay a) 4a pH study where and fluorescence intensity of complex with buffer. pH 3 > pH 2 > pH 9 > pH 7 > pH 5 ≥ pH 9.4 ≥ pH 10 ≈ pH 1.1, b) 4b pH study where and fluorescence intensity of complex with buffer. pH 3 > pH 2 > pH 7 > pH 9 > pH 5 > pH 1.1 ≥ pH 9.4 ≥ pH 10.

Figure D.39. Fluorescence spectroscopy buffer assay a) 4a pH study where and fluorescence intensity of complex with buffer. pH 3 > pH 2 > pH 9 > pH 7 > pH 5 ≥ pH 9.4 ≈ pH 10 ≈ pH 1.1, b) 4b pH study
where and fluorescence intensity of complex with buffer. pH 3 > pH 2 > pH 7 > pH 9 > pH 5 > pH 1.1 ≥ pH 9.4 ≥ pH 10.

Figure D.40. Buffer challenges (50% DMSO, 50% water, pH 1.1 buffer, blank measured pH 1.6) for compound 4d at 100 µM by fluorescence spectroscopy (left) and UV/Vis (right) monitored at time intervals up to 24h at room temperature.

Figure D.41. Buffer challenges (50% DMSO, 50% water, pH 2.0 buffer, blank measured pH 2.5) for compound 4d at 100 µM by fluorescence spectroscopy (left) and UV/Vis (right) monitored at time intervals up to 24h at room temperature.

Figure D.42. Buffer challenges (50% DMSO, 50% water, pH 3.0 buffer, blank measured pH 4.4) for compound 4d at 100 µM by fluorescence spectroscopy (left) and UV/Vis (right) monitored at time intervals up to 24h at room temperature.
**Figure D.43.** Buffer challenges (50% DMSO, 50% water, pH 5.0 buffer, blank measured pH 6.9) for compound 4d at 100 µM by fluorescence spectroscopy (left) and UV/Vis (right) monitored at time intervals up to 24h at room temperature.

**Figure D.44.** Buffer challenges (50% DMSO, 50% water, pH 7.0 buffer, blank measured pH 9.4) for compound 4d at 100 µM by fluorescence spectroscopy (left) and UV/Vis (right) monitored at time intervals up to 24h at room temperature.

**Figure D.45.** Buffer challenges (50% DMSO, 50% water, pH 9.0 buffer, blank measured pH 9.35) for compound 4d at 100 µM by fluorescence spectroscopy (left) and UV/Vis (right) monitored at time intervals up to 24h at room temperature.
Figure D.46. Buffer challenges (50% DMSO, 50% water, pH 10.0 buffer, blank measured pH 12.7) for compound 4d at 100 µM by fluorescence spectroscopy (left) and UV/Vis (right) monitored at time intervals up to 24h at room temperature.

Figure D.47. Water challenges (50% DMSO, 50% water) for compound 4d at 100 µM, by fluorescence spectroscopy (left) UV/Vis (right) monitored at time intervals up to 24h at room temperature.

Figure D.48. 4b FCS assay

Figure D.49. 4d FCS assay
Figure D.50. All Buffer challenges (50% DMSO, 50% water) for compound iid at 100 µM by fluorescence spectroscopy (left) and UV/Vis (right) monitored at 15 minutes at room temperature.

Figure D.51. All Buffer challenges (50% DMSO, 50% water) for compound iid at 100 µM by fluorescence spectroscopy (left) and UV/Vis (right) monitored at 24 hours at room temperature.
Appendix E. Two-photon Time Correlated Single Photon Counting data of DMSO solutions at 10 mM

Table E.1. Two-photon Time Correlated Single Photon Counting data of solutions in DMSO of free ligands, nd = not done.

<table>
<thead>
<tr>
<th></th>
<th>ia</th>
<th>ib</th>
<th>ic</th>
<th>id</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM</td>
<td>$\chi^2 = 1.15$</td>
<td>$\chi^2 = 1.30$</td>
<td>nd</td>
<td>$\chi^2 = 1.13$</td>
</tr>
<tr>
<td>$\lambda_{ex} = 810$ nm</td>
<td>0.568 ns 67.6%</td>
<td>0.468 ns 68.4%</td>
<td>4.1288 ns 64.6%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.6706 ns 32.4%</td>
<td>3.2237 ns 31.6%</td>
<td>0.583 ns 35.4%</td>
<td></td>
</tr>
<tr>
<td>10 mM</td>
<td>$\chi^2 = 1.38$</td>
<td>nd</td>
<td>$\chi^2 = 1.31$</td>
<td>$\chi^2 = 1.34$</td>
</tr>
<tr>
<td>$\lambda_{ex} = 910$ nm</td>
<td>0.388 ns 63.9%</td>
<td>0.413 ns 75.1%</td>
<td>0.339 ns 87.2%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.236 ns 36.1%</td>
<td>5.1734 ns 24.9%</td>
<td>3.848 ns 12.8%</td>
<td></td>
</tr>
</tbody>
</table>

Figure E.1. TCSPC decay curves for mono(substituted) proligands 810 nm (left) 910 nm (right)

Table E.2. Two-photon Time Correlated Single Photon Counting data of solutions in DMSO of free ligands.

<table>
<thead>
<tr>
<th></th>
<th>iia</th>
<th>iib</th>
<th>iic</th>
<th>iid</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM</td>
<td>$\chi^2 = 1.88$</td>
<td>$\chi^2 = 1.63$</td>
<td>$\chi^2 = 1.71$</td>
<td>$\chi^2 = 1.36$</td>
</tr>
<tr>
<td>$\lambda_{ex} = 810$ nm</td>
<td>0.226 ns 95.7%</td>
<td>0.183 ns 98%</td>
<td>0.158 ns 98.7%</td>
<td>0.243 ns 97.8%</td>
</tr>
<tr>
<td></td>
<td>2.576 ns 4.3%</td>
<td>3.969 ns 2%</td>
<td>2.380 ns 1.3%</td>
<td>4.621 ns 2.2%</td>
</tr>
<tr>
<td>10 mM</td>
<td>$\chi^2 = 1.21$</td>
<td>$\chi^2 = 1.14$</td>
<td>$\chi^2 = 1.52$</td>
<td>$\chi^2 = 1.25$</td>
</tr>
<tr>
<td>$\lambda_{ex} = 910$ nm</td>
<td>0.231 ns 89.2 %</td>
<td>0.235 ns 90 %</td>
<td>0.169 ns 99.2%</td>
<td>0.258 ns 94.1%</td>
</tr>
<tr>
<td></td>
<td>2.117 ns 10.8 %</td>
<td>3.102 ns 10 %</td>
<td>4.119 ns 0.8%</td>
<td>6.488 ns 5.9%</td>
</tr>
</tbody>
</table>
Appendix E

Figure E.2. TCSPC decay curves for bis(substituted) proligands 810 nm (left) 910 nm (right)


<table>
<thead>
<tr>
<th></th>
<th>1b λ&lt;sub&gt;ex&lt;/sub&gt; = 810 nm</th>
<th>1b λ&lt;sub&gt;ex&lt;/sub&gt; = 910 nm</th>
<th>1d λ&lt;sub&gt;ex&lt;/sub&gt; = 910 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM</td>
<td>χ&lt;sup&gt;2&lt;/sup&gt; = 1.52</td>
<td>χ&lt;sup&gt;2&lt;/sup&gt; = 1.25</td>
<td>χ&lt;sup&gt;2&lt;/sup&gt; = 1.56</td>
</tr>
<tr>
<td></td>
<td>0.261 ns 98.2%</td>
<td>0.275 ns 100%</td>
<td>0.266 ns 99.7%</td>
</tr>
<tr>
<td></td>
<td>0.597 ns 1.8%</td>
<td></td>
<td>1.201 ns 0.3%</td>
</tr>
</tbody>
</table>

Table E.4. Two-photon Time Correlated Single Photon Counting data of solutions in DMSO of copper bis(thiosemicarbazono)complexes λ<sub>ex</sub> = 910 nm.

<table>
<thead>
<tr>
<th></th>
<th>2a</th>
<th>2c</th>
<th>2d</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM</td>
<td>χ&lt;sup&gt;2&lt;/sup&gt; = 1.70</td>
<td>χ&lt;sup&gt;2&lt;/sup&gt; = 1.13</td>
<td>χ&lt;sup&gt;2&lt;/sup&gt; = 1.24</td>
</tr>
<tr>
<td>λ&lt;sub&gt;ex&lt;/sub&gt; = 910 nm</td>
<td>0.213 ns 90.5%</td>
<td>0.294 ns 91.05%</td>
<td>0.287 ns 91.4%</td>
</tr>
<tr>
<td></td>
<td>1.826 ns 9.5%</td>
<td>1.469 ns 9.76%</td>
<td>1.545 ns 8.6%</td>
</tr>
</tbody>
</table>
**Table E.5.** Two-photon Time Correlated Single Photon Counting data of solutions in DMSO of gallium bis(thiosemicarbazonato) complexes, nd = not done.

<table>
<thead>
<tr>
<th></th>
<th>3a</th>
<th>3b</th>
<th>3c</th>
<th>3d</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM</td>
<td>$\chi^2 = 1.08$</td>
<td>$\chi^2 = 1.15$</td>
<td>$\chi^2 = 1.67$</td>
<td>nd</td>
</tr>
<tr>
<td>$\lambda_{ex} = 810$ nm</td>
<td>0.295 ns 58.3%</td>
<td>0.250 ns 63.7%</td>
<td>0.170 ns 97.4%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.744 ns 41.7%</td>
<td>0.873 ns 36.3%</td>
<td>0.603 ns 2.6%</td>
<td></td>
</tr>
<tr>
<td>10 mM</td>
<td>$\chi^2 = 1.22$</td>
<td>$\chi^2 = 1.25$</td>
<td>$\chi^2 = 1.32$</td>
<td>$\chi^2 = 1.44$</td>
</tr>
<tr>
<td>$\lambda_{ex} = 910$ nm</td>
<td>0.436 ns 57.8%</td>
<td>0.362 ns 52.4%</td>
<td>0.123 ns 85.9%</td>
<td>0.306 ns 69.1%</td>
</tr>
<tr>
<td></td>
<td>0.976 ns 42.2%</td>
<td>0.889 ns 47.6%</td>
<td>4.099 ns 4.1%</td>
<td>0.871 ns 30.9%</td>
</tr>
</tbody>
</table>

**Figure E.3.** TCSPC decay curves for gallium bis(thiosemicarbazonato) complexes 810 nm (left) 910 nm (right)

**Figure E.4.** TCSPC decay curves for indium bis(thiosemicarbazonato) complexes 810 nm (left) 910 nm (right)
Table E.6. Two-photon Time Correlated Single Photon Counting data of 10 mM solutions in DMSO of amine-functionalised bis(thiosemicarbazone) complexes, with high $\chi^2$ indicating precipitation for zinc complexes.

<table>
<thead>
<tr>
<th></th>
<th>1aN</th>
<th>1bN</th>
<th>1cN</th>
<th>1dN</th>
<th>3bN</th>
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</thead>
<tbody>
<tr>
<td>$\lambda_{ex} = 810$ nm</td>
<td>$\chi^2 = 1.83$</td>
<td>$\chi^2 = 1.40$</td>
<td>$\chi^2 = 1.46$</td>
<td>$\chi^2 = 1.84$</td>
<td>$\chi^2 = 1.33$</td>
</tr>
<tr>
<td></td>
<td>0.203 ns, 98.7%</td>
<td>0.208 ns, 99.1%</td>
<td>0.230 ns, 95.1%</td>
<td>0.218 ns, 97.8%</td>
<td>0.640 ns, 50.5%</td>
</tr>
<tr>
<td></td>
<td>1.479 ns, 1.3%</td>
<td>1.502 ns, 0.9%</td>
<td>1.173 ns, 4.9%</td>
<td>1.499 ns, 2.2%</td>
<td>2.373 ns, 49.5%</td>
</tr>
<tr>
<td>$\lambda_{ex} = 910$ nm</td>
<td>$\chi^2 = 1.45$</td>
<td>$\chi^2 = 1.68$</td>
<td>$\chi^2 = 1.42$</td>
<td>$\chi^2 = 1.26$</td>
<td>$\chi^2 = 1.13$</td>
</tr>
<tr>
<td></td>
<td>0.226 ns, 95.9%</td>
<td>0.195 ns, 97.5%</td>
<td>0.210 ns, 95.2%</td>
<td>0.244 ns, 96.3%</td>
<td>0.653 ns, 49.7%</td>
</tr>
<tr>
<td></td>
<td>1.310 ns, 4.1%</td>
<td>0.721 ns, 2.5%</td>
<td>0.828 ns, 4.8%</td>
<td>1.448 ns, 3.7%</td>
<td>2.405 ns, 50.3%</td>
</tr>
</tbody>
</table>

Figure E.5. TCSPC decay curves for amine-functionalised zinc and gallium bis(thiosemicarbazone) complexes 810 nm (left) 910 nm (right)
Appendix F. Two-photon Fluorescence lifetime imaging data of in cancer cells

Table F.1. Two-photon fluorescence lifetime imaging data in HeLa and PC-3 cells of free ligands at 100 µM concentration.

<table>
<thead>
<tr>
<th>Compound</th>
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<th>ia</th>
<th>ic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell line</td>
<td>HeLa</td>
<td>PC-3</td>
<td>HeLa</td>
</tr>
<tr>
<td>$\chi^2$</td>
<td>1.43</td>
<td>1.22</td>
<td>1.16</td>
</tr>
<tr>
<td>$\lambda$/nm</td>
<td>910</td>
<td>910</td>
<td>910</td>
</tr>
<tr>
<td>$\tau_1$/ns</td>
<td>0.176</td>
<td>0.199</td>
<td>0.12</td>
</tr>
<tr>
<td>FWHM/ns</td>
<td>0.052</td>
<td>0.19</td>
<td>0.225</td>
</tr>
<tr>
<td>$\tau_1$ %</td>
<td>51</td>
<td>80</td>
<td>91</td>
</tr>
<tr>
<td>$\tau_2$/ns</td>
<td>2.924</td>
<td>2.583</td>
<td>6.129</td>
</tr>
<tr>
<td>FWHM/ns</td>
<td>1.507</td>
<td>1.114</td>
<td>1.048</td>
</tr>
<tr>
<td>$\tau_2$ %</td>
<td>49</td>
<td>20</td>
<td>8</td>
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</table>

Table F.2. Two-photon fluorescence lifetime imaging data in HeLa and PC-3 cells of ligand precursors, $\lambda_{ex} = 810$ nm, at 100 µM concentration.

<table>
<thead>
<tr>
<th>Compound</th>
<th>iia</th>
<th>iib</th>
<th>iic</th>
<th>iic</th>
<th>iid</th>
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<tbody>
<tr>
<td>Cell line</td>
<td>HeLa</td>
<td>HeLa</td>
<td>HeLa</td>
<td>PC-3</td>
<td>HeLa</td>
</tr>
<tr>
<td>$\chi^2$</td>
<td>1.50</td>
<td>1.26</td>
<td>1.15</td>
<td>1.13</td>
<td>1.23</td>
</tr>
<tr>
<td>$\tau_1$ %</td>
<td>86</td>
<td>81</td>
<td>100</td>
<td>86</td>
<td>80</td>
</tr>
<tr>
<td>$\tau_1$/ns</td>
<td>0.903</td>
<td>0.868</td>
<td>1.803</td>
<td>0.233</td>
<td>0.725</td>
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<tr>
<td>FWHM/ns</td>
<td>0.415</td>
<td>0.642</td>
<td>0.617</td>
<td>0.204</td>
<td>0.358</td>
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<tr>
<td>$\tau_2$ %</td>
<td>14</td>
<td>19</td>
<td>14</td>
<td>14</td>
<td>20</td>
</tr>
<tr>
<td>$\tau_2$/ns</td>
<td>3.549</td>
<td>3.218</td>
<td>2.087</td>
<td>3.128</td>
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<tr>
<td>FWHM/ns</td>
<td>1.479</td>
<td>2.695</td>
<td>1.543</td>
<td>1.712</td>
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</table>
### Table F.3. Two-photon fluorescence lifetime imaging data in HeLa and PC-3 cells of ligand precursors, $\lambda_{ex} = 910\, \text{nm}$, at 100 $\mu\text{M}$ concentration.

<table>
<thead>
<tr>
<th>Compound</th>
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<tbody>
<tr>
<td>Cell line</td>
<td>HeLa</td>
<td>HeLa</td>
<td>HeLa</td>
<td>HeLa</td>
<td>PC-3</td>
<td>PC-3</td>
<td>PC-3</td>
<td>PC-3</td>
</tr>
<tr>
<td>$\chi^2$</td>
<td>1.54</td>
<td>1.05</td>
<td>1.38</td>
<td>1.33</td>
<td>1.48</td>
<td>1.21</td>
<td>1.47</td>
<td>1.21</td>
</tr>
<tr>
<td>$\tau_1$ %</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>92</td>
<td>100</td>
<td>80</td>
<td>100</td>
<td>90</td>
</tr>
<tr>
<td>$\tau_1$ / ns</td>
<td>3.062</td>
<td>2.522</td>
<td>0.344</td>
<td>0.307</td>
<td>2.469</td>
<td>0.081</td>
<td>0.531</td>
<td>0.164</td>
</tr>
<tr>
<td>FWHM / ns</td>
<td>0.42</td>
<td>0.303</td>
<td>0.9</td>
<td>0.575</td>
<td>1.293</td>
<td>0.202</td>
<td>0.506</td>
<td>0.544</td>
</tr>
<tr>
<td>$\tau_2$ %</td>
<td></td>
<td></td>
<td></td>
<td>8</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\tau_2$ / ns</td>
<td></td>
<td></td>
<td></td>
<td>0.436</td>
<td>0.434</td>
<td></td>
<td></td>
<td>0.418</td>
</tr>
<tr>
<td>FWHM / ns</td>
<td></td>
<td></td>
<td></td>
<td>0.653</td>
<td>0.209</td>
<td></td>
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<td>0.455</td>
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</table>

### Table F.4. Two-photon fluorescence lifetime imaging data in cancer cells of copper bis(thiosemicarbazonato) complexes, at 50 $\mu\text{M}$ concentration.

<table>
<thead>
<tr>
<th>Compound</th>
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<th>1b</th>
<th>1c</th>
<th>1d</th>
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</thead>
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<td>HeLa</td>
<td>HeLa</td>
</tr>
<tr>
<td>$\chi^2$</td>
<td>1.67</td>
<td>1.77</td>
<td>1.15</td>
<td>1.30</td>
</tr>
<tr>
<td>$\lambda$ / nm</td>
<td>810</td>
<td>910</td>
<td>910</td>
<td>910</td>
</tr>
<tr>
<td>$\tau_1$ %</td>
<td>93.4</td>
<td>94.8</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>$\tau_1$ / ns</td>
<td>0.152</td>
<td>0.217</td>
<td>0.284</td>
<td>0.266</td>
</tr>
<tr>
<td>FWHM / ns</td>
<td>0.093</td>
<td>0.098</td>
<td>0.087</td>
<td>0.146</td>
</tr>
<tr>
<td>$\tau_2$ %</td>
<td>6.6</td>
<td>5.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\tau_2$ / ns</td>
<td>0.433</td>
<td>0.505</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FWHM / ns</td>
<td>0.625</td>
<td>0.821</td>
<td></td>
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</table>

### Table F.5. Two-photon fluorescence lifetime imaging data in HeLa cells of copper bis(thiosemicarbazonato) complexes, at 50 $\mu\text{M}$ concentration.

<table>
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<th>2d</th>
</tr>
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<td>$\chi^2$</td>
<td>1.19</td>
<td>1.27</td>
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<tr>
<td>$\tau_1$ %</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>$\tau_1$ / ns</td>
<td>0.343</td>
<td>0.297</td>
</tr>
<tr>
<td>FWHM / ns</td>
<td>0.095</td>
<td>0.13</td>
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Table F.6. Two-photon fluorescence lifetime imaging data in HeLa, $\lambda_{ex} = 810$ nm, at 50 µM concentration.

<table>
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<tbody>
<tr>
<td></td>
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<td>1.18</td>
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<td>$\tau_1$</td>
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<tr>
<td>$\tau_1$ / ns</td>
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<td>FWHM / ns</td>
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<td>$\tau_2$</td>
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<td>11</td>
<td>16</td>
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<tr>
<td>$\tau_2$ / ns</td>
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<tr>
<td>FWHM / ns</td>
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Table F.7. Two-photon fluorescence lifetime imaging data, $\lambda_{ex} = 910$ nm in HeLa, PC-3 and MCF-7 cells of gallium bis(thiosemicarbazonato) complexes, at 50 µM concentration, where * and ** refer to before and after irradiation at 488 nm for 9 minutes.

<table>
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<th>3b</th>
<th>3c</th>
<th>3d</th>
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<th>3b**</th>
<th>3c*</th>
<th>3c**</th>
<th>3b*</th>
<th>3b**</th>
<th>3c*</th>
<th>3c**</th>
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<tbody>
<tr>
<td>Cell line</td>
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<td>PC-3</td>
<td>MCF-7</td>
<td></td>
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<td>1.19</td>
<td>1.19</td>
<td>1.17</td>
<td>1.47</td>
<td>1.27</td>
<td>1.18</td>
<td>1.16</td>
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<td>$\tau_1$ / ns</td>
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<td>0.772</td>
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<td>0.449</td>
<td>1.118</td>
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<td>0.475</td>
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<td>FWHM / ns</td>
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Table F.8. Two-photon fluorescence lifetime imaging data in HeLa and PC-3 cells of indium bis(thiosemicarbazonato) complexes, at 50 μM concentration $\lambda_{ex} = 910$ nm.

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<th>4c</th>
<th>4d</th>
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<td>1.37</td>
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<td>0.323</td>
<td>0.653</td>
<td>0.27</td>
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<tr>
<td>FWHM / ns</td>
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<td>0.045</td>
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Table F.9. Two-photon fluorescence lifetime imaging data in HeLa and PC-3 cells of amine-functionalised bis(thiosemicarbazonato) complexes, $\lambda_{ex} = 810$ nm.

<table>
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<tr>
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<th>3bN</th>
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<td>1.43</td>
<td>1.31</td>
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<td>$\tau_2$ / ns</td>
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Table F.10. Two-photon fluorescence lifetime imaging data in HeLa and PC-3 cells of amine-functionalised bis(thiosemicarbazonato) complexes, $\lambda_{ex} = 910$ nm.

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<th>1cN</th>
<th>1dN</th>
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<td>100</td>
<td>100</td>
<td>100</td>
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<td>$\tau_1$ / ns</td>
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<td>0.522</td>
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<tr>
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<tr>
<td>$\tau_2$ / ns</td>
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<td>0.468</td>
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<tr>
<td>FWHM / ns</td>
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<td>0.329</td>
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Appendix G. Fluorescence microscopy in biological cells

Figure G.1. Single-photon confocal fluorescence microscopy of ia at 100 μM, 1% DMSO 30 mins, HeLa. a) DIC micrograph. b) Micrograph with excitation at 488 nm. c) Overlay image of a) and b).

Figure G.2. Single-photon confocal fluorescence microscopy of ia at 50 μM, 0.5% DMSO, 20 mins, HeLa, incubated with mitotracker. a) DIC micrograph. b) Micrograph with excitation at 488 nm. c) Micrograph with excitation at 543 nm. d) Overlay image of a), b) and c).

Figure G.3. Single-photon confocal fluorescence microscopy of ia at 100 μM, 1% DMSO, 20 mins, PC-3, incubated with mitotracker. a) DIC micrograph. b) Micrograph with excitation at 405 nm. c) Micrograph with excitation at 488 nm. d) Overlay image of a), b) and c).
Figure G.4. Single-photon confocal fluorescence microscopy of iid at 50 μM, 0.5% DMSO, 20 minutes, washed then 100 μM, 1% DMSO, incubated for 20 minutes, then washed in FEK-4. a) DIC micrograph. b) Micrograph with excitation at 488 nm. c) Overlay image of a) and b). Scalebar: 20 μm

Figure G.5. Single-photon confocal fluorescence microscopy of iib at 100 μM, 1% DMSO, 20 minutes, in PC-3. a) DIC micrograph. b) Micrograph with excitation at 488 nm. c) Micrograph with excitation at 543 nm, d) Overlay image of a), b) and c). Scalebar: 20 μm

Figure G.6. Single-photon confocal fluorescence microscopy of iib at 100 μM, 1% DMSO, 20 minutes, in HeLa. a) DIC micrograph. b) Micrograph with excitation at 405 nm (green channel). c) Micrograph with excitation at 488 nm (green channel). d) Micrograph with excitation at 488 nm (green channel). Scalebar: 20 μm.
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Figure G.7. Single-photon confocal fluorescence microscopy of iid at 100 μM, 1% DMSO, 20 minutes, in HeLa. a) DIC micrograph. b) Micrograph with excitation at 405 nm (green channel). c) Micrograph with excitation at 488 nm (green channel). d) Micrograph with excitation at 488 nm. (green channel). Scalebar: 20 μm.

Figure G.8. Single-photon confocal fluorescence microscopy of iid at 100 μM, 1% DMSO, 20 minutes, in PC-3. a) DIC micrograph. b) Micrograph with excitation at 405 nm (green channel). c) Micrograph with excitation at 488 nm (green channel). d) Micrograph with excitation at 488 nm. (green channel). Scalebar: 20 μm.

Figure G.9. Confocal microscopy of HeLa cells incubated with 1d at 50 μM, 0.5% DMSO, for 20 minutes, at 37 °C. c) Overlay of a) DIC image and of b) micrograph with excitation at 488 nm.
Figure G.10. Confocal microscopy of HeLa cells incubated with 1b at 100 μM, 1% DMSO, for 20 minutes, at 37. Where (e) Overlay of (a) the DIC image b) micrograph with excitation of 405 nm, (c) the micrograph with excitation of 488 nm and (d) the micrograph with excitation of 543 nm.

Figure G.11. Confocal microscopy of HeLa cells incubated with 2d at 50 μM, 0.5% DMSO, for 60 minutes, at 37 °C. c) Overlay of a) DIC image and of b) micrograph with excitation at 488 nm.
Figure G.12. Complex 3b at 50 μM, 0.5% DMSO, irradiation experiment at 488 nm in PC-3 cells: (a – c) before irradiation and (d – f) after irradiation for ca. 10 minutes, DIC image (a, d), micrograph of cells after excitation at 488 nm (b, e, compound, green channel), micrograph of cells after excitation at 543 nm (d, i, compound, red channel). (c) Overlay of (a) and (b) images, whereas (f) Overlay of images (d) and (e). Scalebar: 20 μm.

Figure G.13. Complex 3d at 50 μM, 0.5% DMSO, 20 minutes irradiation experiment at 488 nm in FEK-4 cells: a) DIC image. b) Micrograph of cells after excitation at 405 nm. c) Micrograph of cells after excitation at 488 nm (compound, green channel). (d) Overlay of (a), (b) and (c). Scalebar: 20 μm.
Figure G.14. Complex 3d irradiation experiment at 488 nm in FEK-4 cells, 100 μM, 1 % DMSO, ca. 3 hours. (a, f) DIC image. (b, g) Micrograph of cells after excitation at 405 nm. (c, h) Micrograph of cells after excitation at 488 nm (compound, green channel). (d) Overlay of (a), (b) and (c) images, scalebar: 20 μm.

Figure G.15. Complex 3c irradiation experiment at 488 nm in HeLa cells: (a – e) with no added stain DIC image (a), micrograph of cells after excitation at 405 nm (b), micrograph of cells after excitation at 488 nm (c, compound, green channel), micrograph of cells after excitation at 543 nm (d, compound, red channel). Image (d) Overlay of (a), (b) and (c) images. Scalebar: 20 μm.

Figure G.16. Complex 3a and Mitotracker in HeLa cells: DIC image (a), micrograph of cells after excitation at 488 nm (b, compound, green channel), micrograph of cells after excitation at 543 nm (c, red channel). Image (d) Overlay of (a), (b) and (c) images suggesting mitochondria localisation of 3. Scalebar: 20 μm.
**Figure G.17.** Complex 3a and Lysotracker in HeLa cells: DIC image (a), micrograph of cells after excitation at 488 nm (b, compound, green channel), micrograph of cells after excitation at 543 nm (c, compound, red channel). Image (d) Overlay of (a), (b) and (c) images suggesting some lysosome localisation. Scalebar: 20 μm

**Figure G.18.** Complex 3a and Lysotracker in PC-3 cells: DIC image (a), micrograph of cells after excitation at 488 nm (b, compound, green channel), micrograph of cells after excitation at 543 nm (c, compound, red channel). Image (d) Overlay of (a), (b) and (c) images suggesting lysosome localisation. Scalebar: 20 μm

**Figure G.19.** Complex 3b and Hoechst (nuclear stain) in HeLa cells at 37°C: DIC image (a), micrograph of cells after excitation at 405 nm (b, Hoechst, blue channel), micrograph of cells after excitation at 488 nm (c, compound, green channel), micrograph of cells after excitation at 543 nm (d, compound, red channel). Image (e) Overlay of (a), (b), (c) and (d) images suggesting some lysosome localisation. Scalebar: 20 μm.
Appendix G

Figure G.20. Single-photon confocal microscopy images of 3b in HeLa cells incubated at 4 °C, (a) DIC image, (b) excitation at 405 nm, emission 420–480 nm (c) excitation at 488 nm, emission >505 nm, (d) Overlay of (a), (b) and (c). Scalebar: 20 μm.

Figure G.21. Single-photon confocal microscopy images of 4a in PC-3 cells, 30 minute incubation (a) DIC image, (b) excitation at 488 nm, emission >505 nm, (c) Overlay of (a) and (b). Scalebar: 20 μm

Figure G.22. Single-photon confocal microscopy images of 4b in PC-3 cells, 20 minute incubation, 50 μM 0.5% DMSO (a) DIC image, (b) excitation at 405 nm, emission 420–480 nm (c) excitation at 488 nm, emission >505 nm, (d) micrograph of cells after excitation at 543 nm and (e) Overlay of (a), (b), (c) and (d) Scalebar: 20 μm
Figure G.23. Single-photon confocal microscopy images of 4c in HeLa cells, 20 minute incubation, 50 μM 0.5% DMSO at 37°C (a) DIC image, (b) excitation at 405 nm, emission 420-480 nm (c) excitation at 488 nm, emission >505 nm, (d) micrograph of cells after excitation at 543 nm and (e) Overlay of (a), (b), (c) and (d) Scalebar: 20 μm

Figure G.24. Single-photon confocal microscopy images of 4a in HeLa cells (a) DIC image, (b) excitation at 488 nm, emission >505 nm, (c) micrograph of cells after excitation at 543 nm and (d) Overlay of (a), (b) and (c). Scalebar: 20 μm
Figure G.25. Single-photon confocal microscopy images of 4b in HeLa cells, 20 minute incubation, 50 µM 0.5% DMSO at 4°C (a) excitation at 405 nm, emission 420–480 nm, (b) excitation at 488 nm, emission >505 nm, (c) micrograph of cells after excitation at 543 nm and (d) Overlay of (a), (b), (c) and (g). (e) is the DIC image from excitation at 405 nm, (f) is the DIC image from excitation at 488 nm, (g) is the DIC image from excitation at 543 nm. Scalebar: 20 µm.

Figure G.26. Single-photon confocal microscopy images of 4c in PC-3 cells, before irradiation at 488 nm 20 minute incubation, 50 µM 0.5% DMSO at 37°C (a) DIC image, (b) excitation at 405 nm, emission 420–480 nm (c) excitation at 488 nm, emission >505 nm, (d) micrograph of cells after excitation at 543 nm and (e) Overlay of (a), (b), (c) and (d) Scalebar: 20 µm
Appendix G

Figure G.27. Single-photon confocal microscopy images of 4c in PC-3 cells, after irradiation at 488 nm 20 minute incubation, 50 μM 0.5% DMSO at 37°C (a) DIC image, (b) excitation at 405 nm, emission 420-480 nm (c) excitation at 488 nm, emission >505 nm, (d) micrograph of cells after excitation at 543 nm and (e) Overlay of (a), (b), (c) and (d) Scalebar: 20 μm.

Figure G.28. Single-photon Epi-fluorescence microscopy images of 4d in HeLa cells, 3 hour incubation, 100 μM, 1% DMSO at 37°C (a) DIC image, (b) excitation at 488 nm, emission >505 nm, (c) Overlay of (a) and (b). Scalebar: 20 μm.

Figure G.29. Single-photon Epi-fluorescence microscopy images of 4d in HeLa cells, 3 hour incubation, 100 μM, 1% DMSO at 4°C (a) DIC image, (b) excitation at 488 nm, emission >505 nm, (c) Overlay of (a) and (b). Scalebar: 20 μm.
Figure G.30. Single-photon confocal microscopy images of 4b in FEK-4 cells, 3 hour incubation, 100 μM, 1% DMSO at 37°C (a) DIC image, (b) excitation at 488 nm, emission >505 nm, (c) Overlay of (a) and (b). Scalebar: 20 μm.

Figure G.31. Single-photon confocal microscopy images of 4b in FEK-4 cells, 3 hour incubation, 100 μM, 1% DMSO at 4°C (a) DIC image, (b) excitation at 488 nm, emission >505 nm, (c) Overlay of (a) and (b). Scalebar: 20 μm.

Figure G.32. Single-photon confocal microscopy images of 4d in PC-3 cells, 30 minute incubation, 50 μM 0.5% DMSO at 37°C (a) DIC image, (b) excitation at 488 nm, emission >505 nm, (c) Overlay of (a) and (b). Scalebar: 20 μm
**Figure G.33.** Single-photon confocal microscopy images of 4d in HeLa cells, 60 minute incubation, 50 µM 0.5% DMSO at 37°C (a) DIC image, (b) excitation at 488 nm, emission >505 nm, (c) Overlay of (a) and (b). Scalebar: 20 µm

**Figure G.34.** Single-photon confocal microscopy images of 4d in, FEK-4 cells 100 µM, 1% DMSO 60 minute incubation, at 37°C (a) DIC image, (b) excitation at 488 nm, emission >505 nm, (c) Overlay of (a) and (b). Scalebar: 20 µm

**Figure G.35.** Single-photon confocal microscopy images of 4a and Hoechst stain in HeLa cells after 20 minutes, (a) DIC image, (b) excitation at 405 nm, emission 420-480 nm (c) micrograph of cells after excitation at 543 nm and (d) Overlay of (a), (b) and (c). Scalebar: 20 µm
Figure G.36. Single-photon confocal microscopy images of 4c and Hoechst 6 hour in PC-3 cells, 20 minute incubation, 50 μM 0.5% DMSO at 37°C (a) DIC image, (b) excitation at 488 nm, emission >505 nm, (c) micrograph of cells after excitation at 543 nm, (d) Overlay of (a), (b) and (c). Scalebar: 20 μm.

Figure G.37. Single-photon confocal microscopy images of 4a and Hoechst stain in HeLa cells, 60 minute incubation (a) DIC image, (b) excitation at 405 nm, emission 420-480 nm (c) excitation at 488 nm, emission >505 nm, (d) Overlay of (a), (b) and (c). Scalebar: 20 μm

Figure G.38. Single-photon confocal microscopy images of 4a and Hoechst stain in PC-3 cells, 60 minute incubation (a) DIC image, (b) excitation at 405 nm, emission 420-480 nm (c) excitation at 488 nm, emission >505 nm, (d) Overlay of (a), (b) and (c). Scalebar: 20 μm
Figure G.39. Single-photon confocal microscopy images of 4d and Hoechst stain in PC-3 cells, 20 minute incubation, 50 µM 0.5% DMSO at 37°C (a) DIC image, (b) excitation at 405 nm, emission 420-480 nm (c) excitation at 488 nm, emission >505 nm, (d) Overlay of (a), (b) and (c). Scalebar: 20 µm

Figure G.40. Single-photon confocal microscopy images of 4d and Hoechst stain in PC-3 cells, 60 minute incubation, 50 µM 0.5% DMSO at 37°C (a) DIC image, (b) excitation at 405 nm, emission 420-480 nm (c) excitation at 488 nm, emission >505 nm, (d) Overlay of (a), (b) and (c). Scalebar: 20 µm

Figure G.41. Single-photon confocal microscopy images of 4a and Hoechst stain in PC-3 cells, 6 hour incubation, 50 µM 0.5% DMSO at 37°C (a) DIC image, (b) excitation at 405 nm, emission 420-480 nm (c) excitation at 488 nm, emission >505 nm, (d) Overlay of (a), (b) and (c). Scalebar: 20 µm
Figure G.42. Single-photon confocal microscopy images of 4d and Hoechst stain in FEK-4 cells, 50 μM, 0.5% DMSO, 30 minutes at 37°C (a) DIC image, (b) excitation at 405 nm, emission 420-480 nm (c) excitation at 488 nm, emission >505 nm, (d) Overlay of (a), (b) and (c). Scalebar: 20 μm

Figure G.43. Single-photon confocal microscopy images of 4b in HeLa cells stained with Hoechst, 20 minute incubation, 50 μM 0.5% DMSO (a) DIC image, (b) excitation at 405 nm, emission 420-480 nm (c) excitation at 488 nm, emission >505 nm, (d) micrograph of cells after excitation at 543 nm and (e) Overlay of (a), (b), (c) and (d) Scalebar: 20 μm.

Figure G.44. Single-photon confocal microscopy images of 4b in PC-3 cells, 6 hour incubation, 50 μM 0.5% DMSO (a) DIC image, (b) excitation at 405 nm, emission 420-480 nm (c) excitation at 488 nm, emission >505 nm, (d) micrograph of cells after excitation at 543 nm and (e) Overlay of (a), (b), (c) and (d) Scalebar: 20 μm
**Figure G.45.** Single-photon confocal microscopy images of 4c and Hoechst 1 hour in PC-3 cells, 20 minute incubation, 50 µM 0.5% DMSO at 37°C (a) DIC image, (b) excitation at 488 nm, emission >505 nm, (c) micrograph of cells after excitation at 543 nm, (d) Overlay of (a), (b) and (c). Scalebar: 20 µm.

**Figure G.46.** Single-photon confocal microscopy images of 4c in HeLa cells stained with ER tracker, 20 minute incubation, 50 µM 0.5% DMSO at 37°C (a) DIC image, (b) excitation at 405 nm, emission 420-480 nm (c) excitation at 488 nm, emission >505 nm, (d) micrograph of cells after excitation at 543 nm and (e) Overlay of (a), (b), (c) and (d) Scalebar: 20 µm.

**Figure G.47.** Single-photon confocal microscopy images of 4a and Mitotracker Red in HeLa cells, 20 minute incubation (a) DIC image, (b) excitation at 488 nm, emission >505 nm, (c) micrograph of cells after excitation at 543 nm, (d) Overlay of (a), (b) and (c). Scalebar: 20 µm.
Figure G.48. Single-photon confocal microscopy images of 4c and Mitotracker in PC-3 cells, 20 minute incubation, 50 µM 0.5% DMSO at 37°C (a) DIC image, (b) excitation at 488 nm, emission >505 nm, (c) micrograph of cells after excitation at 543 nm, (d) Overlay of (a), (b) and (c). Scalebar: 20 µm

Figure G.49. Single-photon confocal microscopy images of 4d and Mitotracker Red in HeLa cells, 20 minute incubation, 50 µM 0.5% DMSO at 37°C (a) DIC image, (b) excitation at 488 nm, emission >505 nm, (c) micrograph of cells after excitation at 543 nm, (d) Overlay of (a), (b) and (c). Scalebar: 20 µm

Figure G.50. Single-photon confocal microscopy images of 4c after a 20 minute incubation in HeLa cells, incubated with Lysotracker Red, 50 µM 0.5% DMSO at 37°C (a) DIC image, (b) excitation at 405 nm, emission 420-480 nm (c) excitation at 488 nm, emission >505 nm, (d) micrograph of cells after excitation at 543 nm and (e) Overlay of (a), (b), (c) and (d) Scalebar: 20 µm.
**Figure G.51.** Single-photon confocal microscopy images of 4d and Lysotracker Red in HeLa cells, 20 minute incubation, 50 μM 0.5% DMSO at 37°C (a) DIC image, (b) excitation at 488 nm, emission >505 nm, (c) micrograph of cells after excitation at 543 nm, (d) Overlay of (a), (b) and (c). Scalebar: 20 μm

**Figure G.52.** Single-photon confocal microscopy images of 4c and ER tracker in PC-3 cells, 20 minute incubation, 50 μM 0.5% DMSO at 37°C (a) DIC image, (b) excitation at 488 nm, emission >505 nm, (c) micrograph of cells after excitation at 543 nm, (d) Overlay of (a), (b) and (c). Scalebar: 20 μm

**Figure G.53.** Single-photon confocal microscopy images of 4c and Mitotracker in PC-3 cells, 20 minute incubation, 50 μM 0.5% DMSO at 37°C (a) DIC image, (b) excitation at 488 nm, emission >505 nm, (c) micrograph of cells after excitation at 543 nm, (d) Overlay of (a), (b) and (c). Scalebar: 20 μm
Figure G.54. Single-photon confocal microscopy images of 4a and Lysotracker Red in HeLa cells, 20 minute incubation (a) DIC image, (b) excitation at 488 nm, emission >505 nm, (c) micrograph of cells after excitation at 543 nm, (d) Overlay of (a), (b) and (c). Scalebar: 20 μm

Figure G.55. (a – c) Single-photon confocal microscopy of complex 1bB reaction mixture, 100 μM, 1% DMSO, incubated in HeLa cells for 20 minutes where a) DIC channel, b) excitation at 488 nm and c) overlay image of (a) and (b). Scalebar = 20 μm. Two-photon fluorescence imaging of complex 2, 100 μM, 1% DMSO, λex = 910 nm, incubated in HeLa cells for 20 minutes where e) is the fluorescence intensity profile, f) is the fluorescence lifetime map of tm and g) is the corresponding lifetime distribution plot in ns of tm.
Figure G.56. Single-photon confocal microscopy of complex 1bB reaction mixture, 100 μM, 1% DMSO, incubated in PC-3 cells for 20 minutes where a) DIC channel, b) excitation at 488 nm and c) overlay image of (a) and (b). Scalebar = 20 μm

Figure G.57. Two-photon fluorescence imaging of complex 1bB reaction mixture, 100 μM, 1% DMSO, λ_{ex} = 910 nm, incubated in PC-3 cells for 20 minutes where a) is the fluorescence intensity profile, b) is the fluorescence lifetime map and c) is the corresponding lifetime distribution plot in ps.

Figure G.58. PC-3 cells stained with Alexa fluor plasma membrane dye, before (above) and after (below) addition of complex 1bB reaction mixture, final concentration after addition was 100 μM, 1% DMSO. a) and e) represent the DIC channel, (b) and (f) correspond to excitation at 405 nm, (c) and (g) excitation at 488 nm and (d) and (h) are an overlay of the three channels.
Figure G.59. MCF-7 cells incubated with complex 1bB reaction mixture for 20 minutes at 100 μM, 1% DMSO before (above) and after (below) addition of Alexa Fluor plasma membrane stain. a) and e) represent the DIC channel, (b) and (f) correspond to excitation at 405 nm, (c) and (g) excitation at 488 nm and (d) and (h) are an overlay of the three channels. (e) – (h) were acquired within 5 minutes of addition and (b) shows background emission before addition of the Alexa Fluor plasma membrane stain. (Precipitation was observable since no cell washing occurred to enable the image to be taken as soon as the compound was added.) Scalebar: 20 μm.

Figure G.60. Two-photon fluorescence imaging of complex 1bB reaction mixture, 100 μM, 1% DMSO, λ<sub>ex</sub> = 910 nm, incubated in MCF-7 cells for 20 minutes where a) is the fluorescence intensity profile, b) is the fluorescence lifetime map and c) is the corresponding lifetime distribution plot in ns.
Figure G.61. Purified compound 1bB, 5% 500 μM in MCF-7 Overlay of DIC image and λ_ex = 543 nm, scalebar = 20 µm.

Figure G.62. Overlay image of purified compound 1dB in PC-3 cells, 5% DMSO 500 μM, DIC and λ_ex = 543 nm channels, scalebar = 20 µm.

Figure G.63. Purified compound 1bB, 1% 100 μM in MCF-7 a) FLIM colour map where lifetime τ_1 = 690 ns, 0.615 FWHM, τ_2 = 0.984 ns, 0.944 ns FWHM, range of FLIM image is 0 to 5 ns, χ^2 = 1.08 where λ_ex = 810 nm and b) Overlay of DIC image and λ_ex = 488 nm, scalebar = 20 µm.
**Figure G.64.** Purified compound 1bB, 1% 100 μM in HeLa a) FLIM colour map where lifetime is 0.872 ns ± 0.490 ns FWHM, range of FLIM image is 0 to 5 ns, $\chi^2 = 1.15$ where $\lambda_{ex} = 810$ nm and b) Overlay of DIC image and $\lambda_{ex} = 488$ nm, scalebar = 20 μm. 1 component.

**Figure G.65.** (a-d) Control experiments in HeLa incubation time 30 min: (a) DIC image, b) ex 488 nm, emission 516-530 nm, c) Mitotracker stain: ex 543 nm, em 605-675 nm, d) overlay (a-c).

**Figure G.66.** (a-d) Control experiments in HeLa incubation time 60 min: (a) DIC image, b) ex 488 nm, emission 516-530 nm, c) Lysotracker stain: ex 543 nm, em 605-675 nm, d) overlay (a-c).
Figure G.67. Single-photon confocal microscopy images of Hoechst in HeLa, (a) DIC image, (b) excitation at 405 nm emission at 451 (c) excitation at 488 nm, emission 516 nm, (c) an overlay of the previous three images.

Figure G.68. Single-photon confocal microscopy images of Hoechst in PC-3, (a) DIC image, (b) excitation at 405 nm emission at 451 (c) excitation at 488 nm, emission 516 nm, (c) an overlay of the previous three images.

Figure G.69. Single-photon confocal microscopy images of DAPI in HeLa (fixed cells), (a) DIC image, (b) excitation at 405 nm, emission 420-480 nm (c) excitation at 488 nm, emission >505 nm, (d) overlay of (a)+(b)+(c)
Figure G.70. Single-photon confocal microscopy images of PC-3 cells stained with Alexa fluor plasma membrane dye, a) represent the DIC channel, (b) correspond to excitation at 405 nm, (c) excitation at 488 nm and (d) are an overlay of the three channels.
Appendix H. MTT MI<sub>50</sub> cytotoxicity investigation

Figure H.1. Scatter graph representing MI<sub>50</sub> in HeLa cells a) of Cis-Platin b) of Cu[ATSM]

Figure H.2. Scatter graph representing MI50 in HeLa cells a) of ia b) of id

Figure H.3. Scatter graph representing MI<sub>50</sub> in HeLa cells a) of iib b) of iic
Appendix H

Figure H.4. Scatter graph representing MI_{50} in HeLa cells a) of 1b) of 1c

Figure H.5. Scatter graph representing MI_{50} in HeLa cells a) of 2b) of 2c

Figure H.6. Scatter graph representing MI_{50} in HeLa cells a) of 3a) of 3b
Figure H.7. Scatter graph representing MI_{50} in HeLa cells a) of 3c b) of 3d

Figure H.8. Scatter graph representing MI_{50} in HeLa cells a) of 4a b) of 4b

Figure H.9. Scatter graph representing MI_{50} in HeLa cells a) of 4c b) of 4d
Figure H.10. Scatter graph representing MI_{50} in FEK-4 cells a) of ia b) of id

Figure H.11. Scatter graph representing MI_{50} in FEK-4 cells a) of 3a b) of 3d

Figure H.12. Scatter graph representing MI_{50} in FEK-4 cells a) of 4a b) of 4d
Figure H.13. Scatter graph representing MI$_{50}$ in HeLa cells a) of 1aN b) of 1bN

Figure H.14 Scatter graph representing MI$_{50}$ in HeLa cells a) of 1cN b) of 1dN

Figure H.15. Scatter graph representing MI$_{50}$ in HeLa cells of 3bN
Appendix I. Miscellaneous

Table 1.1. Table representing the VT-NMR data of 3a.

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Figure 1.1. Van’t Hoff analysis of 3a VT-NMR data.

Figure 1.2. Continuous wave electron paramagnetic resonance (cwEPR) spectrum at the X-band (9.450 GHz) of 2c.
Figure 1.3. DFT optimisation of a symmetric zinc t-boc protected amine bis(thiosemicarbazonato complex in the gas phase by B3LYP 6-31G(d,p). Where grey = carbon, white = hydrogen, blue = nitrogen, red = oxygen, green = zinc.

TD-DFT was calculated for the first 24 excited states using uB3LYP 6-31++ (d,p) methodology, of which excited state 13 had the most significant oscillator strength, f:

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This state for optimization and/or second-order correction.

" Total Energy, E(TD-HF/TD-KS) = -3766.95660896" Copying the excited state density for this state as the 1-particle RhoCI density.

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Note: The values for each transition (e.g., \( L_{137A} \rightarrow L_{140A} \)) represent the transition energy and its corresponding oscillator strength. The \( \langle S^2 \rangle \) value indicates the population of the excited state. 

Reference: Appendix I.
### Appendix I.

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Appendix I.

Excited State 15: 2.319 eV  429.27 nm  \( f=0.0189 \), \( \langle S^2 \rangle=1.094 \)
- 135A -> 140A: 0.16389
- 121B -> 139B: -0.55446
- 123B -> 139B: -0.15587
- 127B -> 139B: -0.32730
- 130B -> 139B: 0.11040
- 131B -> 139B: -0.20337
- 133B -> 139B: -0.16735
- 135B -> 139B: -0.45639
- 135B -> 140B: -0.28937
- 136B -> 139B: 0.22114
- 136B -> 140B: 0.18909
- 138B -> 141B: -0.14996

Excited State 16: 2.130 eV  423.21 nm  \( f=0.0812 \), \( \langle S^2 \rangle=0.884 \)
- 136A -> 140A: 0.47187
- 139A -> 141A: -0.27165
- 121B -> 139B: 0.21841
- 127B -> 139B: 0.11444
- 130B -> 139B: -0.12501
- 131B -> 139B: 0.16854
- 133B -> 139B: 0.15351
- 135B -> 139B: 0.12231
- 135B -> 140B: -0.31493
- 136B -> 140B: 0.59438
- 138B -> 141B: -0.29517

Excited State 17: 3.171 eV  422.39 nm  \( f=0.0106 \), \( \langle S^2 \rangle=2.264 \)
- 134A -> 141A: -0.15688
- 135A -> 140A: 0.63724
- 136A -> 140A: -0.37834
- 139A -> 141A: 0.12868
- 121B -> 139B: 0.20344
- 127B -> 139B: 0.11085
- 134B -> 141B: 0.13880
- 135B -> 139B: 0.11120
- 135B -> 140B: -0.50643

lxxiv
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Appendix I.

Excited State 23: 3.461-A 3.2001 eV 387.44 nm f=0.0014 $<S^{\star2}>$=2.744

- 126A -> 141A -0.10820
- 134A -> 141A 0.52780
- 135A -> 140A 0.14162
- 136A -> 140A 0.10122
- 137A -> 141A -0.20697
- 139A -> 141A 0.32499
- 139A -> 143A -0.10461
- 126B -> 141B 0.10960
- 134B -> 141B -0.52630
- 137B -> 141B 0.16666
- 138B -> 141B -0.31435
- 138B -> 143B 0.10626

Excited State 24: 2.017-B 3.2349 eV 383.27 nm f=0.1058 $<S^{\star2}>$=0.768

- 134A -> 140A 0.62921
- 134B -> 140B 0.75113

Figure 1.4. Flow cytometry studies under normoxia and hypoxia of 4c (a) in EMT6 and (b) in PC-3 cells.
Figure I.5. Biodistribution study in nude mice, of 2c.

Figure I.6. MicroPET images of in nude mice, 2c, where (a), (b), (c) and (d) are at timepoints 1h, 3h, 6h, and 24 h respectively.