Danielle Park

The Noninvasive Imaging of Cell Death

Using an Hsp90 Ligand
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\(^\ddagger\) co-senior authors
Conference Presentations

International:


National:


### Awards and Scholarships

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Turn your face to the sun and the shadows fall behind you.

~ *Maori Proverb*

Dedicated to Tom Bee, my sunshine.

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I would like to thank and acknowledge the contribution of Dr Anthony Don from the Lowy Cancer Research Centre, Sydney, who initiated the project and performed the preliminary experiments. Dr Don was wholly responsible for the kinetic parameters of labelling assays (Figure 12b, 12c and 18). I would also like to thank Dr Jason Wong from the Lowy Cancer Research Centre, Sydney, for his assistance with the mass spectrometry experiments described in Chapter 3, in particular the analysis of LC-MS/MS data.

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me to travel all over the world for collaborations and conferences, encouraged me apply for every course, award or scholarship that would further my career and offered a unique insight into the world of research commercialisation. He conducts his work with the utmost integrity and humility and I can only hope to someday replicate that standard. I could not have asked for a better mentor. Thank you.

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Abstract

Cell death plays an integral role in physiology, including turnover of cells in the gastrointestinal tract, the menstrual cycle and the immune system. Imbalance of this process is also associated with disease. Excessive cell death is characteristic of vascular disorders, neurodegenerative diseases, myelodysplastic syndromes, ischemia/reperfusion injury and organ transplant rejection, among others. Cell death also plays a role in the treatment of disease. In cancer for example, most chemotherapeutics, radiation treatments and anti-hormonal agents act by inducing death of cancer cells. Given the prevalence of cell death in normal physiology and disease, noninvasive imaging of this process is likely to have wide application in biological research and ultimately in patient diagnosis and management.

I have described a small, synthetic organoarsenical GSAO (4-(N-(S-glutathionylacetyl)amino)phenylarsonous acid) that when tagged with various reporter groups can be used to noninvasively image cell death within the body. Tagged GSAO enters the cell during the mid-late stages of cell death coincident with loss of plasma membrane integrity. The probe is retained in the cytosol predominantly by covalent reaction with closely-spaced cysteine thiols near the C-terminus of the 90-kDa heat shock protein (Hsp90). Hsp90 is the most abundant molecular chaperone of the eukaryotic cytoplasm and plays a role in a number of fundamental cellular pathways.

GSAO has been tagged with optical or radioisotope reporting groups and validated in in vitro and in vivo models of cell death. Near infra-red conjugates of GSAO have been used to noninvasively image cell death in mouse tumours and brain cryolesions by fluorescence, while a radio-labelled GSAO has been used to noninvasively image tumour cell death in mice.
using SPECT/CT.
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<thead>
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<tr>
<td>%ID/g</td>
<td>Percentage Injected Dose per Gram</td>
</tr>
<tr>
<td>5FU</td>
<td>5-Fluorouracil</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine Diphosphate</td>
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<td>AIF</td>
<td>Apoptosis Inducing Factor</td>
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<td>Adenine Nucleotide Translocase</td>
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<td>Allophycocyanin</td>
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<td>Adenosine-5'-Triphosphate</td>
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<td>BRAA</td>
<td>4-(N-(bromoacetyl)amino)phenylarsonic acid</td>
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<tr>
<td>BRAO</td>
<td>4-(N-(bromoacetyl)amino)phenylarsonous acid</td>
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<tr>
<td>CAD</td>
<td>Caspase Activated DNase</td>
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<td>CH₃OH</td>
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<td>Computer Tomography</td>
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<td>DIDS</td>
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<td>DISC</td>
<td>Death-Inducing Signal Complex</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
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<td>Dimercaptopropanol</td>
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<td>DNA</td>
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<td>DTPA</td>
<td>Diethylenetriaminepentaacetic Acid</td>
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<td>EDTA</td>
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<td>ER</td>
<td>Estrogen Receptor</td>
</tr>
<tr>
<td>F</td>
<td>Fluorescein</td>
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<tr>
<td>FDG</td>
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<tr>
<td>GSAA</td>
<td>4-(N-((S-glutathionyl)acetyl)amino)benzenearsonic acid</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>GSAO</td>
<td>4-(N-(S-glutathionylacetyl)amino)phenylarsenoxide</td>
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<td>HCl</td>
<td>Hydrogen Chloride</td>
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<td>HER-2</td>
<td>Human Epidermal Growth Factor Receptor 2</td>
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<td>HL</td>
<td>Hodgkin Lymphoma</td>
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<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
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<td>HRP</td>
<td>Horseradish Peroxidase</td>
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<td>Hsp90</td>
<td>Heat Shock Protein 90</td>
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<tr>
<td>HYNIC</td>
<td>Hydrazinonicotinamide</td>
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<tr>
<td>iCAD</td>
<td>Inhibitor of Caspase Activated DNase</td>
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<td>I₂</td>
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<td>LC-MS/MS</td>
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<td>MMTS</td>
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<td>MPTP</td>
<td>Mitochondrial Permeability Transition Pore</td>
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<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
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<tr>
<td>Na₂CO₃</td>
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<td>NEM</td>
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<td>NHS</td>
<td>N-hydroxysuccinimide</td>
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<td>NHL</td>
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<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
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<td>NSCLC</td>
<td>Non-Small Cell Lung Carcinoma</td>
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<td>OG</td>
<td>Oregon Green</td>
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<td>PARP-1</td>
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<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>PE</td>
<td>Phosphatidylethanolamine</td>
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<tr>
<td>Abbreviation</td>
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<tr>
<td>PENAO</td>
<td>4-(N-(S penicillaminylacetyl)amino)phenylarsonous acid</td>
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<td>PET</td>
<td>Positron Emission Tomography</td>
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<td>PI</td>
<td>Propidium Iodide</td>
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<td>PR</td>
<td>Progesterone Receptor</td>
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<td>PS</td>
<td>Phophatidylserine</td>
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<td>PVDF</td>
<td>Polyvinylidene Difluoride</td>
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<td>TG2</td>
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<td>TNFR1</td>
<td>Tumour Necrosis Factor Receptor 1</td>
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<td>TRAIL R1 and R2</td>
<td>TNF-Related Apoptosis Inducing Ligand Receptor 1 and 2</td>
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<td>TRAMP</td>
<td>Translocating Chain-Association Membrane Protein</td>
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<td>TUNEL</td>
<td>Terminal Deoxynucleotidyl Transferase dUTP Nick End Labelling</td>
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<td>RGC</td>
<td>Retinal Ganglion Cell</td>
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<tr>
<td>RIPA</td>
<td>Radio-Immunoprecipitation Assay</td>
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<td>RPMI</td>
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<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<td>ROI</td>
<td>Region of Interest</td>
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<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
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<td>Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis</td>
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<td>Smac/DIABLO</td>
<td>Second Mitochondrial-Derived Activator of Caspase/Direct Inhibitor of Apoptosis Binding protein with Low pI</td>
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<tr>
<td>SPECT</td>
<td>Single Photon Emission Computer Tomography</td>
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<tr>
<td>SPIO</td>
<td>Superparamagnetic Iron Oxide</td>
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<tr>
<td>TLC</td>
<td>Thin Layer Chromatography</td>
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<td>VDAC</td>
<td>Voltage Dependent Anion Channel</td>
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<td>Zn$^{2+}$-DPA</td>
<td>Zinc(II)-Dipicolylamine</td>
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<td>Z-VAD-FMK</td>
<td>Carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone</td>
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The synthesis of 4-(N-(bromoacetyl)amino)phenylarsonous acid (BRAO)  

The synthesis of GSAA  

The synthesis of GSAO  

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Competitive uptake of unlabelled GSAO or GSAA with GSAO-F in apoptotic cells

Fluorescence microscopy of GSAO-OG localisation in dying cells

Identification of the proteins bound to GSAO-biotin in apoptotic cells

The interaction of GSAO-biotin with purified recombinant Hsp90 and β-tubulin

Results

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GSAO-conjugates bind intracellular Hsp90

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*Imaging cell death in vitro and in vivo using near infra-red conjugates of GSAO and GSCA.*

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Cell culture

The *in vitro* validation of near infra-red conjugates of GSAO and GSCA via flow cytometry

Primary tumour growth

*Ex vivo* fluorescence imaging of tumour cell death using GSAO-Cy5.5 and GSCA-Cy5.5

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Murine cryolesion model

Histological validation

**Results**

*In vitro* validation of near infra-red conjugates of GSAO using flow cytometry

GSAO-conjugates label dying tumour cells *in vivo*

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Chapter 1.

Introduction

General Introduction

Cell death plays an integral role in physiology, including turnover of cells in the gastrointestinal tract (Ramachandran et al., 2000), the menstrual cycle (Dahmoun et al., 1999) and the immune system (Ju et al., 1999). Imbalance of this process is also associated with disease (Thompson, 1995). Excessive cell death is characteristic of vascular disorders (Stefanec, 2000), neurodegenerative diseases (Mattson, 2000), myelodysplastic syndromes (Parker and Mufti, 2001), ischemia/reperfusion injury (Gottlieb and Engler, 1999) and organ transplant rejection (Krams and Martinez, 1998), among others. Given the central role of cell death to a broad range of pathologies, it would be highly desirable to have a noninvasive means of detecting this process. An in vivo cell death imaging agent would allow early diagnosis of disease as well as regular and accurate monitoring of disease progression. It would also assist in treatment management when the objective of the therapy is to stimulate cell death.

In oncology for instance, the aim of most anti-tumour therapies such as anti-hormonal agents, chemotherapy and radiation is to induce tumour cell death (Rupnow and Knox, 1999; Thompson, 1995). The more effective a therapy is, the greater the tumour cell death. Although cell death is an immediate and powerful indicator of a tumour's response to therapy, there are currently no means of measuring this clinically. This represents a significant challenge in oncology, particularly to the optimisation of patient care.
Therapy efficacy is currently assessed according to morphological and volumetric changes in the tumour. This is determined 2-3 months into treatment via gross anatomical imaging using computer tomography (CT) or magnetic resonance imaging (MRI). The difficulty with this strategy is that if the treatment proves futile the patient is unnecessarily exposed to serious side-effects whilst the switch to an effective therapy is delayed. In patients with aggressive malignancies this could seriously impact the outcome of treatment.

Anatomical imaging is also insensitive to differences between viable tumour mass and treatment induced necrosis, fibrosis and scarring. The inadequacy of current evaluation methods is exemplified by a number of clinical studies in which patients responded to therapy in the absence of any significant change in tumour size (Weber, 2009). In a study of patients with non-small cell lung carcinoma (NSCLC) treated with concurrent radiation and chemotherapy, 46% of patients displayed a significant histological response whilst evaluation by CT indicated only disease stabilisation (Albain et al., 1995). Similarly, in a phase III trial in 731 patients with NSCLC treated with erlotinib, 8.9% of patients displayed an objective response, however median overall survival improved by 43%. The discrepancy between response rates and survival indicate that anatomical imaging was insensitive to the efficacy of erlotinib in many patients (Shepherd et al., 2005). Clearly, alternative measures of tumour response are required. Ideally they should allow early evaluation of tumour response, and improve on the sensitivity and accuracy of CT and MRI scans.

Cell death is another facet of tumour response to therapy, and visualisation of this process would add a new dimension to the current response evaluation strategies. The noninvasive imaging of tumour cell death could be used in conjunction with CT and MRI scans to
evaluate different aspects of tumour response and so improve accuracy of therapy evaluation. In addition, an imager of cell death would enable evaluation of tumour response within hours of the initial treatment, and allow physicians to customize the treatment program to the individual. Time wasted on ineffectual therapies would be minimized, improving the efficacy of the treatment program and sparing the patient unnecessary side effects.

A cell death imaging agent could also be used to monitor ischemic injury such as stroke and myocardial infarction. A marker of cardiomyocyte death for instance could be used to identify the position and severity of a myocardial infarct. This information is central to determining patient prognosis and would allow patients to be stratified accordingly.

Cell death is accompanied by a broad spectrum of morphological and biochemical changes, from alterations in membrane composition to activation of complex intracellular pathways. Several targeted imaging agents have now been designed around these processes and are in varying stages of pre-clinical and clinical development. In order to understand the mechanism of action of these probes, as well as the rationale behind my own investigations, an overview of cell death is first given. This is followed by a comprehensive review of the cell death imaging agents currently in development. This includes probes directed towards phospholipids, such as Annexin V, Zinc(II)-dipicolylamine, duramycin, and the C2A domain of Synaptotagmin I, as well as caspase inhibitors and substrates, such as isatin sulfonamides and labelled DEVD peptides. The APOMAB antibody and Aposense group of compounds are also explored.
Apoptosis

Apoptosis is a programmed form of cell death essential to the elimination of superfluous or damaged cells in a range of physiological scenarios. Unlike necrosis, apoptosis involves the controlled packaging of cellular constituents into membrane enclosed vesicles called apoptotic bodies and removal by phagocytes without eliciting a full immune response. One of the earliest events characterizing apoptosis is a reduction in cell size due to an efflux of water, followed by membrane blebbing. As the apoptotic process proceeds, phosphatidylserine (PS) is externalized in the outer plasma membrane leaflet, the mitochondrial membrane potential is lost, nuclear chromatin is condensed and the deoxyribonucleic acid (DNA) fragmented (Taylor et al., 2008).

Various apoptotic pathways have been characterised and common to each of these pathways is the end-point activation of a group of cysteine proteases called caspases. Caspases, so named for their cleavage of substrates after an aspartate residue, can be classified as either initiator or executioner caspases. Whilst the role of the former (caspases 8, 9 and 10) is to activate other caspases by cleavage, the later (caspases 3, 6 and 7) are responsible for the degradation of cellular constituents (Kroemer et al., 2007).

Caspases

Over 400 caspase substrates have now been identified and their cleavage mediates the final stages of apoptosis. This includes the cytoskeletal re-arrangements accompanying cellular rounding and retraction as well as membrane blebbing. Caspases have been shown to cleave components of actin microfilaments, microtubular proteins and intermediate filaments as well
as modifying the Rho effector ROCK1, a key regulator of actin cytoskeletal dynamics. Caspase mediated activation of ROCK1 leads to phosphorylation of myosin light chains and actin bundle contraction. Cytosolic pressure in turn induces membrane blebbing in areas where the cytoskeleton is weak (Taylor et al., 2008; Wyllie, 2010).

Cytoskeletal changes such as these are also integral to the re-organisation of the plasma membrane around organelles to form apoptotic bodies. Caspases also target components of focal adhesions sites and cell-cell junctions resulting in detachment from the surrounding extracellular matrix and neighboring cells (Wyllie, 2010).

Chromosomal condensation and cleavage is also mediated by caspases through proteolysis of a DNase inhibitor. In healthy cells, the caspase activated DNase (CAD) is repressed by physical association with its inhibitor iCAD. Cleavage of this inhibitor by caspases releases CAD from its cytoplasmic anchor permitting heterodimerisation and translocation to the nucleus. CAD then targets linker DNA between nucleosomes and degrades genomic DNA into discrete fragments (Reh et al., 2005). Interestingly caspases also appear to be responsible for the release of a chemoattractant lipids and nucleotides that serve as homing devices for surrounding macrophages, eliciting phagocytic clearance of apoptotic bodies (Taylor et al., 2008; Wyllie, 2010).

Caspase activation can occur through two distinct mechanisms, the extrinsic ‘death receptor mediated’ pathway, or the intrinsic ‘mitochondrial mediated’ pathway.
**The extrinsic apoptotic pathway**

In the extrinsic pathway, death receptors on the plasma membrane such as tumour necrosis factor receptor 1 (TNFR1), Fas/CD95, TNF-related apoptosis inducing ligand receptor 1 and 2 (TRAIL-R1 and -R2) and translocating chain-association membrane protein (TRAMP) are bound by ligands leading to receptor aggregation and recruitment of the intracellular Fas-associated death domain protein (FADD). FADD acts an intermediary between the Fas receptor and pro-caspase 8, forming a complex known as the death-inducing signal complex (DISC). The high local concentration of pro-caspase 8 in turn results in its auto-proteolytic activation and subsequent cleavage of effector pro-casapse 3 and 7 (Danial and Korsmeyer, 2004; Moffitt et al., 2010).

**The intrinsic apoptotic pathway**

The intrinsic apoptotic pathway is characterised first and foremost by permeabilisation of the mitochondrial membrane and release of pro-apoptotic factors cytochrome c, apoptosis inducing factor (AIF), endonuclease G, and second mitochondrial-derived activator of caspase/direct inhibitor of apoptosis binding protein with low pI (Smac/DIABLO). Each of these pro-apoptotic factors have different cellular targets, however their cumulative release is considered ‘the point of no return’ in the apoptotic program (Kroemer et al., 2007).

Cytochrome C is perhaps the most well defined mitochondrial mediator of apoptosis. It resides within the intermembrane and is released upon permeabilisation of the mitochondrial membrane. Once in the cytosol, cytochrome c forms a multi-protein complex with apoptosis protease activating factor 1 (APAF-1), adenosine-5’-triphosphate (ATP)/ doxyadenosine-5’-
triphosphate (dATP) and caspase 9 called the ‘apoptosome’. This in turn initiates the
activation of caspase 9 and downstream executioner caspases. Release of Smac/DIABO
indirectly promotes caspase activation via neutralization of endogenous inhibitors of
apoptosis (IAPs) (Vaux and Silke, 2003), whilst AIF and Endonuclease G are involved in
chromatin condensation and DNA fragmentation (Kroemer et al., 2007).

Each of these mitochondrial proteins play a role navigating the final stages of intrinsic
apoptosis, however their function is wholly dependent on mitochondrial membrane
permeabilisation. In the healthy cell the outer mitochondrial membrane is permeable only to
solutes of up to 5 kDa via the voltage dependent anion channel (VDAC), whilst the inner
membrane is impermeable to nearly all ions, allowing formation of a proton gradient that
drives ATP synthesis. The function of most stimuli of the intrinsic apoptotic pathway is thus
to increase membrane permeability (Kroemer et al., 2007). This is predominantly achieved
via the introduction of Bax/Bak associated pores or initiation of the mitochondrial
permeability transition pore (MPTP).

Bax and Bak are members of the multi-domain pro-apoptotic Bcl-2 family of proteins that
each contains three Bcl-2 homology domains. Whilst Bax is predominantly found in the
cytosol or tethered to intracellular membranes, Bak is already embedded in the outer
mitochondrial membrane. Both proteins exert their pro-apoptotic affect by undergoing
conformational changes and forming homo-oligomers in the outer mitochondrial membrane
(Kroemer et al., 2007). The homo-oligomers then either alone or in combination with each
other, form large protein permeable openings in the membrane facilitating cytochrome c
efflux (Kuwana et al., 2002).
It is generally accepted that membrane permeabilisation by Bax and Bak is functionally mediated by another class of Bcl-2 family of proteins, the BH3 domain only proteins, which as the name suggests, share only one Bcl-2 homology domain. Members of this family have two roles, either to bind and activate the multi-domain pro-apoptotic proteins, or to displace anti-apoptotic proteins from the outer mitochondrial membrane. In particular Bid and Bim have been shown to activate Bax and Bak directly, whilst Bad and Bik play a more indirect role displacing anti-apoptotic Bcl-2 proteins (Scorrano and Korsmeyer, 2003). The main function of anti-apoptotic Bcl-2 proteins (Bcl-2 and Bcl-X\(_L\)) is to bind and neutralize Bax and Bak, and so by displacing these anti-apoptotic proteins, Bad and Bik indirectly promote membrane permeabilisation.

Whilst pro-apoptotic Bcl-2 family proteins contribute to cytochrome c release via permeabilisation of the outer mitochondrial membrane, the MPTP is characterised by disruption of both inner and outer membranes. The MPTP is a large, non-specific pore that spans the inner and outer mitochondrial membranes and is permeable to solutes with a molecular mass of up to 1.5 kDa (Kinnally et al., 2011).

Whilst there has been some controversy regarding the constituents of the MPTP, consensus is that the multi-protein complex centers on the adenine nucleotide translocase (ANT) (Halestrap and Brennerb, 2003). The primary physiological function of ANT is to exchange mitochondrial matrix ATP for cytosolic adenosine diphosphate (ADP), fulfilling an essential role in oxidative metabolism. During apoptosis however, ANT forms dynamic interactions with the outer membrane VDAC and matrix dwelling cyclophilin D, to form a pore across the inner mitochondrial membrane (Halestrap et al., 2002).
Formation of the MPTP allows the equilibration of solutes < 1.5 kDa across the inner mitochondrial membrane, an influx of water into the matrix, swelling of the mitochondrial matrix and finally rupture of the outer mitochondrial membrane. This in turn facilitates the release of cytochrome c and dissipation of the mitochondrial transmembrane potential (Halestrap et al., 2002).

A rise in matrix Ca\(^{2+}\) concentration is the primary trigger for opening of the MPTP, however a number of factors are known to sensitise the pore to the affects of Ca\(^{2+}\), including adenine nucleotide depletion, increased inorganic phosphate concentrations, mitochondrial depolarisation, and oxidative stress (Halestrap et al., 2002). Conversely MPTP opening can be inhibited by ligands of ANT such as bongkrekic acid and ADP, and cyclophilin D ligands such as cyclosporin A (Halestrap and Brennerb, 2003; Halestrap et al., 1997).
Necrosis and Secondary Necrosis

Necrosis represents a cell’s response to gross physical or chemical injury, including mechanical stress, osmotic shock, freeze thawing and heat. This form of cell death is characterised by immediate and irreparable damage to the cytoplasmic membrane, resulting in an influx of water and cellular oedema. Consecutive rupture of external and internal membranes leads to the release of harmful lysosomal and cytoplasmic constituents which in turn elicit an inflammatory response (Al-Rubeai and Fussenegger, 2004).

Unlike apoptosis which is likened to programmed cell suicide, necrosis is akin to accidental cell death or ‘murder’. It is also ATP independent and typified by slow dissolution of the nucleus. Biochemically, necrosis is distinguished from apoptosis by random DNA cleavage. Whilst apoptosis results in the formation of an ‘ordered ladder’ of DNA, necrosis is characterised by a heterogeneous mix of DNA degradation products emerging as a ‘smear’ on electrophoretic gels (Al-Rubeai and Fussenegger, 2004).

Necrosis and apoptosis have long been viewed as opposing arms of a classic dichotomy, however it now appears that somewhat of a continuum exists between the two, with the emergence of secondary necrosis following apoptosis (Silva et al., 2008).

Secondary necrosis describes the terminal stages of apoptotic cell death when phagocytic clearance is impeded. Typically apoptotic cells are scavenged early in the apoptotic program by surrounding macrophages and neutrophils. In the absence of macrophages, neighboring epithelial, endothelial or dendritic cells may also perform this role. Sequestration of apoptotic cells can occur prior to DNA fragmentation and perhaps even before significant
morphological changes are observed. This is certainly the case in highly regulated physiological scenarios such as embryogenesis, however in a number of pathologies scavenger cells are often insufficient in number and/or functionally incapacitated (Silva et al., 2008).

Certainly this is the case in tumours where the relative paucity of macrophages to dying tumour cells results in prolonged residency of apoptotic cells in the tumour. In this scenario, the usual morphological and biochemical changes of apoptosis are observed until the completion of apoptotic program. In the absence of phagocytic clearance however, the cell transitions into a secondary necrosis whereby the plasma membrane integrity is lost and the cell eventually ruptures (Elliott and Ravichandran, 2010). Consequently pro-inflammatory constituents are released into the extracellular compartment. Each stage of apoptosis consumes large quantities of energy and the switch between apoptosis and secondary necrosis is thought to be functionally mediated by severe ATP depletion. The generation of reactive oxygen species (ROS) and Ca$^{2+}$ overload have also been implicated (Silva et al., 2008).

It is important to note that although the signalling pathways and biochemical changes accompanying necrosis and secondary necrosis are different, there is little variation in their terminal morphologies. Both conditions are characterized by intense mitochondrial and cytoplasmic swelling as well as rupture of external membranes. As such, it is difficult to discriminate between them based on a single end-point morphological assessment (Silva et al., 2008).
**Imaging cell death**

**18Fluorodeoxyglucose (18F-FDG)**

18F-FDG is a glucose analog in which the 2’ hydroxyl group is replaced with $^{18}$F for positron emission tomography (PET). Phosphorylation of 18F-FDG ensures it is retained in the cell, whilst removal of the 2’ hydroxyl group prevents its metabolism via glycolysis. 18F-FDG imaging is particularly sensitive to neoplastic lesions due to their high metabolic activity, and is routinely used in the clinic for the diagnosis and staging of disease, as well as post-treatment monitoring/re-staging (Kwee et al., 2011).

Mounting evidence now suggests that 18F-FDG could be used to assess tumour response as early as 1-2 cycles into therapy, where a decrease in 18F-FDG uptake supposes a decline in the viable mass of the tumour. This is certainly the case for lymphomas, but also appears to hold for malignancies of the breast, lung, esophagus and colon (Avril et al., 2009; de Geus-Oei et al., 2009; Hicks, 2009; Hutchings and Barrington, 2009; Krause et al., 2009; Weber, 2009).

Early 18F-FDG imaging has been shown to be highly predictive of progression free survival and overall survival in aggressive non-Hodgkin Lymphomas (NHL) and Hodgkin Lymphomas (HL), and can be used to accurately stratify patients into good and poor prognosis groups (Hutchings and Barrington, 2009).

An extension of this is the use of 18F-FDG imaging to identify patients likely to benefit from therapy escalation, as opposed to those that would be cured with a less intensive and less
toxic regime. Over 90% of patients with early stage HLs are cured with standard therapies, however in the long term the treatment itself may give rise to cardiopulmonary disease and secondary cancers. Patients with a low risk of treatment failure are therefore likely to benefit from a less intensive treatment program. Similarly patients with advanced-stage HL and aggressive NHL who do not respond to first-line therapies would benefit from a switch to an intensive salvage regimen as early as possible (Hutchings and Barrington, 2009).

Several phase II and III clinical trials are currently underway investigating $^{18}$F-FDG based response adapted therapy. For early stage HLs this involves the omission of radiotherapy if $^{18}$F-FDG imaging is negative after traditional combinatorial chemotherapy (ABVD), whilst advanced-stage HL and aggressive NHL typically receive treatment intensification if $^{18}$F-FDG imaging is positive (Hutchings and Barrington, 2009). The trials to date appear promising, and have prompted the call to incorporate PET imaging in the standard tumour response evaluation criteria (Wahl et al., 2009).

There is now also considerable momentum to incorporate $^{18}$F-FDG based tumour response measurements in clinical trials evaluating new therapeutics. Indeed, clinical trials for imatinib and sorafenib in which there was a disconnect between patient response and tumour size have highlighted the need for alternative assessment criteria (Brower, 2011). It is argued that the use $^{18}$F-FDG imaging in clinical trials would decrease the length of the evaluation process and improve its accuracy, significantly reducing overall costs (Czernin et al., 2006). It is hoped that $^{18}$F-FDG imaging will soon be FDA approved for this setting (Brower, 2011).

In terms of monitoring tumour response to therapy, $^{18}$F-FDG is certainly the most advanced in its development and will soon be included in routine clinical practice. It is not however
without limitations. Non-specific uptake of $^{18}$F-FDG by surrounding inflammatory cells can result in false positive PET imaging and an underestimation of treatment efficacy (Czernin et al., 2006). Meanwhile a switch to alternative energy sources by the tumour would result in a gross overestimation of treatment response. Treatments affecting the glucose metabolism of the tumour are also likely to confound interpretation of PET images. Hormonal therapies such as tamoxifen for instance induce a transient increase in glucose metabolism. The so called ‘metabolic flare’ is typically observed in estrogen receptor positive breast cancers 7-10 days into treatment (Dehdashti et al., 1999; Mortimer et al., 2001). There are also questions regarding the applicability of $^{18}$F-FDG imaging to slowly growing tumours with low baseline uptake, in which only a small dynamic range is likely to be observed (Grimberg et al., 2009). Beyond these very specific limitations however, $^{18}$F-FDG imaging is likely to have a wide scope of applications clinically.
**Phospholipid binding agents**

The execution phase of apoptosis is marked by the externalization of PS and phosphatidylethanolamine (PE) in the membrane of the apoptotic cell. In healthy cells, ATP-dependent enzymes aminophospholipid ‘translocase’ and ‘floppase’ work in concert to maintain lipid bilayer asymmetry; whereby cationic phospholipids such as phosphatidylcholine and sphingomyelin are pumped to the outer leaflet and PS is restricted to the inner leaflet (Blankenberg, 2008a, b; Fadeel, 2004).

In the early stages of apoptosis, the enzymes translocase and floppase are inactivated in a calcium dependent manner, whilst scramblase is simultaneously activated, leading to redistribution of PS and PE across the membrane. This event is preceded by cell shrinkage and decreased packing of the lipid membrane, and occurs independently of cytochrome c release, caspase activation and DNA fragmentation (Blankenberg, 2008a, b). The main purpose of PS externalisation in the apoptotic program is to serve as a recognition signal for surrounding macrophages, facilitating controlled disposal of apoptotic bodies without inciting an inflammatory response (Wang, 2009). PS recognition triggers the release of anti-inflammatory cytokines which suppress dendritic cell activation (Gaipl et al., 2003).

A number of apoptotic imaging agents detect dying cells by binding externalized phospholipids such as PS and PE. They constitute between 10-20% of all membrane phospholipids, and are thus a relatively abundant target (Wang, 2009; Zhao et al., 2008). Apoptotic tumour cells for instance have in the range of 6-24 x 10^6 PS binding sites per cell. As the phospholipids are localized on the external surface of the cell, they are also readily available to the imaging agent (Nguyen and Aboagye, 2010; Zhao, 2009).
**Annexin V**

Annexin V is a 36 kDa endogenous human protein which binds to PS with nanomolar affinity. The protein is thought to exhibit a disc like structure with four PS binding sites on the convex side. At the bottom of each binding pocket 1-2 calcium ions facilitate interaction with the PS head group (Zhao, 2009).

Annexin is abundant in the placenta, and expressed at lower concentrations in the kidneys, myocardium, skeletal muscle, platelets, monocytes and endothelial cells. It functions primarily as an anti-coagulant, however recent evidence suggests that it may also promote a pro-inflammatory environment through ligation of PS residues on apoptotic cells. It has also been shown to inhibit phospholipase A$_2$ and protein kinase C (Blankenberg, 2008a).

Labelled Annexin V is the most widely utilized and well documented cell death imaging agent. It has been evaluated in numerous phase I, II and III clinical trials for acute myocardial infarction, cardiac transplant rejection, non-hemorrhagic stroke, Alzheimer’s disease, rheumatoid arthritis and tumour response to chemo or radiotherapy (Belhocine and Blankenberg, 2006; Blankenberg, 2008a).

Annexin V was initially labelled with $^{99m}$Tc through an N$_2$S$_2$ amide and used by Belhocine et al. in a trial of 15 cancer patients with late stage small and NSCLC, HL, NHL and metastatic breast cancers. Of the 9 patients who had a clinically significant response to chemotherapy, 7 displayed increased uptake of $^{99m}$Tc-N$_2$S$_2$-Annexin V by the tumour. The remaining 6 patients showed no change in tumour uptake concordant with a negligible response to chemotherapy. Although the study confirmed the potential utility of $^{99m}$Tc-N$_2$S$_2$-Annexin V
in the clinic, the labelling process was particularly cumbersome with very low labelling efficiency. The compound also demonstrated a high degree of non-specific uptake in the kidneys, liver and urinary bladder, as well as biliary excretion into the bowel precluding imaging of the abdominal region (Belhocine et al., 2002).

Follow up studies have thus centred on alternate labelling methods to improve yield and biodistribution (see Wang, 2009 for a full review). The most ubiquitous derivative of $^{99m}$Technetium labelled Annexin V employs a hydrazinonicotinamide (HYNIC) linker, eliminating concentration of the compound in the intestines. Unfortunately, non-specific uptake in the kidneys, liver and urinary bladder remains. Despite this shortcoming, $^{99m}$Tc-HYNIC-Annexin V has been used for a majority of the clinical trials conducted with the protein (Blankenberg, 2008a).

In one such study, $^{99m}$Tc-HYNIC-Annexin V was used to evaluate the response of malignant lymphomas, leukaemia, NSCLC and head and neck small cell carcinoma (H&NSCC) to radiation or chemotherapy in 33 patients. Increased tumour uptake of $^{99m}$Tc-HYNIC-Annexin V was associated with partial or complete tumour response to therapy (Kartachova et al., 2004). Similar results were also observed in a study of 16 patients with NSCLC subjected to platinum based therapy (Kartachova et al., 2007).

Despite early success with $^{99m}$Tc-HYNIC-Annexin V, the closure of Theseus Imaging Corporation has resulted in a lack of clinical grade compound for completion of clinical trials. This alongside a lack of commercial funding has halted further progression of the compound (Kapty et al., 2010).
The main criticism of the agent is its large size, which at 36 kDa results in slow delivery to site of interest, slow clearance, non-specific uptake in non-target tissues and poor biodistribution (Smith et al., 2009; Zeng and Miao, 2009). Prolonged residency of the tracer also results in high effective doses of radiation for the patient (Nguyen and Aboagye, 2010).

There are also concerns regarding the specificity of Annexin V for dying cells given PS is externalised in activated platelets, macrophages, endothelial cells and aging erythrocytes (Nguyen and Aboagye, 2010; Schutters and Reutelingsperger, 2010; Smith et al., 2009). Activated platelets and endothelial cells are reported to express $2 \times 10^5$ and $8.8 \times 10^6$ PS sites per cell respectively, where PS externalisation initiates coagulation and thrombin formation (Schutters and Reutelingsperger, 2010; Wang, 2009). In activated macrophages, PS exposure may influence the phagocytic capacity of the cell (Hamon et al., 2000).

$^{99m}$Tc-HYNIC-Annexin V has even been used to image platelet activation in bacterial induced endocarditis in rats and rabbits. Tracer uptake reflected platelet activation in the luminal layer of platelet-fibrin vegetations (Rouzet et al., 2008). It has also been used to visualise platelet and macrophage activation in a human patient presenting with endocarditis (Kietselaer et al., 2007).

Another difficulty with PS directed imaging agents is that PS externalisation does not necessarily represent cells committed to the cell death pathway. Indeed PS can be externalised in a reversible fashion independent of caspase activation, cytochrome c release and DNA fragmentation (Balasubramanian et al., 2007). This is thought to occur in situations of physiological stress such as growth factor deprivation, nitric oxide production and p53 activation, where PS is again internalised upon removal of the offending stressor (Blankenberg, 2008a, 2009). For instance the ischemic generation of the free radical nitric
oxide has been associated with reversible PS exposure in neurons (Lin et al., 2000). The phenomenon is also observed upon activation of the tumour suppressor p53. p53 is activated by a range of physiological stressors including hypoxia, heat shock, DNA damage and nucleotide depletion (Geske et al., 2001). Hypertonic shock has also been shown to induce in reversible PS expression in blood granulocytes and monocytes (Yang et al., 2002).

Although PS expression is considerably less under these conditions, large quantities of stressed cells are likely to confuse interpretation of data, and contribute to an inaccurate depiction of the extent of cell death within the lesion. Indeed much of the Annexin V uptake observed in ischemic reperfusion injury is thought to be due to a large number of stressed cells with low PS expression, opposed to only few cells truly committed to die with high PS expression (Blankenberg, 2009).

Annexin V binding of PS is also highly dependent on calcium. Whilst optimal binding occurs at concentrations around 25 mM in vitro, the highest physiological calcium concentration is 10 fold less at 2.5 mM. Attempts to enhance the binding activity of Annexin V have been made via mutation of the protein, however they have largely been unsuccessful (Schoenberger et al., 2008).

The most promising development in this regard is a self chelating Annexin V with equivalent binding affinity for PS but superior biodistribution. $^{99m}$Tc-Annexin V-128 and $^{99m}$Tc-Annexin V-117 incorporate an endogenous chelation site through addition of 6 amino acids at the N-terminus of the protein. The internal cysteine residue at position 316 is mutated to serine, allowing site specific conjugation to $^{99m}$Technetium through the N terminal cysteine. In pre-clinical models this has decreased renal retention by over 80% (Tait et al., 2000; Tait et al.,
De Saint-Hubert et al. have similarly introduced a single thiol at positions 2 and 116 of Annexin V for site specific conjugation to various radioisotopes. Both residues reside on the concave side of the protein, distal from the phospholipid and calcium binding domains such that PS binding affinity is not affected. The modified Annexin V appears to have rapid renal clearance and relatively lower liver uptake than its forerunners (Bauwens et al., 2011; De Saint-Hubert et al., 2010).

A number of $^{18}$F conjugates developed for PET imaging have also shown promise in experimental models. This imaging modality has the advantage of greater sensitivity and resolution, and quantification of tracer uptake. The compound demonstrated lower uptake in the liver, spleen and kidney compared to $^{99m}$Tc-HYNIC-Annexin V (Murakami et al., 2004). The V-128 mutant has also been labelled with $^{18}$F for PET imaging, although it has yet to be tested in vivo (Li et al., 2008).

No doubt these derivatives of Annexin V represent the best approach for future development of the probe, however further pre-clinical studies are still required (Blankenberg, 2008b). Indeed mutation of the protein poses the risk of generating antigenic sites, as well as changing both the intrinsic activities and biological half life of the protein (Schoenberger et al., 2008). Due to the biodistribution of the compound, the primary indications of $^{99m}$Tc-HYNIC-Annexin V in its current form are likely to be in H&NSCC, NSCLC and NHL following cisplatin therapy (Blankenberg, 2008b).
C2A

The C2A domain of Synaptotagmin I is another protein that binds anionic phospholipids such as PS in a calcium dependent manner. At 14.7 kDa it is less than half the size of Annexin V and is thus predicted to clear from non target tissues faster. It is also expected to penetrate the tumour more efficiently, providing a greater signal to noise ratio (Alam et al., 2010). C2A was originally conjugated to superparamagnetic iron oxide (SPIO) nanoparticles for MRI of tumour cell apoptosis following chemotherapeutic treatment. Specifically, C57BL/6 mice bearing subcutaneous lymphomas were treated with etoposide and cyclophosphamide prior to imaging with C2A-SPIO (Zhao et al., 2001). Due to the large size of the reporter molecule (30 nm) and its less than optimal contrast, this was later replaced with gadolinium (5-10 nm), however the chelate significantly reduced the affinity of the agent for PS. Current efforts are therefore directed towards modification of the C2A protein to allow labelling with fluorescent, radionuclide and MR-detectable probes. This has been achieved via mutation of an internal serine to cysteine. The resultant C2Am protein has significantly less binding to viable cells in vitro and thus greater specificity for dying cells (Alam et al., 2010).

A $^{99m}$Technetium labelled form of the protein has been successfully used in mice bearing subcutaneous NSCLC. Mice treated with paclitaxel displayed a 4 fold increase in tumour uptake 72 hours after treatment, as determined by region of interest (ROI) analysis of single photon planar images and gamma counting of dissected tumour specimens. Importantly this also correlated with a higher apoptotic index in chemotherapy treated tumours (Wang et al., 2008).
In a study by Zhao et al, the protein was also conjugated to $^{99m}$Tc for single photon planar imaging of acute myocardial infarction in rats. The target to background ratio between the injured and healthy myocardium was 30.4 for $^{99m}$Tc-C2A-glutathione-S-transferase (GST), and 6.5 for the inactive control $^{99m}$Tc-C2A-GST-N-hydroxysuccinimide (NHS). The latter reflects non-specific diffusion of the compound due to elevated vascular permeability and increased interstitial space following ischemic challenge (Zhao et al., 2006). It has also been employed in porcine models of acute myocardial infarction, where target to background ratios were $10.2 \pm 5.7$ when determined by gamma counting of specimens ex vivo, and $3.22 \pm 0.85$ when determined by ROI analysis of single photon planar images (Fang et al., 2007).

One of the major criticisms of the agent is its low affinity to PS with a dissociation constant between 90-300 nM. This is significantly higher than the 1-2 nM observed for Annexin V (Blankenberg, 2008b; Zeng and Miao, 2009). The authors argue however that this may reduce non-specific binding to viable cells with low PS expression, and thus be advantageous in an imaging capacity (Alam et al., 2010).

Although C2A is expected to have faster clearance and lower background than Annexin V on account of its size, all of the in vivo studies to date have used a GST conjugate of protein, which at 37kDa is actually larger than Annexin V. This likely explains the high renal and hepatic uptake, precluding imaging of the abdominal area. The agent must therefore be optimised before it reaps the expected benefits of reduced size (Wang et al., 2008).

The C2Am derivative shows the most promise in this regard, as preliminary data using a near infra-red conjugate displays minimal liver accumulation and rapid kidney clearance in mice (approximately 86% in 24 hours). In vivo analysis etoposide treated mice bearing murine
lymphomas also displayed a 52% higher uptake of C2Am in treated vs non treated tumours, which increased to 82% upon ex vivo analysis (Alam, Neves et al. 2010).

**Duramycin**

Duramycin is a 19 amino acid peptide which exclusively binds PE with high affinity in a calcium independent manner (Marki et al., 1991). Like PS, PE is externalized to the outer leaflet of the membrane early in the apoptotic program, however it differs significantly in function. PE is thought to facilitate apoptotic blebbing via reorganization of actin filaments and extensive membrane remodelling (Mills et al., 1998; Umeda and Emoto, 1999).

As the second most abundant phospholipid in the plasma membrane (after phosphatidylcholine) PE represents an attractive target for cell death imaging, however as duramycin binds the phospholipid head group in 1:1 ratio it is unlikely to generate a greater signal than PS directed agents. As many as 8 Annexin V molecules are known to bind one PS head group. Nonetheless, $^{99m}$Technetium labelled duramycin has shown a great deal of promise in a rat model of myocardial infarction. Total infarct uptake of duramycin was 4 times that of an inactive control compound, whilst target to background ratios were $4.8 \pm 0.4$ (infarct:lung) and $12.2 \pm 1.3$ (infarct:muscle) (Zhao et al., 2008).

Due to its small size, duramycin clears rapidly from the circulation with a blood half life of less than 4 min, highlighting an infarct as early as 10 min post injection. The agent is cleared via the renal system, excreted un-metabolised and displays very low hepatic and gastrointestinal uptake. Given its favorable biodistribution and stability in vivo, duramycin
represents a promising molecular probe for the noninvasive imaging of apoptosis (De Saint-Hubert et al., 2009; Zhao et al., 2008).

**Zinc(II)-dipicolylamine (Zn²⁺-DPA)**

Zn²⁺-DPA is a small synthetic compound that selectively recognizes anionic membranes of apoptotic cells. In much the same way that calcium facilitates Annexin V binding to apoptotic membranes, Zn²⁺ ions mediate cooperative association of DPA with PS head groups (Koulov et al., 2003; Smith et al., 2011b). Zn²⁺-DPA was conjugated to the near infra-red carbocyanine fluorophore 794 to form PSS-794, and has been used to image cell death *in vivo* in multiple experimental models.

In one study, muscle cell death was induced via local injection of a synthetic ionophore, ethanol or ketamine. PSS-794 was administered intravenously, and the mice imaged 24 hours later via planar whole body fluorescence imaging. The target to background ratio (cytotoxin injected leg:saline injected leg) was 5.97 ± 0.42 for the ionophore, 4.15 ± 0.25 for ethanol and 2.83 ± 0.09 for ketamine. This was significantly higher than Annexin 750, which due to its large size and slow clearance displayed high background signal, particularly in the kidneys and bladder (Smith et al., 2011a).

In more clinically relevant models, PSS-794 has also been used to image tumour cell death. The innate apoptosis of prostate adenocarcinomas were imaged in rats 24 hours after intravenous administration of PSS-795. The target to background ratio (tumour:opposite shoulder) was 2.2, and the signal intensity was approximately twice that of control fluorophores lacking the Zn²⁺-DPA targeting ligand (Smith et al., 2010). Increased uptake
was also observed following tumour radiation. *Ex vivo* analysis of tumours displayed a two fold increase in PSS-794 uptake following radiation treatment, which was approximately eight fold more than the control compound (Smith et al., 2011b). The optimal window for imaging is around 24 hours.
**Caspase binding agents**

As discussed previously, the intrinsic and extrinsic apoptotic pathways converge on the activation of effector caspases 3, 6 and 7, facilitating reorganization of the cytoskeleton and plasma membrane to form apoptotic bodies, and DNA fragmentation. One approach to apoptotic imaging is detecting activation of this pathway via labelled caspase inhibitors or substrates. Unlike PS probes, caspase reagents are specific for apoptosis, and will not label necrotic cells. Those directed toward end stage effector caspases will also give a more accurate assessment of cell death by highlighting only those cells truly committed to apoptosis.

**Caspase inhibitors**

The first approach to caspase imaging involved radioiodination of the pan caspase inhibitor carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (Z-VAD-FMK). Apoptotic Morris hepatoma cells displayed only modest uptake of the compound highlighting the difficulties of probing intracellular targets in apoptotic cells. Alternative probes that enter cells reversibly were later identified via high-throughput screening. This group of compounds, known as isatin sulfonamides, display remarkable selectivity for effector caspases 3 and 7 over initiator caspases. They bind their target with nanomolar affinity by forming a thiohemiketal with nucleophilic cysteine and histidine residues in the catalytic site (Lee et al., 2000).

A $^{18}$F conjugate known as $^{18}$F-WC-11-89 was first used to assess liver cell death in rats following cyclohexamide treatment. Uptake increased two fold in treated animals as
determined by PET imaging and *ex vivo* analysis (Faa et al., 1994). A next generation compound with improved stability and moderate lipophilicity, $^{18}$F-ICMT-11, was also used to image tumour cell death following chemotherapy. PET imaging revealed a 2 fold increased uptake in 38C13 murine lymphoma xenografts treated with cyclophosphamide (Nguyen et al., 2009). This is comparable to recent data presented at the American Association for Cancer Research Annual Meeting, 2011, in which a 1.5 fold increase was observed in HCT116 colon carcinomas treated with TL32711, a SMAC mimetic (Nguyen, Smith et al. 2011).

In the studies to date the isatin sulfonamides have exhibited high uptake in the liver and intestines, reflecting the areas in which the compound is metabolised and eliminated respectively. Unfortunately this will likely preclude imaging of the abdominal region (Nguyen et al., 2009).

One of the major drawbacks of caspase inhibitors is their cross-reactivity with cathepsins, a family of cysteine proteases constitutively expressed in numerous tissues and organs such as the liver, kidney and spleen. One group have developed a fluorescently labelled ‘activity-based probe’- AB50 that covalently binds activated caspases via an acyloxymethyl ketone (AOMK) functional group. Crucially, AB50 displays no reactivity towards cathepsins. To enhance cell penetration, AB50 was conjugated to a string of positively charged amino acids known as the Tat sequence, and both compounds were assessed *in vivo* in mouse models of cell death. In dexamethasone treated mice, thymic cell death and caspase activation mirrored uptake of AB-50-Cyanine 5 (Cy5) and tAB50-Cy5. Overall uptake of the compounds was significantly higher in the thyri of dexamethasone treated mice relative to the vehicle treated group, however a similar trend was also observed with control compounds lacking the
AOMK active group, indicating a high degree of non-specific uptake (Edgington et al., 2009).

A similar pattern was observed in COLO205 colon carcinomas of mice treated with drozitumab (APOMAB), a chemotherapeutic currently in Phase II clinical trials for lung cancer. The monoclonal antibody induces tumour cell death by binding death receptor-5 and activating the extrinsic apoptotic pathway (Adams et al., 2008; Ashkenazi, 2008). This was largely attributable to high background uptake of the control compounds, however a significant difference was still seen between treated and vehicle treated tumours when imaged with AB-50-Cy5 and tAB50-Cy5. A 3.2 fold and 4.5 fold increased uptake was observed for the two compounds respectively. One potential drawback of the compound is that it maintains cross-reactivity with legumain, another off target cysteine protease. Addition of a Glu-Pro-Asp sequence to AB-50 abolished legumain binding, however poor cell permeability precluded further development of this derivative (Edgington et al., 2009).

**Caspase substrates**

Because labelled caspase inhibitors trap their target, the signal generated is attenuated upon binding site saturation. Caspase substrates conversely have the inherent advantage of cycling through the activated caspase resulting in amplification of the radiotracer and greater signal intensity (Zeng and Miao, 2009). A number of small peptides containing the recognition sites of effector caspases have been developed for this purpose. The preferred cleavage sequence of caspases 3 and 7 is four amino acids with aspartate residues at positions P1 and P4, a hydrophobic amino acid at position P2 and a hydrophilic amino acid residue at P3, otherwise known as DEVD (Ganesan et al., 2006).
Initial work focused on conjugation of the DEVD peptide to the fluorescent 7-amino-4-methylcoumarin via an amide bond. The amide bond reduces electron donation to the aromatic ring quenching the fluorescent signal until cleaved by activated caspases. In order to reduce interference from background auto-fluorescence the peptide was then conjugated to the near infra-red rhodamine 110. It was further optimised by replacing one of the two DEVD peptides with a more lipophilic octyl amide or carboxy morpholine group, so that only a single cleavage event was required for generation of the fluorescent signal.

Another novel approach, TcapQ, flanked the DEVD peptide with a near infra-red Alex Fluor 647 probe and a QSY-21 fluorescent quencher. The proximal orientation of the two leads to signal quenching until the peptide is cleaved by effector caspases and the fluorophore liberated. A Tat sequence was also added to the N terminal end of the peptide to improve cellular penetration (Bullok and Piwnica-Worms, 2005). Peptide cleavage therefore not only results in dequenching of the fluorophore, but also separates it from the cell penetrating moiety trapping it within the intracellular compartment.

Although TcapQ successfully labelled apoptotic KB3-1 and Jurkat cells in vitro it displayed only moderate success in vivo in human colon xenografts subjected to *E. Histolytica* (Bullok et al., 2007; Smith et al., 2009). It has also been applied to a rat model of glaucoma, where retinal ganglion cell (RGC) death was induced by intravitreal injection of N-methyl-D-aspartate (NMDA). Fluorescent microscopy revealed a dose dependent activation of TcapQ in the retinas of NMDA treated rats. TcapQ activation correlated with RGC apoptosis as indicated by terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining, and pre-treatment with the caspase-3 inhibitor DEVD-FMK significantly attenuated
the fluorescent signal. Animals injected with the non-cleavable control compound also displayed little to no fluorescent labelling in the retina (Barnett et al., 2009).

Similar results were observed with the second generation compound KcapQ which has the intrinsic advantage of improved cell permeability, reduced toxicity and greater specificity for effector caspases (Maxwell et al., 2009). The obvious advantage of this approach is that the probe is not fluorescent in its native state, theoretically decreasing the background signal associated with non-specific binding and vascular pooling (Barnett et al., 2009). The disadvantage is that the near infra-red fluorescence signal is unable to penetrate more than a few centimeters of tissue and so the probe is limited to pre-clinical investigations in small animals with superficial lesions. Radio-labelled caspase substrates have been reported, however low relative uptake in apoptotic vs healthy cells in vitro has hindered further development (Bauer et al., 2005).

An aminoluciferin conjugate has also been produced for bioluminescent imaging. Whilst attached to the DEVD peptide the luciferin cannot be oxidized by firefly luciferase. Cleavage of the peptide by activated caspases is required to generate a bioluminescent signal. This strategy has the obvious advantage of no background signal as the light is generated via biochemical reaction rather than irradiation from an external light source (Smith et al., 2009). Due to limited penetration of the bioluminescent signal however it is restricted in vivo to small animal imaging applications.

One criticism of caspase based imaging agents is that caspase activation is not strictly specific to apoptosis (Blankenberg, 2008b). Low level caspase 3 activation is also thought to play a role in more general cellular activities such as platelet aggregation and amylase
secretion from pancreatic acinar cells (Rosado et al., 2006). This could potentially increase general background signal or complicate interpretation of data.
Other

Aposense Compounds

The Aposense compounds are a family of small molecule probes that accumulate in the cytosol of apoptotic cells coincident with PS externalization, loss of mitochondrial membrane potential and caspase activation. Although a great deal of ambiguity surrounds their mechanism of action it is hypothesized that the hydrophobic moiety common to all Aposense probes anchor them to the plasma membrane, whilst the charged moiety inhibits translocation across the membrane of viable cells. The redistribution of phospholipids in the early stages of apoptosis reduces the energetic barrier, allowing passage across the perturbed, though intact membrane (Aloya et al., 2006; Cohen et al., 2007; Reshef et al., 2007).

This family of compounds consists of five individual members, N,N’-didansyl-L-cystine (DDC) (MW = 700), NST-732 (MW = 368), NST-729, ML-9 (MW = 173), and ML-10 (MW = 206), labelled with the fluorescent dansyl group or the radioactive tritium or $^{18}$F. DDC, NST-729 and NST-732 have been used primarily in proof of concept studies in rodent models of renal ischemia and reperfusion, cerebral stroke, closed head injury, Alzheimer’s, and tumour cell death following chemotherapy or radiation (Aloya et al., 2006; Cohen et al., 2007; Damianovich et al., 2005; Reshef et al., 2007; Reshef et al., 2008a; Shirvan et al., 2009). Chemotherapy induced apoptosis in the intestinal mucosa has also been observed (Levin et al., 2009).

In these studies, fluorescent microscopy of the affected tissue sections revealed co-localisation of DDC, NST-729 and NST-732 with apoptotic cells, as indicated by TUNEL
and activated caspase staining. Analysis of total fluorescence in homogenised tissue also revealed a 5.5 fold increased uptake of DDC in tumours treated with cyclophosphamide, and a 12 fold increased uptake of NST-732 in irradiated tumours (Aloya et al., 2006; Cohen et al., 2007). Meanwhile ${^3}$H-DDC displayed a 4.65 fold increased uptake in the infarcted brain and a 2 fold increase in the ischemic kidney, as determined by β counting \textit{ex vivo} (Damianovich et al., 2005; Reshef et al., 2007).

Whilst these studies have been useful as proof of principle of the Aposense probes, their capacity to image apoptosis \textit{in vivo} is limited by the use of a dansyl or tritium reporter group. Conceivably the fluorine of NST-732 could be replaced with $^{18}$F for PET imaging however this is yet to be realised. The most promising member of the family in this sense is ML-10 which was conjugated to $^{18}$F and employed in a mouse model of cerebral stroke. \textit{Ex vivo} analysis of tissues by $\gamma$ counting displayed 0.16 ± 0.04 % ID/g uptake in the damaged cerebral hemisphere, compared to 0.07 ± 0.02 % ID/g in the healthy contralateral hemisphere (a 2.29 fold increase). \textit{Ex vivo} phosphor imaging of tissue sections also showed between 6-10 fold higher signal density in the infarcted area compared to the corresponding region in the contralateral hemisphere.

The compound exhibits favourable biodistribution in rats with a blood half life of 23 min and rapid clearance through the kidneys. Unfortunately the study failed to provide biodistribution data in mice to allow comparison of the infarcted brain with non target tissues. It is thus difficult to assess the potential of $^{18}$F-ML-10 in an \textit{in vivo} capacity, particularly with regard to signal to background ratio. Blood uptake in the rat at the same time point was 0.36% ID/g, raising the question of high systemic background signal. As only one \textit{in vivo} PET image of
the head was published in this study, it is difficult to determine whether or not this was the case (Reshef et al., 2008b).

A study involving the sister compound $^{3}$H-ML-9 may go some way toward answering these questions. Tumour uptake following chemotherapy was determined by $\beta$ counting of tissues ex vivo, revealing an 18 fold increase in tumour uptake following doxorubicin treatment and a 7.7 fold increase in tumour uptake following combination therapy with BiCNH and 5-fluorouracil (5FU). This was also verified by autoradiography and densitometry analysis of tumour sections. Signal to noise ratios were between 3-6.6 for the treated tumour compared to the blood, liver, lung, muscle, intestine and kidney. Uptake in the treated tumours also appeared reasonably specific, as unlabelled ML-9 exhibited a competitive effect, blocking uptake of $^{3}$H-ML-9 by almost 50% (Grimberg et al., 2009). If the biodistribution of $^{3}$H-ML-9 is indicative of $^{18}$F-ML-10, then $^{18}$F-ML-10 could very well represent a promising tool for the in vivo detection of apoptosis.

One of the major criticisms of these studies is the particularly low uptake values in apoptotic tissues, no more than 1.6 % ID/g and 1.20% ID/g in the cerebral stroke and tumour cell death model respectively. Furthermore the doses administered appear to be excessive on both a weight and molar basis compared to those typically observed in small animal imaging experiments. DDC and NST-732 for example were administered at 70 mg/kg, approximately 70 fold more than most other fluorescent probes. This has raised some concern about potential toxicity (Blankenberg, 2008a, b).

Ambiguity surrounding the mechanism of action of these compounds also makes it difficult to determine their suitability to other cell death scenarios in vivo (Edgington et al., 2009).
Nonetheless, $^{18}$F-ML-10 has entered clinical trials and preliminary data is encouraging. Ongoing Phase I trials so far indicate favourable biodistribution and dosimetry. Phase II trials are now recruiting for evaluation of cerebral infarct in patients with ischemic stroke, and response of brain tumour metastases to radiation (Reshef et al., 2010). In the latter study, $^{18}$F-ML-10 uptake was observed 10 days into whole brain radiotherapy and compared with changes in tumour size at 6-8 weeks (as determined by MRI). In the 7 patients that have completed the study to date a significant correlation has been observed (Allen et al., 2009).

One of the major advantages of the Aposense compounds is their cytosolic accumulation. The accumulation of PS directed agents to apoptotic cells is limited by both the surface area of the plasma membrane and the abundance of externalised of PS. The cytosolic volume of the cell is substantially greater, and agents that accumulate in this compartment should exhibit greater signal amplification and thus superior detection and resolution of cell death. Indeed each apoptotic cell is thought to harbour $1.5 \times 10^8$ molecules of NST-732, two orders of magnitude more than the cells capacity for Annexin V (Aloya et al., 2006).

**APOMAB**

APOMAB is a monoclonal antibody that specifically recognises the La ribonucleoprotein. La is a chaperone protein that facilitates many facets of RNA synthesis and is upregulated in malignant cells. In response to DNA damaging agents such as radiation and cytotoxic drugs, La expression increases and it is translocated from the nucleus to the cytoplasm of the dying cell. In the late stages of apoptosis loss of plasma membrane integrity facilitates entry of APOMAB where it binds the cytosolic target. The probe is then retained in the dying cell through the actions of type 2 transglutaminase (TG2). TG2 is induced early in apoptosis and
facilitates the polymerisation of various cytoskeletal components to form apoptotic bodies. It also crosslinks the La antigen such that APOMAB is fixed within the dying cell (Al-Ejeh et al., 2007a; Al-Ejeh et al., 2009).

Radio-labelled APOMAB has been used to image tumour cell death in vivo. Mice bearing subcutaneous EL4 lymphomas were treated with cyclophosphamide and etoposide and administered the $^{111}$Indium labelled 3B9 probe. $^{111}$In-3B9 accumulation in treated tumours peaked at approximately 50% of the injected dose per gram of tumour tissue, more than two fold more than that of untreated mice. Uptake also correlated with tumour cell apoptosis as determined by ex vivo 7-AAD staining. Comparatively low uptake was observed in normal organs (Al-Ejeh et al., 2007b). A similar pattern was observed with an $^{111}$Indium labelled DAB4 clone. Scintigraphic imaging showed that $^{111}$In-DAB4 accumulated in chemotherapy treated tumours in a linear dose-dependent manner. $^{111}$In-DAB4 uptake was proportional to tumour cell apoptosis as determined by ex vivo staining for poly ADP-ribose-1 (PARP-1) cleavage. $^{111}$In-DAB4 accumulation also correlated with an extension in median survival time (Al-Ejeh et al., 2009).

Large molecules such as antibodies are more likely to exhibit a long circulatory half life and slow clearance resulting in high background signals. In an effort to improve signal to background ratios an $^{111}$Indium labelled (Fab)$_2$ fragment of the full IgG antibody was also investigated. Accumulation of radio-labelled 3B9 and DAB4 (Fab)$_2$ fragments were antigen specific and chemotherapy dose dependent. They were also cleared significantly faster than their full IgG counterparts enhancing the signal to noise ratio, however total tumour uptake was reduced. Thus the prolonged residency of large antibodies in the blood facilitates greater
tumour uptake but also reduces tumour to background ratios (Al-Ejeh et al., 2007b; Al-Ejeh et al., 2009).

One of the primary advantages of the APOMAB technology is that it is specific for apoptotic tumour cells. Thus dying cells in chemo-sensitive tissues such as the gut and bone marrow do not complicate the image. Also because the agent is specific for cell death induced by DNA damaging agents, only treatment induced apoptosis will be detected (Al-Ejeh et al., 2009).

**GSAO**

4-(N-(S-glutathionylacetyl) amino)phenylarsenoxide (GSAO) is a small synthetic compound composed of phenylarsine oxide fused to glutathione (GSH) via an N-acetyl linker. The trivalent arsenic moiety of GSAO mediates interaction with closely spaced thiols, whereby both sulphur atoms are complexed to arsenic in a stable dithioarsenite ring. The synthesis and initial characterisation of the compound was conducted in the Hogg laboratory and has been described previously (Donoghue et al., 2000).

GSAO can be conjugated to various reporter molecules including fluorophores and radioisotopes through the primary amine of the γ-glutamyl residue (Figure 1). In this form it selectively labels dead and dying cells. Given the prevalence of cell death in normal physiology and disease, such an agent is likely to have many applications in biological research and patient diagnosis and management. In order for GSAO to progress to clinical trials however, further pre-clinical validation is required. Specifically the mechanism behind its accumulation in dying cells needs to be elucidated. Similarly its application to *in vivo* models of cell death must also be demonstrated.
This thesis therefore has three central aims, to investigate the means through which GSAO-conjugates enter the dying cell, to determine the mechanism through which they are retained and to demonstrate their application to an *in vivo* setting.

Figure 1. The structure of GSAO. The different reporter groups are linked through the amine of the γ-glutamyl residue.
Chapter 2.

Tagged GSAO accumulates in the mid-late stages of apoptosis

**Introduction**

Apoptosis is characterised by a multi-step cascade of morphological and biochemical changes, including phosphatidylserine externalisation, loss of mitochondrial membrane potential, caspase activation, DNA fragmentation and loss of plasma membrane integrity (Taylor et al., 2008). The timeline of these events is well established, and the apoptotic program can be divided into early, mid and late stages according to these specific hallmarks. A number of fluorescent probes have been developed to detect these processes *in vitro*, allowing the analysis of large populations of cells by flow cytometry. This methodology also allows the differentiation of specific sub-populations of early apoptotic, late apoptotic, necrotic and healthy cells (Darzynkiewicz et al., 1997; Krysko et al., 2008).

The most well known molecular probes for this purpose are propidium iodide (PI) and fluorophore conjugates of Annexin V. As discussed previously, Annexin V is a 36 kDa protein that detects PS externalisation in the plasma membrane. The re-distribution of phospholipids in the apoptotic membrane typically occurs 1-2 hours after the initiation of apoptosis, coincident with caspase activation and mitochondrial depolarisation. Fluorophore conjugates of Annexin V are therefore effective markers of early stage apoptosis. As plasma membrane rupture during necrosis also exposes PS residues, Annexin V also detects necrotic...
cell death (Al-Rubeai and Fussenegger, 2004; Darzynkiewicz et al., 1997; Krysko et al., 2008).

PI is a charged cationic dye that is impermeable to healthy cells. During the late stages of apoptosis the plasma membrane integrity is compromised allowing PI to enter the cell and intercalate with DNA. PI can also be used to detect necrosis and secondary necrosis as plasma membrane rupture also permits PI entry (Al-Rubeai and Fussenegger, 2004; Krysko et al., 2008).

The combination of these probes therefore provides a timeline for the progression of apoptosis, whilst also differentiating between different types of cell death. Healthy cells for instance are negative for both PI and Annexin V (Figure 2a). During the early stages of apoptosis the plasma membrane retains the capacity to exclude PI, however PS externalisation enables labelling by Annexin V. The early apoptotic population is thus Annexin V positive but PI negative (Figure 2b). During the late stages of apoptosis, or, as the cell transitions to secondary necrosis, the plasma membrane integrity is compromised facilitating PI entry. This population of cells is positive for both Annexin V and PI (Figure 2c) (Al-Rubeai and Fussenegger, 2004; Darzynkiewicz et al., 1997; Krysko et al., 2008).
Figure 2. The differentiation of healthy, early apoptotic and late apoptotic/secondary necrotic cells by flow cytometry using Annexin V-Allophycocyanin (APC) and PI. Representative scatter plots of cells in various stages of cell death. a) Healthy cells are negative for both PI and Annexin V-APC and sit in the bottom left quadrant. b) Early apoptotic cells are positive for Annexin V-APC, but continue to exclude PI and sit in the bottom right quadrant. c) Late apoptotic and secondary necrotic cells are positive for both Annexin V-APC and PI and sit in the top right quadrant.

The terminal stages of cell death in necrosis, late apoptosis and secondary necrosis are characterised by rupture of the plasma membrane, giving rise to dual Annexin V and PI staining. A single end-point assessment is therefore incapable of discriminating between the different cell death pathways. This can only be achieved by progressive time course analysis. During apoptosis PS externalization occurs early in cell death program, separate from plasma membrane disruption. There is consequently a considerable lag between Annexin V and PI labelling. During necrosis however, PS exposure coincides with permeabilisation of the plasma membrane. Annexin V and PI labelling therefore occur simultaneously, and at no
point during the time course is an intermediate Annexin V only population observed (Kryska et al., 2008).

The primary aim of Chapter 2 was to characterise the accumulation of GSAO in \textit{in vitro} models of cell death, and determine those cellular changes responsible for its entry. To this end, GSAO was conjugated to Oregon Green (OG) and its specificity for dying cells observed via flow cytometry. To demonstrate the independence of the outcome on the reporting group, GSAO was also conjugated to Fluorescein (F) and Cy5.5.

GSAO-F was then used to determine the kinetic parameters of labelling. Specifically the time and concentration dependence of GSAO-F accumulation were investigated. The uptake of GSAO-conjugates was also observed in the context of landmark apoptotic events such as phosphatidylserine externalisation and loss of plasma membrane integrity, indicated by Annexin V-APC and PI respectively. This enabled me to determine at which point in the apoptotic cascade GSAO-conjugates labelled dying cells. For these experiments fluorescently tagged 4-(N-(S-glutathionylacetyl)amino)benzoic acid (GSCA) was used as a control compound. GSCA contains a chemically inert carboxylic acid group in place of the chemically reactive trivalent arsenic in GSAO.

As discussed in Chapter 1, the end stage activation of caspases during apoptosis can occur through two distinct mechanisms, the intrinsic or ‘mitochondrial mediated’ pathway, and the extrinsic or ‘death receptor mediated’ pathway. In the extrinsic pathway, death receptors on the plasma membrane such as TNFR1 and Fas/CD95 are bound by ligands, and caspase activation is mediated by formation of the death inducing signalling complex (Danial and Korsmeyer, 2004). The intrinsic apoptotic pathway conversely centers upon permeabilisation
of the mitochondrial membrane and release of cytochrome c. Cytochrome c forms a multi-
protein complex called the ‘apoptosome’ and this in turn activates the executioner caspases
(Kroemer et al., 2007).

To investigate whether GSAO accumulation was specific to one of these apoptotic pathways,
accumulation of fluorescently labelled GSAO or GSCA was observed in Jurkat cells treated
with different apoptotic inducers. In the first instance, Jurkat cells were treated with the
microbial alkaloid staurosporine, a broad spectrum protein kinase inhibitor. Staurosporine
occupies the ATP binding sites of protein kinases and induces the intrinsic apoptotic pathway
(Scarlett et al., 2000; Tafani et al., 2001; Tamaoki et al., 1986). A monoclonal antibody that
binds the Fas/CD95 death receptor on Jurkat cells was used to trigger the extrinsic apoptotic
pathway. To demonstrate the application of GSAO to clinically relevant models of tumour
cell death, the chemotherapeutic doxorubicin was also employed. Doxorubicin triggers G2/M
growth arrest and apoptotic cell death and is commonly used in the treatment of a number of
solid tumours, including soft-tissue sarcomas, aggressive lymphomas, and tumours of the
breast, oesophagus, and liver (Gewirtz, 1999; Minotti et al., 2004). GSAO accumulation was
also investigated in models of necrosis by subjecting cells to repeated freeze-thaw cycles
(Reshef et al., 2008b).
Materials and Methods

The following chemicals were purchased and used without further purification:

\(p\)-aminobenzoic acid, bromoacetyl bromide (Acros Organics), \(p\)-arsanilic acid, dimethylformamide, GSH, dimethyl sulfoxide (DMSO) (Sigma-Aldrich), L-ascorbid acid, potassium hydroxide, sodium bicarbonate, sodium carbonate (Mallinckrodt), acetonitrile, dichloromethane, 37% hydrochloric acid, iodine, methanol, 98% sulphuric acid (Fisher).

Unless otherwise specified, all other chemicals were purchased from Sigma-Aldrich.

The synthesis of GSAO and 4-((S-glutathionyl)acetyl)amino)benzenearsonic acid (GSAA)

The synthesis of 4-(N-(bromoacetyl)amino)phenylarsonic acid (BRAA)

Potassium hydroxide (5.1 g, 91.8 mmol) was dissolved in 125 mL deionized water (H\(_2\)O).

\(P\)-arsanilic acid (10.0 g, 46.3 mmol) and sodium carbonate (14.5 g, 137.1 mmol) were added slowly to the solution whilst stirring. The warm solution was cooled in ice-water bath and bromoacetyl bromide (6 mL, 13.9 g, 68.9 mmol) in 25 mL dichloromethane was added dropwise into the cooled solution. The reaction mixture was stirred for 15 min and the ice-water bath removed. The cloudy reaction mixture was transferred to a separatory funnel and the organic layer separated by draining off. The aqueous solution was acidified with 98% sulphuric acid to pH 1 with precipitation of product. It was filtered by suction, dried in air and then held under vacuum overnight to give BRAA (11.2 g, 33.0 mmol, 71.4%).

\(^1\)H NMR (300 MHz, DMSO-\(d_6\)) \(\delta\) (ppm): 4.08 (s, 2H), 5.78 (br s, 2H), 7.71 (d, 2H), 7.79 (d, 2H), 10.74 (s, 1H).
The synthesis of 4-\((N\)-(bromoacetyl)amino)phenylarsonous acid (BRAO)

BRAA (10.0 g, 29.6 mmol) and L-ascorbic acid (16.1 g, 91.4 mmol) were stirred in 100 mL de-aerated methanol under argon. Iodine (2.5 g, 9.7 mmol) was added and the solution stirred under argon at room temperature. The reaction mixture turned clear after 30 min. Stirring was continued for 2 hours, and the solution filtered by suction to remove the insoluble material. The filtrate was evaporated under reduced pressure to approximately 50 mL. 50 mL of 1M hydrochloric acid was added to the filtrate whilst stirring to precipitate the product. Stirring was continued for a further 20 min. The solid was filtered by suction, rinsed with 30 mL methanol:water: (1:2), and dried under vacuum overnight to give BRAO (10.2 g, quantitative).

\(^1\)H NMR (300 MHz, DMSO-\(d_6\)) \(\delta\)(ppm): 3.85 (s, 2H), 4.06 (s, 2H), 7.66 (s, 4H), 10.45 (s, 1H).

\(^{13}\)C NMR (75.4 MHz, DMSO-\(d_6\)) \(\delta\)(ppm): 30.2, 118.5, 118.7, 130.3, 140.4, 166.7.

Figure 3. Overview of the synthesis of BRAA and BRAO from \(p\)-Arsanilic acid. Potassium hydroxide (KOH). Sodium carbonate (Na\(_2\)CO\(_3\)). Water (H\(_2\)O). Sulphuric acid (H\(_2\)SO\(_4\)). Methanol (CH\(_3\)OH). Iodine (I\(_2\)).
The synthesis of GSAA

BRAA (1.0 g, 3.0 mmol) was dissolved in 10 mL dimethylformamide. A solution of GSH (1.1 g, 3.6 mmol) in 10 mL of 1M sodium bicarbonate was added to the BRAA in dimethylformamide, and the reaction mixture stirred at room temperature overnight. The reaction mixture was acidified with 20 mL of 1M hydrogen chloride and evaporated to dryness. The solid residue was dissolved in hydrogen chloride (aq) to pH 1 (15 mL). The crude product solution was purified by C\textsubscript{18} flash chromatography (70 g column) with water-acetonitrile gradient as eluant (40 mL/min flow rate monitoring at 257 nm) to yield product (1.1 g, 2.0 mmol, 67.2%) upon lyophilization.

\textsuperscript{1}H NMR (300 MHz, D\textsubscript{2}O) δ(ppm): 1.93 (q, 2H), 2.32 (t, 2H), 2.80 (m, 1H), 2.98 (m, 1H), 3.30 (m, 2H), 3.66 (t, 1H), 3.78 (s, 2H), 4.44 (m, 1H), 7.48 (d, 2H), 7.56 (d, 2H).

\textsuperscript{13}C NMR (75.4 MHz, D\textsubscript{2}O) δ(ppm): 25.8, 31.1, 33.6, 36.3, 41.3, 52.8, 53.4, 121.0, 125.4, 131.1, 142.1, 170.7, 172.3, 173.0, 173.2, 174.4.
Figure 4. Overview of the synthesis of GSAA from BRAA. Glutathione (GSH). Sodium bicarbonate (NaHCO₃). Water (H₂O). Dimethylformamide (DMF).

The synthesis of GSAO

BRAO (1.0 g, 3.1 mmol) was dissolved in 10 mL dimethylformamide by warming to 50°C under argon. After the solution cooled to room temperature, it was cooled in an ice-water bath. A solution of GSH (1.0 g, 3.3 mmol) in 15 mL of 0.5M sodium bicarbonate (prepared with de-aerated deionized water) was added to the BRAO, the ice-water bath removed and the reaction mixture stirred at room temperature overnight. The reaction mixture was acidified to pH 2 with 37% hydrogen chloride and evaporated to dryness. The solid residue was dissolved in hydrogen chloride (aq) to pH 1 (12 mL). The crude product solution was purified by C₁₈ flash chromatography (25 g column) in three portions with water-acetonitrile gradient as eluant (40 mL/min flow rate monitoring at 257 nm) to yield product (0.92 g, 1.7 mmol, 53.3%) upon lyophilization.

¹H NMR (300 MHz, D₂O) δ(ppm): 1.92 (q, 2H), 2.33 (t, 2H), 2.83 (m, 1H), 3.02 (m, 1H), 3.33 (m, 2H), 3.60 (t, 1H), 3.79 (s, 2H), 4.45 (m, 1H), 7.41 (d, 2H), 7.56 (d, 2H).

¹³C NMR (75.4 MHz, D₂O) δ(ppm): 26.2, 31.4, 33.8, 36.4, 41.7, 53.2, 53.9, 121.6, 130.2, 139.0, 144.3, 171.1, 172.5, 173.4, 173.5, 174.7.
Figure 5. Overview of the synthesis of GSAO from BRAO, Glutathione (GSH). Sodium bicarbonate (NaHCO₃). Water (H₂O). Dimethylformamide (DMF).

**The synthesis of 4-((N-(bromoacetyl)amino)benzoic acid (BRCA)**

Sodium carbonate (70.1 g, 661.7 mmol) was dissolved in 300 mL deionized water, and p-aminobenzoic acid (30.5 g, 222.3 mmol) was added slowly to the solution while stirring. 400 mL water was added and the solution cooled in an ice-water bath until it reached 3°C. Bromoacetyl bromide (27 mL, 62.6 g, 309.9 mmol) was dripped quickly into the cooled solution and rinsed with 10 mL acetonitrile. The reaction mixture was stirred for 10 min and the ice-water bath removed. After a further 10 min of stirring, 98% sulphuric acid was carefully added until the suspension was pH 1. The white product was filtered by suction and air dried overnight. The solid was resuspended in 250 mL water and acidified with 98% sulphuric acid to pH 1. It was then filtered, dried in air held under vacuum overnight to give BRCA (39.6 g, 153.6 mmol, 69.1%).

¹H NMR (300 MHz, DMSO-d₆) δ(ppm): 4.05 (s, 2H), 6.59 (d, 1H), 7.68 (d, 2H), 7.88 (d, 2H), 10.64 (s, 1H).

¹³C NMR (75.4 MHz, DMSO-d₆) δ(ppm): 30.3, 118.4, 130.3, 130.9, 142.4, 165.1, 166.6.
The synthesis of GSCA

BRCA (1.2 g, 4.5 mmol) was dissolved in 10 mL dimethylformamide. A solution of GSH (1.5 g, 4.93 mmol) in 25 mL of 0.5 M sodium bicarbonate was added to BRCA and the reaction mixture stirred at room temperature overnight. The reaction mixture was evaporated and the residue dissolved in 50 mL water. The resulting solution was acidified with 37% hydrogen chloride to pH 0.7 and filtered by suction to remove impurities. The filtrate was adjusted to pH 2.8 with 50% (w/w) sodium hydroxide solution. After standing overnight, the product was filtered by suction, dried in air and then held under vacuum overnight to give GSCA (1.2 g, 2.56 mmol, 56.7%).

$^1$H NMR (300 MHz, DMSO-$d_6$) δ(ppm): 1.92 (m, 2H), 2.35 (m, 2H), 2.79 (m, 1H), 3.05 (m, 1H), 3.42 (s, 2H), 3.42 (m, 1H), 3.71 (d, 2H), 4.52 (m, 1H), 7.72 (d, 2H), 7.86 (d, 2H), 8.55 (m, 2H), 10.66 (s, 1H).

$^{13}$C NMR (75.4 MHz, DMSO-$d_6$) δ(ppm): 26.8, 31.6, 34.0, 36.0, 41.2, 52.4, 53.0, 118.3, 125.2, 130.1, 142.9, 166.7, 168.2, 170.2, 170.3, 170.7, 171.7.
Figure 6. Overview of the synthesis of BRCA and GSCA from \( p \)-Aminobenzoic acid.

Sodium carbonate (\( \text{Na}_2\text{CO}_3 \)). Sulphuric acid (\( \text{H}_2\text{SO}_4 \)). Water (\( \text{H}_2\text{O} \)). Glutathione (GSH).

Sodium bicarbonate (\( \text{NaHCO}_3 \)). Dimethylformamide (DMF).

**The conjugation of GSAO and GSCA to Fluorescein and Oregon Green**

A solution of Fluorescein-5-EX succinimidyl ester, Oregon Green 488-X succimidyl ester (Molecular Probes, Invitrogen) (10 mg per mL in DMSO) was added to GSAO or GSCA (10 mg per mL in 0.1 M bicarbonate buffer, pH 8.3) and incubated for 60 min in the dark in the presence of nitrogen or argon. The molar ratio of pendant to fluorophore was \(~11:1\). The conjugate was separated from unreacted fluorophore by passing the reaction through eight Sephadex G10 spin columns (Sigma-Aldrich) equilibrated with 0.1 M bicarbonate buffer, pH 8.3. The Sephadex matrix has a fractionation range of \(<700\), so that compounds with a higher molecular weight are excluded from the pore volume. Unreacted GSAO, GSCA, Fluorescein and Oregon Green with molecular weights of 548, 484, 590.56 and 509.38 respectively are thus maintained in the matrix, whilst the conjugate elutes in the void volume.
Purity of the conjugates was confirmed by high performance liquid chromatography (HPLC) (1200 Series; Agilent Technologies) on a Zorbax Eclipse XDB-C18 column (4.6 x 150 mm, 5 µm; Agilent Technologies) gradient mobile phase of acetonitrile-water (from 5:95 to 95:5 vol/vol over 10 min, followed by a 7 min equilibration), flow rate of 0.3 mL per min and detection by absorbance at 256 nm. Purity was also assessed by thin layer chromatography (TLC). Aliquots of GSAO-conjugates were collected after each column separation and pipetted onto silica gel-60A TLC plates (Fluka) alongside unconjugated fluorophore. TLC plates were resolved in butanol/acetic acid/water (3:1:1) and covered until samples had separated. Samples were visualised using the LAS4000 (Fujifilm) imaging system.

The concentration of the active arsenical of fluorescent GSAO-conjugates was measured by titrating with dimercaptopropanol and calculating the remaining free thiols with 5,5’-dithiobis(2-nitrobenzoic acid) (Don et al., 2003). The concentration of GSAO and GSCA fluorescent conjugates was calculated by measuring the fluorescence on a Cary 300 UV-Vis spectrophotometer (Varian) employing Beer’s law within the range 200 nm to 600 nm.

The characterization of GSAO and GSCA fluorescent conjugates in in vitro models of cell death

Jurkat A3 cells (ATCC) were cultured in Roswell Park Memorial Institute medium (RPMI) medium supplemented with 10% foetal bovine serum, 2 mM L-glutamine, and 1 ug per mL penicillin/streptomycin. Cell culture plasticware was from Techno Plastic Products (Trasadingen). All other cell culture reagents were from Gibco.
Jurkat A3 cells were seeded at a density of $5 \times 10^5$ cells per mL and incubated without or with 0.1 µg per mL anti-human Fas (CD95) antibody (Medical and Biological Laboratories), 4 µM staurosporine (Sigma-Aldrich) or 20 µM doxorubicin (Pfizer) for discrete times up to 24 h. In the in vitro model of necrosis, cells were subjected to three freeze-thaw cycles in liquid nitrogen (Reshef et al., 2008b). Cells were washed twice with ice cold phosphate-buffered saline (PBS), resuspended in ice cold 0.1 M Hepes, pH 7.4 buffer containing 0.14 M NaCl and 25 mM CaCl$_2$ and incubated at room temperature with 1 µM of the GSAO-fluorophore conjugates for 15 min with shaking. Cells were washed twice with ice cold PBS and then incubated with 5 µL per 100 µL of Annexin V-APC (BD Pharmingen) and either 1 µg per mL PI (Molecular Probes, Invitrogen) or 1 µM Sytox Blue Dead Cell Stain (Molecular Probes, Invitrogen) for 15 min in the dark. Flow cytometry was performed using a BD™ FACS Canto II Flow Cytometer (BD Biosciences) and data analysed using FlowJo software version 8.7.

$10^4$ events were collected and cellular debris was excluded from the analysis by gating out events with a FSC and SSC < 50. Unstained and single stained controls were used to establish compensation matrices and to correct spectral overlap between OG/FITC, PI, Sytox Blue, and APC/Cy5.5. Positive events were determined by gating around unstained and untreated populations of cells.

**The time and concentration dependent uptake of GSAO-F in dying cells**

Human fibrosarcoma HT1080 cells (ATCC) were cultured in Dulbecco's Modified Eagle Medium (DMEM) medium supplemented with 10% foetal bovine serum, 2 mM L-glutamine,
and 1 ug per mL penicillin/streptomycin. Cells were treated for 20 h with 1 μg per mL camptothecin (Calbiochem), then detached with PBS containing 2.5 mM ethylenediaminetetraacetic acid (EDTA) and combined with cells that had detached during incubation. To determine the time dependence of GSAO-F uptake, cells were incubated with 1 μM of GSAO-F for discrete times up to 45 min, washed twice with ice cold PBS, stained with 1 μg per mL PI and analysed by flow cytometry. The mean fluorescence of the apoptotic cells at each time point was determined by gating the 50% of cells with the highest fluorescein fluorescence, and determining the mean fluorescence of the gated cells (50% was chosen based on the observation that 60% of cells in the camptothecin-treated population were PI and GSAO-F positive after a 15 min incubation with these dyes). To determine the concentration dependence of GSAO-F uptake, cells were incubated for 15 min with varying concentrations of GSAO-F and the mean fluorescence determined as described above.
Results

Conjugation of GSAO to fluorophores

In order to characterise the accumulation of GSAO in *in vitro* models of cell death, it was conjugated to Fluorescein, Oregon Green and Cy5.5. GSAO was incubated with an 11 fold molar excess of the fluorophore, and the conjugate separated from unreacted fluorophore by size exclusion column chromatography. In this technique, separation is based on differences in the size and shape of the analyte particles, which governs their access to the pore volume of the Sephadex matrix. The exclusion limit of the G10 Sephadex matrix is 700 so that the conjugate is excluded from entering the pores and elutes in the void volume, whilst unreacted fluorophore and GSAO are retained in the matrix. Consecutive column elutions yield an increasingly pure sample of the conjugate.

TLC analysis is an effective means of visualising this process. This technique separates analytes according to the rate at which they ascend a thin layer of adsorbent material such as silica gel, known as the ‘stationary phase’. Samples are applied to the sheets of silica and a solvent or ‘mobile phase’ is drawn up the plate via capillary action. Separation of the sample is based on the varying affinity of analytes for the stationary phase, and their solubility in the mobile phase. For example, if the mobile phase is less polar than the stationary phase, polar analytes will have a greater affinity for the stationary phase and ascend the plate slower than more polar constituents. Conjugation of GSAO to the fluorophore changes the properties of the compound and its affinity to the stationary phase. Consequently, it ascends the plate slower than free fluorophore. TLC thus allows visualisation of the relative quantities of conjugated and unconjugated fluorophore in the sample (Figure 7).
Figure 7. TLC analysis of GSAO-OG formation. Excess GSAO and fluorophore was removed from initial preparations of GSAO-OG by size exclusion column chromatography. Aliquots collected from each column elution (columns 2-5) were run alongside pure fluorophore (column 1) to show the relative concentration of unconjugated fluorophore in the solution. A sequential decrease in free fluorophore can be seen following consecutive column elution, finally yielding a pure GSAO-OG sample (column 6). The formation of Fluorescein and Cy5.5 conjugates were also verified by TLC analysis (results not shown).
Figure 8. HPLC analysis of GSAO, GSCA, GSAO-OG and GSCA-OG. To confirm successful conjugation of Oregon Green to GSAO and GSCA, GSAO-OG and GSCA-OG (second and fourth panel respectively) were compared to a sample containing GSAO or GSCA only (first and third panel respectively) using HPLC. Addition of the fluorophore to GSAO and GSCA changes the time it elutes from the column, resulting in an additional peak at 11.87 min and 12.23 min respectively. The formation of Fluorescein and Cy5.5 conjugates were also verified by HPLC analysis (results not shown).
In vitro characterisation of GSAO-conjugates

The specificity of GSAO-conjugates for dying cells was characterised in vitro using flow cytometry. Apoptosis was induced in Jurkat A3 cells using an antibody against the CD95/Fas receptor. Cells were then incubated with Annexin V-APC, PI and either GSAO-OG or the control compound GSCA-OG. Following treatment with Fas Ab, a majority of cells entered apoptosis as indicated by dual Annexin V-APC and PI staining. GSAO-OG but not GSCA-OG labelled these cells (Figure 9).

The mean Oregon Green fluorescence of cells treated with Fas Ab was 927 (±123), approximately 9 fold higher than untreated cells at 102 (±42). 56% (±2%) of treated cells were positive for GSAO-OG as opposed to only 14% (±6%) of untreated cells. The small proportion of cells positive for GSAO-OG in the untreated condition were also positive for Annexin V-APC and PI, indicating normal cellular turnover in culture. GSCA-OG fluorescence was negligible in both the untreated and treated conditions, despite the fact that the same apoptotic profile was observed. GSAO-OG, but not GSCA-OG therefore selectively labels dying cells in vitro.
Figure 9. GSAO-OG but not GSCA-OG selectively labels dying cells. Jurkat A3 cells were untreated or treated with Fas antibody (Ab) for 24 h to induce apoptosis, incubated with the control compound GSCA-OG (lower panels) or GSAO-OG (upper panels), Annexin V-APC, and PI (all panels), and analysed by flow cytometry. After 24 hours a majority of cells had entered apoptosis as indicated by dual Annexin V-APC and PI staining. GSAO-OG but not GSCA-OG labelled these cells. The numbers on the histograms or scatter plots are the percentage of cells in the gate or quadrant, respectively.
To demonstrate the independence of the outcome on the reporter group, GSAO and GSCA were also conjugated to Fluorescein and Cy5.5. Jurkat cells were treated with an apoptotic inducer and the labelling of apoptotic cells with GSAO-F and GSAO-Cy5.5 compared to GSAO-OG. GSCA-F and GSCA-OG were included to control for non-specific binding. As the emission spectra of PI displays considerable overlap with that of Cy5.5, Sytox Green was used in place of PI wherever Cy5.5 conjugates were employed. Like PI, Sytox Green is a nucleic acid stain that is impermeable to the cell unless the plasma membrane is compromised. The two probes were used to ensure that the proportion of apoptotic cells did not vary between samples, and thus any difference in labelling could be attributed to the GSAO-conjugate alone.

GSAO-Cy5.5 and GSAO-F labelled the same proportion of cells as GSAO-OG in both treated and untreated samples. Labelling was proportional to the number of PI and Sytox Green stained cells in both conditions (Figures 10 and 11). Like GSCA-OG, GSCA-Cy5.5 and GSCA-F did not label dying cells. The conjugates were therefore used interchangeably in the following experiments.
Figure 10. GSAO-conjugates label dying cells independent of the reporter group. Jurkat A3 cells were treated or untreated with staurosporine for 24 h and incubated with PI and either GSAO-F or GSAO-OG. Cells were then analyzed by flow cytometry. Fluorescein (F) and Oregon Green (OG) conjugates of GSAO labelled the same proportion of cells in both treated and untreated samples (green). Labelling was proportional to the number of PI positive cells (red) in both conditions. Results are the mean and standard deviation of duplicate samples from two independent experiments.
Figure 11. GSAO-conjugates label dying cells independent of the reporter group. Jurkat A3 cells were treated or untreated with Fas antibody (Ab) for 24 h and incubated with either GSAO-Cy5.5 and Sytox Green, or GSAO-OG and PI. Cells were analyzed by flow cytometry. a) Cyanine 5.5 (Cy5.5) and Oregon Green (OG) conjugates of GSAO labelled the same number of cells in both untreated and treated samples. Labelling was proportional to the number of PI/Sytox Green positive cells (red) in both conditions. Results are the mean and standard deviation of duplicate samples.

The kinetic parameters of labelling

To determine the kinetic parameters of labelling, the influence of time and concentration on GSAO-F uptake were then investigated. Camptothecin treated human fibrosarcoma HT1080 were incubated with GSAO-F and PI and analysed by flow cytometry (Figure 12 a). The mean fluorescence of the apoptotic cells was used to quantitate GSAO-F uptake. Uptake of GSAO-F was consistent with rate-limited binding to one or more targets. A half-maximal uptake time of 1.6 minutes was determined by fitting the data to a one phase exponential
association curve (Figure 12b). Extrapolating the data from the non apoptotic cell population, an equivalent fluorescence value would have been reached after 1500 minutes, indicating that the rate of GSAO-F uptake was approximately 3 orders of magnitude higher in the apoptotic population.

The influence of GSAO-F concentration on uptake into apoptotic cells over 10 minutes was determined (Figure 12c). The data were fitted to a one site binding hyperbola, and half-maximal uptake of GSAO-F was estimated to occur at 3.4 μM. This value is within one order of magnitude of previously determined dissociation constants, for the binding of GSAO to synthetic and protein dithiols (Donoghue et al., 2000). GSAO-F uptake appeared to saturate at around 10 μM.
Figure 12. Time and concentration dependent uptake of GSAO-F into apoptotic cells. (a) HT1080 fibrosarcoma cells were treated with camptothecin for 20 h, incubated with PI GSAO-F and analysed by flow cytometry. The numbers on scatter plots are the percentage of cells in the quadrant. b) HT1080 fibrosarcoma cells were treated with camptothecin for 20 h and incubated with 1 μM GSAO-F for discrete times up to 45 min. The mean fluorescence of the GSAO-F positive populations is shown. (c) HT1080 fibrosarcoma cells were treated with camptothecin for 20 h and incubated with varying concentrations of GSAO-F for 15 minutes. The mean fluorescence of the GSAO-F positive populations is shown. Results are the mean and range of two independent measurements.
GSAO-conjugates enter the cell during the late stages of apoptosis

To determine the point at which GSAO accumulated in apoptotic cascade, GSAO-OG uptake was observed with respect to PS externalisation and plasma membrane disruption, as indicated by Annexin V-APC and PI respectively. Jurkat cells were treated with Fas Ab for discrete periods up to 24 hours. Cells were then incubated with Annexin V-APC, PI and either the control compound GSCA-OG or GSAO-OG. The proportion of positively labelled cells at each time point was determined by flow cytometry.

A majority of cells were positive for Annexin V-APC as early as 4 hours into treatment, indicating the induction of early apoptosis. GSAO-OG displayed minimal uptake at this time point. At 8, 16 and 24 hours whilst approximately 90% of cells were positive for Annexin V-APC, only 40-50% were positive for GSAO-OG. This indicates that GSAO-OG accumulation does not coincide with PS exposure, and thus early stage apoptosis. The induction of late stage apoptosis or secondary necrosis was observed 8 hours into treatment, with the emergence of a PI positive population of cells. GSAO-OG uptake mirrored PI staining at 4, 8, 16 and 24 hours. GSAO-OG entry therefore occurs during the late stages of cell death coincident with a loss of plasma membrane integrity. There was no labelling of cells with the control compound GSCA-OG at any time point (Figure 13).
Figure 13. GSAO accumulates during the late stages of apoptosis following loss of plasma membrane integrity. Jurkat A3 cells were incubated in the presence or absence of Fas Ab for discrete times up 24 h. The cells were then incubated with Annexin V-APC, PI and either the GSCA-OG control or GSAO-OG. The cells were then analysed by flow cytometry. The proportion of positively labelled cells at each time point is shown. GSAO-OG (green) accumulates at the same time as PI (red), following loss of plasma membrane integrity. The control compound GSCA-OG did not label any cells. Results are the mean and standard deviation of samples in duplicate from two independent experiments.

The uptake of GSAO-conjugates is independent of the mechanism of cell death

Fas antibody binds the CD95 receptor on Jurkat cells and activates the extrinsic apoptotic pathway. To investigate whether the accumulation of GSAO-conjugates was dependent on the cell death pathway activated, the same assay was performed with different apoptotic inducers. The broad spectrum protein kinase inhibitor staurosporine was used to induce the intrinsic apoptotic pathway. The chemotherapeutic doxorubicin was also employed to mimic
a clinically relevant model of tumour cell death. The agent triggers G2/M growth arrest and apoptotic cell death. Doxorubicin fluoresces with an emission spectrum between 520-700 nm (Perez, 2001) overlapping with the emission spectra of Oregon Green, Fluorescein and PI. Cy5.5 conjugates of GSAO and GSCA were therefore employed, and Annexin V-APC labelling omitted. An alternate marker of plasma membrane integrity, Sytox Blue, was also utilised.

The uptake of GSAO-conjugates was identical between cells treated with Fas Ab, staurosporine and doxorubicin (Figure 14). GSAO-conjugates accumulated in dead cells during the late stages of apoptosis coincident with a loss of plasma membrane integrity. Uptake of the agent is therefore independent of the apoptotic pathway activated.
Figure 14. GSAO-Fluorophore accumulation is independent of the mechanism of apoptotic cell death. Jurkat A3 cells were also incubated with a) the broad spectrum kinase inhibitor staurosporine, or b) doxorubicin, for discrete times up to 24 h. The cells were then incubated with either GSCA-OG or GSCA-Cy5.5 (control), GSAO-OG or GSAO-Cy5.5, PI or Sytox Blue, and Annexin V-APC. The proportion of positively labelled cells at each time point is shown. GSAO-OG and GSAO-Cy5.5 labels cells independent of the cell death pathway activated. Results are the mean and standard deviation of samples in duplicate.

To investigate the application of GSAO to other cell death scenarios, tagged GSAO was also applied to an *in vitro* model of primary necrosis in which cells are subjected to repeated cycles of freeze-thawing. There was a significant reduction in forward scatter following freeze-thaw treatment, to the extent that only cellular debris remained. Almost all events in the treated condition were PI positive. GSAO-OG, but not the control compound GSCA-OG, labelled the necrotic debris (Figure 15).
Figure 15. GSAO-OG labels primary necrotic cells \textit{in vitro}. Jurkat A3 cells were untreated or subjected to three freeze-thaw cycles and stained with PI and either GSAO-OG (top panels) or the control compound GSCA-OG (lower panels). GSAO-OG but not GSCA-OG labelled PI positive necrotic cells. Data is representative of duplicate samples from two independent experiments.
Discussion

In this chapter I have characterised the accumulation of GSAO-fluorophore conjugates in *in vitro* models of cell death. I have shown that GSAO-conjugates specifically label dead and dying cells, whilst a control compound GSCA, containing a chemically inert carboxylic acid in place of the trivalent arsenic, does not. GSAO was also conjugated to Oregon Green, Fluorescein and Cy5.5 to demonstrate the independence of the outcome on the reporter group.

The kinetic parameters of labelling have also been explored, and the influence of time and concentration on GSAO-fluorophore uptake determined. The rate of uptake of GSAO conjugates was approximately 1000 fold higher in apoptotic vs healthy cells. Labelling with GSAO-conjugates is therefore rapid, saturable, and highly specific for dying cells.

The uptake of GSAO-conjugates was also explored in the context of landmark apoptotic events such as PS externalisation and plasma membrane disruption. I have shown that uptake of GSAO-conjugates coincide with PI staining, and thus occurs during the late stages of cell death when the plasma membrane integrity is compromised. To demonstrate the independence of the cell death detection on the mechanism of cell death *in vitro* models of intrinsic apoptosis, extrinsic apoptosis and necrosis were utilised. The accumulation of GSAO-conjugates was identical amongst these scenarios and thus occurs independent of the cell death pathway activated. This bodes well for the application of tagged GSAO to a broad spectrum of cell death scenarios *in vivo*. 
Chapter 3.

GSAO-conjugates are retained in the cytosol of the dying cell by covalent reaction with Heat Shock Protein 90 (Hsp90).

Introduction

In Chapter 2 I demonstrated the means through which GSAO-conjugates enter the dying cell. Changes in membrane permeability during the mid-late stages of cell death facilitate its uptake. The aim of Chapter 3 was to determine the mechanism through which GSAO-conjugates are then retained in the dying cell.

Trivalent arsenicals, such as the lipophilic phenylarsenoxide from which GSAO is derived, bind vicinal cysteine thiols with high affinity, forming stable cyclic dithioarsinites in which both sulphur atoms of the cysteine thiols are complexed to arsenic. This metalloid has very low reactivity for single protein thiols, as the non cyclic products formed with monothiols are much less stable (Adams et al., 1990; Stocken and Thompson, 1946; Whittaker, 1947). I reasoned that tagged GSAO was binding intracellular proteins within the dying cell in order to be retained and that this interaction was characterised by dithiol reactivity. To confirm this, GSAO-OG accumulation assays were conducted in the presence of dimercaptoproponal (DMP), a synthetic dithiol that mimics the protein target and sequesters the trivalent arsenic moiety of GSAO (Donoghue et al., 2000). GSAO-OG accumulation assays were also conducted in the presence of dideoxystilbenesulfonic acid (DIDS), a thiol cross-linker that
mimics GSAO and sequesters protein targets with closely spaced cysteine thiols
(Kowaltowski et al., 1997).

The reactivity of GSAO-conjugates with protein dithiols was also confirmed by observing
GSAO-F accumulation in the presence of unlabelled GSAO or GSAA. In GSAA the active
trivalent arsenic is replaced with a chemically inert pentavalent arsenic. I hypothesised that
unlabelled GSAO would compete with GSAO-F for the intracellular protein target, whilst the
compound lacking dithiol reactivity, GSAA, would not.

These experiments allowed me to confirm the nature of the interaction between tagged
GSAO and its protein targets. I then investigated the location and identity of these targets.
The distribution of GSAO-OG in apoptotic cells was visualized using fluorescence confocal
microscopy. A biotinylated form of GSAO was then used to isolate the protein targets
allowing their identification by mass spectrometry. To confirm the interaction of tagged
GSAO with the targets identified, assays were also conducted with purified recombinant
forms of the protein.
Materials and Methods

GSAO-OG labelling of dying cells pre-treated with a synthetic dithiol or a thiol cross-linker

Jurkat A3 cells were seeded at a density of 5 x 10^5 cells per mL and incubated with or without 0.1 µg per mL anti-human Fas (CD95) antibody for 24 hours. Cells were washed twice with ice cold phosphate-buffered saline, and resuspended in 0.1 M Hepes, pH 7.4 buffer containing 0.14 M NaCl and 25 mM CaCl_2. Cells were then incubated with or without 600 µM of the thiol cross-linker, DIDS (Sigma-Aldrich), or 50 µM of the synthetic dithiol, DMP (Sigma-Aldrich), for 10 min shaking at room temperature. Cells were stained with Annexin-V APC, PI and GSAO-OG as described in Chapter 2. Flow cytometry was performed using a FACS Canto II Flow Cytometer (Becton Dickinson) and data analysed using FlowJo software version 8.7.

Competitive uptake of unlabelled GSAO or GSAA with GSAO-F in apoptotic cells

Human fibrosarcoma HT1080 cells were treated with camptothecin for 20 hours as described above. Cells were co-incubated with 1 µM GSAO-F and varying concentrations of unlabelled GSAO or GSAA for 5 min. The mean fluorescence of the apoptotic population was determined by flow cytometry, and expressed as fraction of the mean fluorescence of the control (which contained no GSAO or GSAA).
Fluorescence microscopy of GSAO-OG localisation in dying cells

Jurkat A3 cells were seeded at a density of $5 \times 10^5$ cells per mL and incubated with or without 4 μM staurosporine for 24 hours. Cells were washed and stained with Annexin V-APC, DAPI and GSAO-OG as described above. $1 \times 10^5$ cells were seeded into FD35-100 Fluorodish Cell Culture Dishes (Coherent Scientific) and images captured using a Leica TCS SP5 inverted laser-scanning confocal microscope running Leica LAS software.

Identification of the proteins bound to GSAO-biotin in apoptotic cells

GSAO-biotin was prepared as described by Donoghue et al. (2000). $1 \times 10^8$ Jurkat A3 cells were seeded at a density of $5 \times 10^5$ cells per mL and treated with 4 μM staurosporine for 24 hours. Cells were washed twice, suspended in 10 mM Hepes, pH 7.4 buffer containing 0.14 M NaCl and 2.5 mM CaCl$_2$ and incubated with or without 50 μM GSAO-biotin for 15 min. Washed cells were resuspended in 50 mM Tris, pH 8 buffer containing 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA and a cocktail of protease inhibitors (Roche) and incubated at 4 °C for 20 min. The cells were then sonicated on ice and the lysate clarified by centrifugation at 5250 g for 20 minutes at 4 °C. Approximately 3.5 mg of total protein was incubated with 0.5 mg of streptavidin agarose beads (Sapphire Bioscience) at 4 °C for 2 hours on a rotating wheel. Beads were washed 5 times with radio-immunoprecipitation assay (RIPA) buffer (Sigma-Alrdich), and the bound proteins eluted at 4 °C overnight into 50 μl of 50 mM Tris, pH 8 buffer containing 150 mM NaCl, protease inhibitor cocktail (Roche) and 2 mM DMP. The eluted proteins were separated on a 4-12% gradient NUPAGE Bis-Tris gel (Invitrogen), and stained with Sypro Ruby Protein Gel Stain (Molecular Probes, Invitrogen).
Identification of proteins eluted from the beads was performed essentially as previously described (Wong et al., 2009). Briefly, the proteins were separated on a Sypro Ruby stained gel and the bands of interest excised. Alternatively, the following steps were performed in solution on the total protein eluate. The samples were reduced with dithiothreitol, cysteines alkylated with iodoacetamide (IAM) (Sigma-Aldrich) and digested with modified trypsin (Promega) (1:100 enzyme:protein) for 3 h at 37°C. Following digestion, tryptic peptides were separated by nano-liquid chromatographic system (Dionex) over a C18 column using a 0.5% gradient for 60 min at a flow rate of 250 nL per min. Eluting peptides were directly analysed using a Micromass Q-ToF 2 (Waters Corporation) mass spectrometer equipped with a nanospray source. The mass spectrometer was operated in data dependent acquisition mode to generate MS/MS data for protein identification. Mass spectral data were searched using Inspect (v20090202) 4 against the Uniprot/Swiss-Prot database (release 15.4). Search parameters were: Precursor tolerance 100 ppm and product ion tolerances ± 0.4 Da, variable modification of Met-O and Cys-carboxyamidomethyl, full tryptic cleavage and up to 1 missed cleavage. The significance threshold was set at p < 0.05.

**The interaction of GSAO-biotin with purified recombinant Hsp90 and β-tubulin**

1 µg of purified β-tubulin (Cytoskeleton Inc) and 5 µg of reduced Hsp90 (Prospec) was incubated with or without thiol alkylators, 20 mM IAM, 10 mM methylmethanethiosulfonate (MMTS) or 10 mM N-ethylmaleimide (NEM) (Sigma-Aldrich) for 30 minutes. Proteins were then labelled with 200 µM GSAO-biotin for 1 h, resolved on a 4-12% gradient NUPAGE Bis-Tris gel (Invitrogen), and transferred to polyvinylidene difluoride (PVDF) membrane. Complex formation between GSAO-biotin and either Hsp90 or β-tubulin was detected by
blotting with streptavidin horseradish peroxidase (HRP) (1:2000 dilution; Dako Cytomation). The blot was developed using chemiluminescence (NEN). To control for non-specific binding of streptavidin-peroxidase, GSAO-biotin was omitted in one experiment.
Results

**GSAO-conjugates are retained in the dying cell by binding protein dithiols**

It was hypothesised that GSAO-conjugates are retained in apoptotic cells by binding proteins with closely spaced cysteine thiols. To investigate this, Jurkat cells were treated with Fas Ab and the accumulation of GSAO-OG observed in the presence of the synthetic dithiol DMP, or the thiol cross-linker DIDS. The mean Oregon Green fluorescence of the PI positive population was used as a measure of GSAO-OG accumulation in apoptotic cells.

DMP sequesters the trivalent arsenic moiety through which GSAO interacts with protein dithiols. If the retention of GSAO-OG in apoptotic cells is dependent on its reactivity with protein dithiols, pre-treatment with DMP should inhibit GSAO-OG accumulation. DIDS conversely cross-links and thus sequesters closely spaced protein dithiols. If GSAO-OG binds protein targets at these residues, DIDS should also inhibit GSAO-OG accumulation in apoptotic cells (Figure 16).
Figure 16. The interaction of DIDS with protein dithiols, and DMP with GSAO. a) DIDS cross-links closely spaced thiols on the protein target (red). b) The synthetic dithiol DMP sequesters the trivalent arsenic moiety of GSAO.

The mean OG fluorescence of apoptotic PI positive cells was 1674 ± 52. Pre-treatment of the apoptotic cells with DMP and DIDS completely inhibited GSAO-OG accumulation with a mean OG Fluorescence of 17 ± 1 and 14 ± 3 respectively (Figure 17). Neither DMP nor DIDS affected Annexin V staining. GSAO-OG retention in apoptotic cells is therefore dependent on its reactivity with protein dithiols.
Figure 17. DIDS and DMP inhibit GSAO-OG retention in dying cells. Jurkat A3 cells were treated with Fas Ab for 24 h to induce apoptosis. Cells were then incubated with or without DMP or DIDS, stained with GSAO-OG and PI, and analysed via flow cytometry. Pretreatment with DIDS and DMP inhibited GSAO-OG labelling of dying cells. The numbers on the scatter plots are the percentage of cells in the gate.

The nature of this interaction was also confirmed by observing GSAO-F uptake in apoptotic cells in the presence of unlabelled GSAO or GSAA. Camptothecin-treated HT1080 cells were co-incubated with GSAO-F and varying concentrations of GSAO or GSAA as shown. The mean Fluorescein fluorescence of the apoptotic population was used as a measure of GSAO-F accumulation. GSAA contains an inert pentavalent arsenic in place of the trivalent arsenic, and is therefore unable to bind cysteine thiols. It was expected that unlabelled GSAO would compete with GSAO-F for the protein target and inhibit labelling of apoptotic cells, whereas GSAA would not.

Indeed GSAO but not GSAA inhibited accumulation of GSAO-F in apoptotic cells in a concentration dependent manner. The addition of 100 μM GSAO blocked GSAO-F uptake by
as much as 80%, whilst the same concentration of GSAA resulted in only a 20% drop in GSAO-F labelling (Figure 18). The retention of GSAO-conjugates in apoptotic cells is therefore dependent on its reactivity with protein dithiols.

Figure 18. Competitive uptake of GSAO-F into apoptotic cells by GSAO but not GSAA.
Camptothecin-treated HT1080 cells were incubated for 5 min with 1 μM GSAO-F and GSAO or GSAA as shown (x-axis). Uptake of GSAO-F into apoptotic cells was measured by flow cytometry and is expressed as a fraction of the mean fluorescence in the control (no GSAO or GSAA). Unlabelled GSAO, but not GSAA competed with GSAO-F for uptake into apoptotic cells. Results are the mean and range of two independent measurements.
GSAO-conjugates accumulate in the cytoplasm of the dying cell

To determine the location of the proteins bound by GSAO-conjugates, Jurkat A3 cells were incubated with GSAO-OG and the nucleic acid stain, DAPI, and visualised via fluorescence confocal microscopy. Annexin V-APC was used as an independent marker of apoptotic cells within the sample. GSAO-OG accumulated intracellularly and distributed throughout the cytoplasm of the dying cell. GSAO-OG did not co-localise with the nucleic acid stain DAPI (Figure 19).
Figure 19. GSAO-OG localises in the cytoplasm dying cells. Fluorescence confocal image (left panels) and brightfield (BF) image (right panel) and of a Jurkat A3 cell treated with staurosporine for 24 hours and incubated with GSAO-OG (green), DAPI (blue) and Annexin V-APC (red). GSAO-OG distributes throughout the cytoplasm of the dying cell and is excluded from the nucleus.
GSAO-conjugates bind intracellular Hsp90

To identify the intracellular protein targets of GSAO-conjugates, GSAO was biotinylated and incubated with apoptotic cells. Labelled proteins were collected on streptavidin agarose beads, and eluted using the synthetic dithiol DMP (Figure 20). In the first instance, the proteins were separated on a Sypro Ruby stained gel and the bands of interest excised, trypsin digested and analysed separately by liquid chromatography mass spectrometry (LC-MS/MS). In the second instance, a trypsin digest of the total eluate was analysed directly by LC-MS/MS. GSAO-biotin was omitted from one sample to control for non-specific binding of proteins to streptavidin agarose beads.

Figure 20. The experimental protocol used to isolate the intracellular protein targets of GSAO-biotin. GSAO-biotin was used to label the intracellular protein targets of apoptotic cells which were collected on streptavidin agarose beads. The proteins were then eluted using the synthetic dithiol DMP, which competes with the protein target for GSAO-biotin. The proteins were identified using mass spectrometry.
A number of distinct bands were observed in a Sypro Ruby stained SDS-PAGE gel of the protein eluate when GSAO-biotin was present. These bands were not observed in a sample lacking GSAO-biotin, confirming the specificity of the reaction (Figure 21). Densitometry analysis revealed the most prominent protein had a molecular weight of ~90 kDa. This band was excised, trypsin digested and identified as Hsp90 by mass spectrometry.

**Figure 21. GSAO-biotin binds intracellular Hsp90.** The intracellular protein targets of GSAO-biotin were eluted from streptavidin beads, resolved on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Sypro Ruby. GSAO-biotin was omitted in one sample to control for non-specific binding of proteins to streptavidin beads. The major labelled band was identified as Hsp90 by mass spectrometry. The positions of molecular weight markers are indicated at the left. Data is representative of three independent experiments.
LC-MS/MS analysis of the entire protein eluate was also undertaken. On the basis of mass-spectral counts, Hsp90 was identified as the most abundant protein bound by GSAO-biotin (Liu et al., 2004). It is 1.5 times as abundant as the next most abundant protein, tubulin, and roughly twice as abundant as eukaryotic translation elongation factor 2 and filamin A (110 spectral counts vs 74, 58 and 57 respectively) (Table 1). LC-MS/MS analysis of the protein eluate of samples containing no GSAO-biotin confirmed that the proteins did not bind non-specifically to streptavidin beads.
<table>
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<td>EEF2, eukaryotic translation elongation factor 2</td>
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<td>TUBA1B, tubulin, alpha 1b</td>
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<td>P13796</td>
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<td>Tubulin beta chain (Tubulin beta-5 chain)</td>
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Table 1. LC-MS/MS analysis of GSAO-biotin labelled proteins in apoptotic cells. The intracellular protein targets of GSAO-biotin were eluted from streptavidin beads, trypsin digested and analysed by LC-MS/MS. Proteins are arranged in descending order according to mass spectral counts. Hsp90 (green) and tubulin (blue) were identified as the most abundant proteins bound by GSAO-biotin, with a total of 110 and 74 spectral counts respectively.
To confirm the interaction of GSAO-biotin with Hsp90 and tubulin, assays were then conducted with purified recombinant forms of the protein. Hsp90 and tubulin were incubated with or without GSAO-biotin, resolved on SDS-PAGE and transferred to a PDVF membrane. Complex formation between GSAO-biotin and the protein was then detected by blotting with streptavidin HRP (Figure 22). GSAO-biotin was omitted from one sample to control for non-specific binding of streptavidin HRP to Hsp90 and tubulin (Figure 23).

Figure 22. The experimental protocol used to confirm the interaction of GSAO-biotin with Hsp90 and tubulin. Complex formation between GSAO-biotin and either Hsp90 or tubulin (red) was detected using streptavidin HRP.
Figure 23. GSAO-biotin binds purified recombinant Hsp90 and tubulin. Complex formation between GSAO-biotin and either Hsp90 or tubulin was detected by blotting with streptavidin HRP. To control for non-specific blotting with streptavidin peroxidise GSAO-biotin was omitted in the experiment shown in the left lanes. The positions of molecular weight markers are indicated at the left. The appearance of bands approximately 70 kDa and 90 kDa in size are consistent with the expected size of tubulin and Hsp90 respectively. Data is representative of two independent experiments.

To confirm that the interaction of GSAO-biotin with Hsp90 was mediated by cysteine thiols in the protein, purified Hsp90 was also incubated with or without the small thiol alkylators IAM, NEM, MMTS and then labelled with GSAO-biotin. Complex formation was determined by blotting with streptavidin HRP. NEM, MMTS and IAM covalently modify cysteine thiols within the protein and should therefore inhibit binding of GSAO-biotin. In the
presence of these alkylators a Hsp90: GSAO-biotin complex could not be detected (Figure 24).

Figure 24. GSAO-biotin reacts with cysteine thiols in Hsp90. Purified Hsp90 was incubated with or without the small thiol alkylators IAM, NEM, MMTS and then labelled with GSAO-biotin. The protein was resolved on SDS-PAGE and complex formation measured by blotting with streptavidin peroxidase. To control for non-specific blotting with streptavidin HRP, GSAO-biotin was omitted in the experiment shown in the last lane. The positions of molecular weight markers are indicated at the left. * indicates an Hsp90 degradation product. Data is representative of two independent experiments.
**Discussion**

In Chapter 3 I have shown that GSAO-conjugates are retained in the dying cell by covalent reaction with protein dithiols. GSAO-OG labelling of apoptotic cells was completely inhibited by a synthetic dithiol DMP that sequesters the trivalent arsenical of GSAO. It was also blocked by a thiol cross-linker DIDS that sequesters closely spaced thiols on the protein target. Furthermore, unlabelled GSAO but not GSAA competed with GSAO-F uptake in apoptotic cells. The trivalent arsenic of unlabelled GSAO reacts with protein dithiols and inhibits GSAO-F uptake in dying cells, whilst the pentavalent arsenic of GSAA does not. Very few proteins react with trivalent arsenic in the extracellular milieu, as closely spaced protein cysteine thiols are usually oxidized to a cysteine disulfide bond in this environment. The intracellular milieu, in contrast, is reducing in nature, and thus contains a number of proteins that react with trivalent arsenic (Dilda and Hogg, 2007). I therefore anticipated that GSAO-conjugates would be retained in the dying cell by binding intracellular protein targets.

This notion was first explored using fluorescence confocal microscopy to visualise localisation of the probe in dying cells. Indeed, GSAO-conjugates accumulated intracellularly and distributed throughout the cytoplasm of the dying cell. The identity of these protein targets were then determined using mass spectrometry. The main protein to which GSAO-conjugates bound was Hsp90.

Hsp90 is the most abundant chaperone of the eukaryotic cytoplasm and accounts for 1-2% of total cellular protein. It is expressed ubiquitously and under normal conditions mediates the folding, stabilisation, assembly and activation of various client proteins. Hsp90 is also a key component of the cellular response to stress, and synthesis of the protein increases in
response to hypoxia, heat and acidosis (Whitesell and Lindquist, 2005). Tumour cell malignancies are also dependent on higher than normal activity of Hsp90. The protein is reportedly upregulated between 2-10 fold in these cells (Ferrarini et al., 1992). Here it mediates the folding and stabilisation of unstable mutant oncoproteins, and/or over-expressed proteins that would otherwise be lethal to the cell. Hsp90 therefore buffers both the hostile external tumour microenvironment, as well as the numerous genetic lesions within (Whitesell and Lindquist, 2005).

A prime example of this is the SRC tyrosine kinase. Most oncogenic mutations of the kinase involve deletion of a crucial regulatory domain, resulting in a constitutively active but conformationally unstable protein (Falsone et al., 2004). Association of Hsp90 with the mutant kinase stabilises the protein, promoting continuous pro-proliferative signalling and facilitating cellular transformation (Oppermann et al., 1981; Whitesell and Lindquist, 2005). Hsp90 has also been shown to bind and stabilise the membrane bound human epidermal growth factor receptor 2 (HER-2) which is over-expressed in approximately 30% of breast cancers (Sidera et al., 2008). Through physical association with Hsp90 these proteins are protected from proteasomal degradation, and thus tolerated by the cell.

Hsp90 contains a highly conserved Cys-Cys motif in the C-terminal domain. This pair of cysteines (Cys589 and Cys590) are involved in redox reactions in the cytoplasm and are cross-linked by the trivalent arsenical arsenite (Nardai et al., 2000). The optimal spacing of cysteine thiolates for reaction with As(III) is 3-4 Å (Adams et al., 1990; Bhattacharjee and Rosen, 1996). As Cys589 and Cys590 are approximately 7.2 Å apart, it is likely that GSAO-conjugates bind Hsp90 at these residues (Figure 25).
Figure 25. Human Hsp90α (residues 293-732, 3Q6M). Hsp90 contains a pair of highly conserved cysteines at positions 589 and 590 7.2 Å apart (yellow). Tagged GSAO likely cross-links these residues.

The abundance of Hsp90 within the cytosol bodes well for the suitability of GSAO-conjugates to imaging cell death. The effectiveness of an imaging agent is determined in part by how much of the agent accumulates in a given volume. A high concentration of imaging agent at the target results in better limits of detection and resolution. The abundance of Hsp90 in the cytosol allows for high levels of GSAO-conjugates in apoptotic cells and therefore superior detection and resolution of cell death. This is likely to be particularly true in instances of tumour cell death due to the elevated levels of Hsp90 in these cells.

Given the role of Hsp90 in tumour cells, it would also be of interest to investigate the ability of GSAO to functionally inhibit the chaperone protein. A number of Hsp90 inhibitors are
currently in clinical trials for the treatment of cancer, and have been particularly effective in patients with melanoma, breast and prostate cancer (Powers and Workman, 2006). These compounds bind the N-terminal ATPase domain of Hsp90 and prevent shuttling between ADP and ATP bound conformations. Drug bound Hsp90 also recruits E3 ubiquitin ligases, targeting client proteins for degradation (Whitesell and Lindquist, 2005). Although Hsp90 is moderately upregulated in tumour cells, the selectivity of the compounds relates to the affinity of tumour cell Hsp90 for its inhibitors. Due to the oncogenic load of mutant client proteins, tumour cell Hsp90 is more likely to be found in ATPase active multi-protein complexes, and this exhibits a 100 fold greater affinity for its inhibitors than Hsp90 from normal cells (Kamal et al., 2003; Whitesell and Lindquist, 2005).

As GSAO binds the C-terminus of Hsp90 it is unlikely to affect Hsp90 in the same capacity as N-terminal inhibitors. However as Hsp90 dimerisation is mediated through the C-terminus, it is conceivable that GSAO could sterically hinder dimerisation, induce separation of Hsp90 monomers and induce Hsp90 substrates to be released. Indeed a coumarin based Hsp90 inhibitor, coumerrymycin A1, is thought to function in this manner (Allan et al., 2006; Sgobba et al., 2010). The C-terminus of Hsp90 is also known to contain a second, cryptic C-terminal ATPase domain as well as a conserved pentapeptide sequence for binding co-chaperones (Allan et al., 2006; Garnier et al., 2002). Novobiocin and its derivatives induce degradation of Hsp90 client proteins in tumour cells through this mechanism (Donnelly and Blagg, 2008). It is possible that GSAO could exhibit a similar inhibitory effect upon binding of C-terminal cysteine residues. Future studies will investigate this hypothesis.

As tagged-GSAO does not enter cells with an intact membrane, future investigations will necessarily involve cold GSAO. Hsp90 inhibition may be assessed by colorimetric assays for
ATPase activity, as described by Rowlands and colleagues (Rowlands et al., 2004).

Alternatively inhibition of Hsp90 will be determined by western blot or ELISA by observing the down-regulation of known client proteins such as RAF-1 and Hsp70 (Aherne et al., 2003).

In summary, Chapters 2 and 3 have demonstrated the mechanism through which GSAO-conjugates selectively enter and accumulate in dying cells. The plasma membrane of viable cells is impermeable to GSAO-conjugates, however changes in membrane integrity during the late stages of cell death allow the probe to enter. GSAO-conjugates are then retained in the cytosol by covalent reaction with intracellular proteins such as Hsp90. Specifically GSAO-conjugates cross-link closely spaced thiols on the protein target.
Chapter 4.

Imaging cell death \textit{in vitro} and \textit{in vivo} using near infra-red conjugates of GSAO and GSCA.

\textbf{Introduction}

In Chapters 2 and 3 the mechanism through which GSAO-conjugates specifically accumulate in dying cells was elucidated. Notably, changes to membrane integrity during the late stages of cell death facilitate uptake of GSAO-conjugates, whilst covalent reaction with intracellular proteins such as Hsp90 allow them to be retained. The agent may have applications in patient diagnosis and management, however if it is to be introduced into a clinical setting, \textit{in vivo} validation is first required.

The aim of Chapter 4 was to demonstrate the application of GSAO-conjugates to pre-clinical mouse models of cell death. Optical imaging techniques such as fluorescence confocal microscopy and whole body fluorescence imaging were employed.

In fluorescent imaging, a fluorophore is excited by a light source of an appropriate wavelength triggering the release of photons with a specific emission spectrum. The emitted photons are subsequently captured by an optical detector such as a charge-coupled device camera. In order to maximise both the image resolution and signal to noise ratio, two factors should be considered in regard to the choice of fluorophore, namely the depth of tissue
penetration and the contribution of auto-fluorescence at that particular wavelength. The depth of tissue penetration refers to the attenuation of light (both excitation and emission) as a result of both absorption and scattering by the surrounding tissue. In the first instance, endogenous chromophores found in living tissues such as lipid, melanin and haemoglobin absorb light as it enters and exits the specimen (Kovar et al., 2007). In the second instance, photons are absorbed and re-emitted by cellular structures without a loss of energy, but potentially in a different direction. Although the photon is likely to travel in the same direction in most instances, the accumulation of multiple scattering events results in the gradual randomisation of propagation direction. This not only attenuates the strength of the signal but also affects the resolution of the image, resulting in ‘blurring’ (Ntziachristos, 2010).

As a general rule, both light scattering and light absorption decrease with increasing wavelength. The greatest tissue penetration is thus achieved at the upper end of the optical spectrum in the near-infrared range (Kovar et al., 2007). In relatively transparent tissues with low scattering and absorption, such as the breast, this may extend to as much as 10-12 cm. In tissues such as the brain or muscle however, the light is unlikely to penetrate more than 3-6 cm (Ntziachristos, 2010).

Auto-fluorescence is the emission of light from biological structures when subjected to the same source of excitation as the fluorophore. At a cellular level this is usually the result of pyridinic (NADPH) and flavin coenzymes as well as aromatic amino acids and lipopigments. At the level of whole tissues, extracellular matrix proteins such as collagen and elastin account for most of the auto-fluorescent signal (Monici, 2005).
Auto-fluorescence is likely to confound the interpretation of data if it exhibits a similar emission spectrum to the fluorophore of interest, particularly if the signals are of comparable magnitude. Whilst this tends to be a problem at the ultra violet and visible end of the spectrum, the higher wavelength ranges are typically spared from interference. The use of near-infrared fluorophores thus lends to a better signal to noise ratio in vivo (Kovar et al., 2007).

In order to maximise tissue penetration and signal to noise ratio, GSAO was therefore conjugated to the near-infrared fluorophores Cy5.5 and Alexa Fluor 750 with emission maximums of 694 nm and 775 nm respectively. These probes have the added advantage of chemical and optical stability, low toxicity and non-specific binding, and rapid clearance of free dye from the body (Kovar et al., 2007). The activity of the conjugates was verified in in vitro models of cell death using flow cytometry, and dual fluorescence/bioluminescence imaging.

Bioluminescence is a naturally occurring form of chemiluminescence, whereby a chemical reaction generates light. Cells containing a luciferase reporter gene are incubated with luciferin and light is generated in an ATP dependent fashion. Due to a lack of ATP dying cells are unable to produce a bioluminescent signal in the presence of the substrate. The accumulation of fluorescently labelled GSAO in dying cells should thus be met with a simultaneous loss in bioluminescence.

To demonstrate the specific accumulation of the probe in dying cells in vivo, Balb/c nude mice bearing a subcutaneous murine colon carcinoma were injected intravenously with GSAO-Cy5.5 following treatment with chemotherapy. The hairless phenotype of nude mice
is ideal for optical imaging as hair blocks, absorbs and scatters light. Intravenous administration of the probe was chosen to allow rapid systemic distribution and ultimately clearance. This in turn minimises the likelihood of background uptake due to long exposure to the probe (Kovar et al., 2007). Following administration of GSAO-Cy5.5, tumours were excised and the tissue sections analysed \textit{ex vivo} by fluorescence confocal microscopy. A fluorescent antibody for active caspase 3 was used to demarcate apoptotic cells and the relative localisation of GSAO-Cy5.5 observed. GSCA-Cy5.5 was used to control for non-specific accumulation of the probe in apoptotic cells.

GSAO and GSCA were also tagged with Alexa Fluor 750 and applied \textit{in vivo} to a mouse model of brain trauma (Grzybicki et al., 1998; Hotta et al., 2005). A cryolesion was induced in the mouse brain by applying a liquid nitrogen cooled probe to the skull. The freezing injury induces cell death via two distinct mechanisms. Firstly, intracellular ice crystals rupture the cell membrane damaging the cell directly (Meryman, 1956). Secondly, damage to endothelial cell junctions results in vascular permeability, oedema, thrombosis and ultimately failure of the microcirculation. The consequence is a secondary ischemic assault (Gage et al., 2009). Whilst the centre of the lesion is characterised by necrosis, apoptosis is also observed in the periphery as early as 8 hours post injury (Steinbach et al., 1999). The latter is thought to occur via mitochondrial permeabilisation following increased expression of Bax (Wen et al., 2007). Developed in the 1960s, the technique is well established and relatively easy to perform experimentally.

Following induction of the brain cryolesion GSAO-AF750 or GSCA-AF750 was injected intravenously and the mice imaged at various times using small animal fluorescence imaging.
The relative Alexa Fluor 750 fluorescence in the lesion was quantified. The influence of lesion size and probe concentration on Alexa Fluor 750 signal was also investigated.

At the experiment endpoint the brains were also excised and imaged ex vivo. The brains were then fixed, paraffin embedded and sectioned. Accumulation of the probe in the tissue sections was observed by fluorescence imaging whilst adjacent sections were stained with TUNEL to indicate the presence of apoptotic cells. Co-localisation of the two confirmed the specific accumulation of GSAO-AF750 in dying cells in vivo.
Materials and Methods

The conjugation of GSAO and GSCA to near infra-red fluorophores

A solution of Alexa Fluor 750 succinimidyl ester (Molecular Probes, Invitrogen) or Cy5 N-Hydroxysuccinimide ester (GE Healthcare) (10 mg per mL in DMSO) was added to GSAO (4.2 mg or 6.7 mg per mL respectively) or GSCA (5.9 mg or 4.1 mg per mL respectively) in 0.1 M bicarbonate buffer, pH8.3 and incubated for 60 min in the dark in the presence of nitrogen. The molar ratio of fluorophore to pendant was ~1:1. Unreacted fluorophore was quenched by adding 10 mM glycine.

Cell culture

Jurkat A3 cells, CT26.WT cells (ATCC) and 4T1-luc2 cells expressing the codon-optimised luciferase gene luc2 (Caliper Life Sciences) were cultured in RPMI-1640 medium supplemented with 10% foetal bovine serum, 2 mM L-glutamine, and 1 ug per mL penicillin/streptomycin.

The in vitro validation of near infra-red conjugates of GSAO and GSCA via flow cytometry

The in vitro validation of near infra-red GSAO and GSCA conjugates was performed as described in Chapter 2 and 3. For Alexa Fluor 750 conjugates flow cytometry was performed using a BD™ LSR II Flow Cytometer (BD Biosciences) equipped with a high powered 200 mV 628 nm red excitation laser.
Primary tumour growth

7-9 week old Balb/c nude mice (Covidien Imaging Solutions) were held in groups of 3 to 5 at a 12 h day and night cycle and were given animal chow and water *ad libidum*. A suspension of $0.5 \times 10^6$ human colorectal carcinoma CT26.WT cells in 0.1 mL of PBS was injected subcutaneously in the proximal midline (Ojo-Amaize et al., 2007). 9 days post tumour cell implantation mice were randomised into two groups. Tumour volume and animal weight was measured every day. Tumour volume was calculated using the relationship width x length x height (mm) x 0.523.

*Ex vivo* fluorescence imaging of tumour cell death using GSAO-Cy5.5 and GSCA-Cy5.5

Mice bearing subcutaneous CT26.WT colorectal carcinomas were treated with a single tail vein injection of 10 mg/kg doxorubicin 9 days post tumour cell implantation (Wilmanns et al., 1992). The following day the mice received a tail vein injection of either GSAO-Cy5.5 or GSCA-Cy5.5 (5 mg/kg in 100 µl PBS) and 1 h later the tumours were excised. Tumours were embedded in paraffin, 4 micron sections prepared and stained with cleaved caspase-3 (Asp175) rabbit primary monoclonal antibody (5A1E, 1:200 dilution, Cell Signalling), secondary Cy2 conjugated AffiniPure donkey anti-rabbit IgG (1:200 dilution, Jackson ImmunoResearch) and 4',6-diamidino-2-phenylindole (DAPI)/AntiFade Reagent-Prolong Gold (Invitrogen). Immunohistochemistry was performed according to the Cell Signalling Technology Immunohistochemistry Protocol. Images were collected with an Olympus FV1000 Laser Scanning Microscope (Excitation λ: DAPI (405 nm); Cy2 (488 nm); and Cy5.5 (633 nm).
**In vitro cryo-induced cell death assay**

4T1-luc2 cells were seeded onto a 12-well plate and grown to confluency in RPMI-1640 medium. The medium was discarded and a bar of dry ice 3-5 mm in diameter applied to the underside of the culture well for 15 seconds. GSAO-AF750 or GSCA-AF750 (0.1 µM or 0.4 µM in 300 ul PBS) was added to the cells and incubated for 15 min at 37 °C. The cells were gently washed with PBS and imaged with the IVIS Spectrum (Caliper Life Sciences) (Fluorescence: excitation filter: 710 nm, emission filter: 780 nm). Subsequently, luciferin (Gold Biotechnology) (1.25 mg/kg in 100 ul PBS) was added to each well and bioluminescence images acquired using the same machine (bioluminescence: open filter). The conversion of luciferin to oxyluciferin and light is ATP dependent. Whilst healthy 4T1-luc2 cells produce a bioluminescent signal in the presence of the luciferin substrate, dying cells deficient in ATP do not.

**Murine cryolesion model**

Balb/c nude mice (Charles River Laboratories, France) were sedated with 2-3% isoflurane and a cryolesion induced in the parietal lobe of the right cerebral hemisphere. A metal rod 1 mm in diameter was cooled with liquid nitrogen and applied to the skull for 5 or 60 seconds. Mice were allowed to recover from the anaesthetic and observed for any neurological signs or changes in behaviour as a result of the injury (Grzybicki et al., 1998). Mice were then injected intravenously with either GSAO-Alexa Fluor 750 or GSCA-Alexa Fluor 750 (0.1 mg/kg or 1mg/kg in 100ul PBS) and imaged with the Pearl® Impulse Small Animal Imaging System (LI-COR Biosciences) at 1, 3, 6 and 24 hours. Prior to imaging mice were wiped with 80% v/v ethanol to remove any of the compound excreted in the urine and subsequently
contaminating the skin. At the experiment endpoint the mice were sacrificed by cervical
dislocation and the brains excised for \textit{ex vivo} imaging. Images were acquired at 800 nm at a
resolution of 85 $\mu$m.

The fluorescent signal was digitized and electronically displayed as a pseudocolor overlay on
a gray scale white light image of the animal. Each real value was mapped to a colour in a
look up table and applied to the image. The data was analysed using the Pearl® Impulse
Software, Version 2.0. Total Alexa Fluor 750 Fluorescence was determined by drawing a
ROI over the area in which the cryolesion was induced. The size and shape of the ROI was
the same for each time point. An ROI of equivalent size was then drawn over the adjacent
hemisphere to determine the background signal and this was subtracted from the lesion
signal. Where mean Alexa Fluor 750 Fluorescence is shown, the value has been corrected for
the area of the ROI. The same look up table has been applied to the images shown where
indicated in the figure legends to allow direct comparison.

\textbf{Histological validation}

Brains were fixed in 4\% formaldehyde and embedded in paraffin. 8 micron sections were
prepared and imaged using the LI-COR Odyssey Infra-red Imager 9120 (LI-COR
Biosciences) at 800 nm. Adjacent sections were subjected to TUNEL staining to confirm
accumulation of GSAO-AF750 in cells undergoing apoptosis.
Results

*In vitro validation of near infra-red conjugates of GSAO using flow cytometry*

In order to demonstrate the application of GSAO-conjugates to *in vivo* models of cell death, GSAO was first conjugated to the near infra-red fluorophores Cy5 and Alexa Fluor 750. The conjugates were validated in *in vitro* models of cell death as described in Chapters 2 and 3. Briefly, Jurkat cells were treated with Fas Ab or staurosporine to induce apoptosis, and incubated with GSAO-conjugates and the late stage apoptotic marker PI or Sytox Green. GSAO conjugates labelled PI/Sytox Green positive cells whilst the control GSCA conjugates, did not (Figure 26 and 27). The mean AF750 fluorescence of PI positive cells was more than 200 fold more in cells stained with GSAO-AF750, than the control GSCA-AF750 compound. The near infra-red conjugate therefore retained its specificity for dead and dying cells. The binding is therefore specific to the trivalent arsenic moiety of the compound.
Figure 26. GSAO-AF750 selectively labels dying cells in vitro. Jurkat A3 cells were untreated or treated with staurosporine for 24 h to induce apoptosis, incubated with the control compound GSCA-AF750 (lower panels) or GSAO-AF750 (upper panels) and PI (all panels), and analysed by flow cytometry. After 24 hours a majority of cells had entered late apoptosis as indicated by PI staining. GSAO-AF750 but not GSCA-AF750 labelled these cells. The numbers on the histograms or scatter plots are the percentage of cells in the gate or quadrant, respectively. Data is representative of duplicate samples from three independent experiments.
Figure 27. GSAO-Cy5.5 selectively labels dying cells *in vitro*. Jurkat A3 cells were untreated or treated with Fas antibody (Ab) for 24 h to induce apoptosis, incubated with the control compound GSAO-Cy5.5 (upper panels) or GSCA-Cy5.5 (lower panels) and Sytox Green (all panels), and analysed by flow cytometry. After 24 hours a majority of cells had entered late apoptosis as indicated by Sytox Green staining. GSAO-Cy5.5 but not GSCA-Cy5.5 labelled these cells. The numbers on the histograms or scatter plots are the percentage of cells in the gate or quadrant, respectively. Data is representative of duplicate samples from two independent experiments.
**GSAO-conjugates label dying tumour cells *in vivo***

Once the conjugates had been validated *in vitro*, Cy5.5 conjugates were used to demonstrate the specificity of the probe for dying tumour cells *in vivo*. Mice bearing subcutaneous murine CT26 colorectal carcinoma tumours were treated with doxorubicin by tail-vein injection to induce tumour cell death. GSAO-Cy5.5 or the control compound GSCA-Cy5.5 was injected intravenously the day after treatment and the tumours excised 1 h later. The tumours were then embedded in paraffin, sectioned and stained with the nucleic acid stain DAPI and a fluorescent antibody targeted toward activated caspase 3. The latter was used to distinguish apoptotic cells in the specimen. Fluorescence confocal microscopy revealed co-localisation of GSAO-Cy5.5 and active caspase 3 in the tissue sections analysed (Figure 28a). Consistent with the fluorescence confocal microscopy of dying cells *in vitro*, GSAO-Cy5.5 had a cytoplasmic distribution and did not co-localise with the nucleic acid stain DAPI. GSCA-Cy5.5 was not observed in any of the tumour sections analysed (Figure 28b). Uptake of GSAO-Cy5.5 in apoptotic cells is therefore specific.

To control for tissue auto-fluorescence and non-specific staining of the secondary Cy2 fluorescent antibody, adjacent tissue sections were treated with the secondary antibody in the absence of the primary caspase 3 antibody. Caspase 3 positive cells were not observed in these samples (Figure 28c).
a)  
DAPI  
Caspase 3  
GSAO-Cy5.5  
Merged  

b)  
DAPI  
Caspase 3  
GSCA-Cy5.5  
Merged
Figure 28. GSAO-Cy5.5 labels apoptotic tumour cells in mice. Balb/c mice bearing CT26 colorectal carcinoma tumours were treated with doxorubicin and the following day were administered 5 mg/kg GSAO-Cy5.5 (a) or the control compound GSCA-Cy5.5 (b). After 1 h, the tumours were excised, sectioned, stained for activated caspase 3, and counterstained with a nucleic acid stain (DAPI).(a & b) Fluorescence confocal microscopy showed that cells positive for activated caspase 3 (green) were also positive for GSAO-Cy5.5 (red), but not for GSCA-Cy5.5. c) Adjacent tissue sections treated with the secondary antibody in the absence of the primary caspase 3 antibody confirmed the specificity of the caspase 3 staining.
Validation of GSAO-conjugates in an in vitro cryolesion model of cell death

Validation was also performed in a cryolesion model of cell death in-vitro. Murine breast cancer 4T1 cells expressing the luc 2 luciferase reporter gene, were subjected to a dry ice lesion prior to staining with varying concentrations of GSAO-Alexa Fluor 750 or GSCA-Alexa Fluor 750. Dry ice was applied to the centre of the well on the underside of the culture dish so that an external ring of healthy cells remained. This was confirmed by bioluminescence imaging following incubation with luciferin. Dying cells lacking ATP were unable to produce light in the presence of this substrate (Figure 29a). Fluorescence imaging revealed that GSAO-Alexa Fluor 750 but not GSCA-Alexa Fluor 750 labelled dying cells at the centre of the well in a concentration dependent manner (Figure 29b).
Figure 29. The accumulation of GSAO-AF750 or GSCA-AF750 in cells subjected to dry ice.
4T1-luc2 cells in the centre of each well were subjected to a dry ice lesion and incubated with luciferin and either GSAO-AF750 or GSCA-AF750. The concentration of the probe is indicated to the left of the figure. a) Bioluminescence imaging confirmed cell death in the centre of each well as a result of the dry ice lesion. b) Fluorescence imaging revealed that GSAO-AF750 but not GSCA-AF750 labelled dying cells in a concentration dependent manner. Data is representative of three independent experiments.
The noninvasive imaging of brain trauma in mice using GSAO-conjugates

To demonstrate the *in vivo* utility of near infra-red GSAO-conjugates, GSAO-AF750 and GSCA-AF750 were also applied to a murine model of brain trauma. A 60 second cryolesion was induced in the right parietal lobe of Balb/c nude mice followed by an intravenous injection of 1 mg/kg GSAO-AF750 or GSCA-AF750. The mice showed no evident neurological signs or changes in behaviour after injury. Fluorescence was measured at 1, 3, 6 and 24 hours using a small animal fluorescence imager. GSAO-AF750 and GSCA-AF750 cleared from non target tissues within 3 hours of administration, with the exception of the kidneys. GSAO-AF750 but not GSCA-AF750 was observed in the lesion at all time points (Figure 30 and 31).
Figure 30. Time serial of three individual mice bearing a brain cryolesion and injected with GSAO-AF750. Mice bearing a 60 second cryolesion were injected with 1 mg/kg GSAO-AF750 and imaged at 1, 3 and 6 hours. Three representative mice are shown. GSAO-AF750 was observed in the lesion at all time points, and was cleared from all non target tissues, except the kidneys, 3 hours after administration.
Figure 31. Time serial of three individual mice bearing a brain cryolesion and injected with the control GSCA-AF750. Mice bearing a 60 second cryolesion were injected with 1 mg/kg GSCA-AF750 and imaged at 1, 3 and 6 hours. Three representative mice are shown. GSCA-AF750 was cleared from the body 3 hours after administration, and was not observed in the lesion at any time point. The same look up table has been applied to the GSAO-AF750 and GSCA-AF750 images for direct comparison.
For quantification of Alexa Fluor 750 fluorescence an ROI was drawn over the area in which the cryolesion was induced. The size and shape of the ROI was consistent for each time point. An equivalent ROI was also drawn over the adjacent hemisphere (background) and this value subtracted from the lesion signal. To account for variation in lesion size, this value was then corrected for area. GSAO-AF750, but not GSCA-AF750 could be detected in the lesion at all time points (p value < 0.001) (Figure 32). The GSAO-AF750 signal is consistent in the lesion for the first 6 hours, and drops significantly by 24 hours. The signal to background ratio for GSAO-AF750 (lesion: adjacent healthy hemisphere) was highest at 3, 6 and 24 hours, at 3:1.

Figure 32. GSAO-AF750/GSCA-AF750 fluorescence in the brain cryolesion over time. Mice bearing a 60 second cryolesion were injected with 1 mg/kg GSAO-AF750 or GSCA-AF750 and imaged at 1, 3, 6 and 24 hours. GSAO-AF750 (green), but not GSCA-AF750 (blue) was detected in the lesion. Results are the mean and standard deviation of two independent experiments. GSAO-AF750 (1 h n = 6, 3 h n = 11, 6 h n = 11, 24 h n = 5), GSCA-AF750 (1 h n = 5, 3 h n = 10, 6 h n = 10, 24 h n = 5).
The brains of the mice were also excised at 6 hours for *ex vivo* imaging (Figure 33). A small amount of GSCA-AF750 could be detected in the lesion, however GSAO-AF750 fluorescence was over 7 fold higher (p value < 0.001) (Figure 34).

**Figure 33.** *Ex-vivo* imaging of brains with cryolesion. Mice bearing a 60 second cryolesion were injected with 1 mg/kg GSAO-AF750 (left panel) or GSCA-AF750 (right panel). 6 hours later brains were excised and imaged *ex vivo*. 11 representative brains are shown. GSAO-AF750 but not GSCA-AF750 could be detected in the lesions.
Figure 34. GSAO-AF750/GSCA-AF750 fluorescence in the brain cryolesion *ex-vivo*. Mice bearing a 60 second cryolesion were injected with 1 mg/kg GSAO-AF750 or GSCA-AF750. 6 hours later the brains were excised and imaged *ex vivo*. A small amount of GSCA-AF750 (blue) could be detected in the lesion, however GSAO-AF750 fluorescence (green) was significantly higher (p value < 0.001). Results are the mean and standard deviation of two independent experiments. GSAO-AF750 n = 6, GSCA-AF750 n = 5.

The excised brains were also fixed, paraffin embedded and sectioned for fluorescence imaging. GSAO-AF750 but not GSCA-AF750 could be detected in the lesion. The presence of dying cells in the lesion was confirmed by TUNEL staining of adjacent tissue sections. This method labels the terminal ends of nucleic acids and thus detects DNA fragmentation in apoptotic cells. GSAO-AF750 co-localised with TUNEL positive apoptotic cells in the lesion (Figure 35).
Figure 35. AF750 fluorescence and TUNEL staining of brain sections. Mice bearing a 60 second cryolesion were injected with 1 mg/kg GSAO-AF750 or GSCA-AF750. 6 hours later the brains were excised, fixed, paraffin embedded, sectioned and imaged *ex vivo*. Fluorescence imaging revealed that GSAO-AF750 (upper left panel) but not GSCA-AF750 accumulated in the lesion (upper right panel). TUNEL staining confirmed the presence of apoptotic cells in the lesion (bottom panel).
GSAO-conjugates accumulate in the brain cryolesion in a concentration dependent manner.

The concentration dependence of GSAO-AF750 accumulation was also investigated. Mice bearing a 60 second cryolesion were administered 0.1 mg/kg GSAO-AF750 and imaged at 1, 3 and 6 hours (Figure 36). GSAO-AF750 was observed in the lesions at all time points, however the total fluorescence was 31-52 fold less than mice injected with 1 mg/kg (Figure 37). The signal to noise ratio (lesion: adjacent healthy hemisphere) also dropped to 1.3:1 at 1 and 3 hours, and 1.6:1 at 6 hours. Applying the same look up table to the 0.1 mg/kg and 1 mg/kg groups results in saturation of the latter. This does not impede quantification of the data, however a direct visual comparison cannot be made between whole body images of the two groups. The images shown in Figure 36 are provided to demonstrate GSAO-AF750 accumulation in the lesion.
Figure 36. Time serial of three individual mice bearing a brain cryolesion and injected with 0.1 mg/kg GSAO-AF750. Mice bearing a 60 second cryolesion were injected with 0.1 mg/kg GSAO-AF750 and imaged at 1, 3 and 6 hours. Three representative mice are shown. GSAO-AF750 was observed in the lesion at all time points.
Figure 37. The concentration dependent accumulation of GSAO-AF750 in the brain cryolesion over time. Mice bearing a 60 second cryolesion were injected with 0.1 mg/kg (blue) or 1 mg/kg GSAO-AF750 (green) and imaged at 1, 3, and 6 hours. GSAO-AF750 could be detected in the lesion in both groups at all time points, however GSAO-AF750 fluorescence was significantly lower in mice injected with 0.1 mg/kg (p value < 0.01 at 1 and 6 hours and < 0.05 at 3 hours). Results are the mean and standard deviation of 5-11 independent measurements. 1 mg/kg GSAO-AF750 (1 h n = 6, 3 h n = 11, 6 h n = 11), 0.1 mg/kg GSAO-AF750 (1 h n = 5, 3 h n = 5, 6 h n = 5).

Mice injected with 0.1 mg/kg GSAO-AF750 were also sacrificed at 6 hours and their brains imaged ex vivo (Figure 38). The same look up table cannot be applied to both 0.1 mg/kg and 1 mg/kg groups for reasons detailed above. Consequently a direct visual comparison should not be made between the ex vivo images from each group. The images are provided only to demonstrate GSAO-AF750 accumulation in the lesion. GSAO-AF750 fluorescence was 29 fold higher in mice injected with 1 mg/kg GSAO-AF750 than 0.1 mg/kg GSAO-AF750 (Figure 39).
Figure 38. *Ex-vivo* imaging of brains with cryolesion. Mice bearing a 60 second cryolesion were injected with 0.1 mg/kg GSAO-AF750. 6 hours later brains were excised and imaged *ex vivo*. 3 representative brains are shown. GSAO-AF750 could be detected in the lesions.

Figure 39. GSAO-AF750 fluorescence in the brain cryolesion *ex-vivo*. Mice bearing a 60 second cryolesion were injected with 1 mg/kg or 0.1 mg/kg GSAO-AF750. 6 hours later the brains were excised and imaged *ex vivo*. GSAO-AF750 fluorescence was significantly higher in the cryolesions of mice injected with 1 mg/kg GSAO-AF750 (green) than 0.1 mg/kg GSAO-AF 750 (blue) (p value < 0.01). Results are the mean and standard deviation of 5-11 independent measurements. 1 mg/kg GSAO-AF750 n = 11, 0.1 mg/kg GSAO-AF750 n = 5.
The excised brains were also fixed, paraffin embedded and sectioned for fluorescence imaging. AF750 fluorescence in the lesion was lower in mice injected with 0.1 mg/kg compared to 1 mg/kg GSAO-AF750 (Figure 40).

![Image](image_url)

**Figure 40. GSAO-AF750 fluorescence in *ex vivo* brain sections.** Mice bearing a 60 second cryolesion were injected with 0.1 mg/kg or 1 mg/kg GSAO-AF750. 6 hours later the brains were excised, fixed, paraffin embedded, sectioned and imaged *ex vivo*. GSAO-AF750 fluorescence was higher in the cryolesion of mice injected with 1 mg/kg GSAO-AF750 than 0.1 mg/kg GSAO-AF750.
The accumulation of GSAO-conjugates in the brain cryolesion is dependent on the size of the lesion.

The influence of lesion size on GSAO-AF750 accumulation was also investigated. Mice bearing a 5 second cryolesion were administered 1 mg/kg GSAO-AF750 and imaged at 1, 3 and 6 hours (Figure 41). GSAO-AF750 was observed in the lesions at all time points, however the total fluorescence was 4.5-7 fold less than mice bearing a 60 second lesion (Figure 42). The same look up table cannot be applied to the 5 second and 60 second groups for the reasons mentioned above. The images shown in Figure 38 are provided to demonstrate GSAO-AF750 accumulation in the lesion.
Figure 41. Time serial of three individual mice bearing a 5 second brain cryolesion injected with 1 mg/kg GSAO-AF750. Mice bearing a 5 second cryolesion were injected with 1 mg/kg GSAO-AF750 and imaged at 1, 3 and 6 hours. Three representative mice are shown. GSAO-AF750 was observed in the lesion at all time points.
Figure 42. GSAO-AF750 accumulation in the brain cryolesion is dependent on the size of the lesion. Mice bearing either a 5 second (blue) or 60 second cryolesion (green) were injected with 1 mg/kg GSAO-AF750 and imaged at 1, 3, and 6 hours. GSAO-AF750 could be detected in the lesion in both groups at all time points, however GSAO-AF750 fluorescence was significantly lower in mice bearing a 5 second lesion (p value < 0.05). Results are the mean and standard deviation of 5-11 independent measurements. 60 second (1 h n = 6, 3 h n = 11, 6 h n = 11), 5 second (1 h n = 5, 3 h n = 5, 6 h n = 5).

Mice bearing a 5 second lesion were also sacrificed at 6 hours and their brains imaged ex vivo (Figure 43). The same look up table cannot be applied to both 5 second and 60 second groups for reasons detailed above. Consequently a direct visual comparison should not be made between the ex vivo images from each group. The images are provided only to demonstrate GSAO-AF750 accumulation in the lesion. GSAO-AF750 fluorescence was 17 fold higher in mice bearing a 60 second lesion than mice bearing a 5 second lesion (Figure 44). The excised brains were also fixed, paraffin embedded and sectioned for fluorescence imaging (Figure...
GSAO-AF750 fluorescence was higher in mice bearing a 60 second cryolesion than a 5 second cryolesion.

**Figure 43. Ex-vivo imaging of brains with a 5 second cryolesion.** Mice bearing a 5 second cryolesion were injected with 1 mg/kg GSAO-AF750. 6 hours later brains were excised and imaged ex vivo. 3 representative brains are shown. GSAO-AF750 could be detected in the lesions.

**Figure 44. GSAO-AF750 Fluorescence in the 5 second brain cryolesion ex-vivo.** Mice bearing either a 5 second (blue) or 60 second cryolesion (green) were injected with 1 mg/kg GSAO-AF750. 6 hours later the brains were excised and imaged ex vivo. GSAO-AF750
fluorescence was significantly higher in the 60 second cryolesions than the 5 second cryolesions (p value < 0.01). Results are the mean and standard deviation of 5-11 independent measurements. 60 second n = 11, 5 second n = 5.

Figure 45. GSAO-AF750 Fluorescence in *ex vivo* brain sections. Mice bearing either a 5 second or 60 second cryolesion were injected with 1 mg/kg GSAO-AF750. 6 hours later the brains were excised, fixed, paraffin embedded, sectioned and imaged *ex vivo*. GSAO-AF750 fluorescence was higher in mice bearing a 60 second cryolesion than a 5 second cryolesion.
Discussion

In Chapter 4 I have explored the *in vivo* utility of GSAO-conjugates. To maximise tissue penetration and minimise the contribution of tissue auto-fluorescence, GSAO was conjugated to the near infra-red fluorophores Cy5.5 and Alexa Fluor 750. Using flow cytometry, the probes were verified in *in vitro* models of cell death. GSAO-conjugates specifically labelled dying cells *in vitro*, whilst GSCA-conjugates did not. Labelling was also inhibited by the synthetic dithiol DMP which sequesters the trivalent arsenic of GSAO.

Dual fluorescence/bioluminescence imaging was also employed in an *in vitro* model of cryo injury. GSAO-AF750 labelled bioluminescent negative cells subjected to a dry ice lesion, whereas GSCA-AF750 did not. Healthy bioluminescent positive cells labelled with neither.

Following *in vitro* validation, the probes were then applied to mouse models of tumour cell death and brain trauma. Mice bearing a subcutaneous colon carcinoma were treated with chemotherapy and injected with Cy5.5 conjugates. *Ex vivo* analysis of the tumours by fluorescence confocal microscopy revealed co-localisation of GSAO-Cy5.5 with caspase positive apoptotic cells. GSCA-Cy5.5 was not observed in any of the tumour sections analysed. GSAO-Cy5.5 therefore specifically labels dying tumour cells *in vivo*.

Mice bearing a brain cryolesion were also injected with Alexa Fluor 750 conjugates and imaged using small animal fluorescence imaging. GSAO-AF750 accumulated in the lesion and was retained for up to 72 hours. GSCA-AF750 could not be detected in the lesion at any time point. The probes cleared rapidly from non target organs lending to an optimal signal to noise ratio as early as 3 hours post injection. The prolonged retention of GSAO-AF750 in the
kidneys indicates that it may be cleared from the body through the renal system. Both the concentration of the probe and the size of the cryolesion influenced accumulation of GSAO-AF750 in the lesion. The specificity of GSAO-AF750 for dying cells was also confirmed via \textit{ex vivo} analysis of tissue sections. Fluorescence imaging and immunohistochemistry revealed co-localisation of GSAO-AF750 with TUNEL positive apoptotic cells in the lesion.

The success of an optical imaging agent \textit{in vivo} is dependent on efficient delivery of the probe to target cells and rapid elimination of unbound ligand so that a high signal to noise ratio is achieved. Non-specific binding of the probe to non target tissues naturally decreases this ratio and confounds interpretation of data (Kovar et al., 2007). In Chapter 4, I have demonstrated that near infra-red conjugates of GSAO retain specificity for dying cells both \textit{in vitro} and \textit{in vivo}. The Alexa Fluor 750 conjugates display favourable biodistribution in that they are rapidly cleared from non target tissues resulting in a high signal to noise ratio. The near infra-red conjugates are therefore likely to have many applications to the noninvasive monitoring of cell death experimentally.
Chapter 5.

Imaging tumour cell death \textit{in vivo} with radio-labelled GSAO.

\textbf{Introduction}

In Chapter 4 I explored the application of near infra-red GSAO-conjugates to pre-clinical mouse models of cell death. Optically tagged GSAO retained its specificity for dying cells \textit{in vitro} and \textit{in vivo} and was used to noninvasively image cell death in a mouse model of brain trauma.

Optical imaging is now starting to be incorporated into clinical practice. This has recently become possible due to advances in camera systems for real time, intraoperative visualisation of near infra-red probes (Schaafsma et al., 2011). Two such probes are currently registered by the Food and Drug Administration and the European Medicines Agency, and have found clinical application in sentinel lymph node mapping for breast, skin, gastro-intestinal, non-small cell lung, oropharyngeal and gynaecological cancer (Bredell, 2010; Kusano et al., 2008; Tagaya et al., 2008; Tanaka et al., 2009; Troyan et al., 2009; Yamashita et al., 2011). Targeted near infra-red probes are also being trialled in image guided surgery to delineate tumour margins and aid resection (Keereweer et al., 2011).

Optical probes have a number of advantages over radiopharmaceutical tracers. The patient is not subjected to radiation and the associated infrastructure (radiochemist, cyclotron and
specialised waste processing facility) is not required (Keereweer et al., 2011). Nonetheless, optical imaging is limited to superficial applications in tissues with reasonable transparency. Near infra-red light cannot penetrate more than a couple of centimetres in most tissues (Kovar et al., 2007; Ntziachristos, 2010).

If tagged GSAO is to be applied to deep seated tissues or organs it must be adapted for more penetrative imaging modalities. The most sensitive imagers, such as single photon emission computed tomography (SPECT), utilise radiotracers. This technology uses a gamma camera to capture the emission of photons from radionuclides injected into the patient. Multiple 2D images are acquired and reconstructed by a computer into a 3D dataset.

To explore the application of GSAO to nuclear imaging, GSAO and GSCA were tagged with \(^{111}\text{In}\) via a diethylenetriaminepentaacetic acid (DTPA) linker. The choice of radionuclide and chelator is crucial to the development of the imaging agent. Ideally the radionuclide should have wide commercial availability and be relatively inexpensive to produce. Its half life should also be long enough to accommodate the chelation chemistry, but short enough to minimise the patient’s exposure to radiation. As many hospitals do not have a cyclotron onsite, the half life should also facilitate transport from an offsite facility. With a half life of 67 hours \(^{111}\text{In}\) satisfies these requirements. This is assisted by the DTPA linker which exhibits fast complex association rates and straightforward chelation chemistry (Boswell and Brechbiel, 2007). Finally, the radionuclide should be linked to the ligand without impairing the functionality of either. To confirm that conjugation to \(^{111}\text{In}\) did not affect the specificity of the probe for dying cells, \(^{111}\text{In}\)-GSAO was therefore validated in \emph{in vitro} models of cell death.
The success of any imaging agent *in vivo* is largely dependent on the biodistribution of the compound. Non-specific binding to non target tissues and prolonged retention in clearance organs significantly reduces the signal to background ratio, contrast and thus resolution. Although near infra-red conjugates of GSAO displayed favourable biodistribution, the use of different reporter molecules can dramatically change the way the probe interacts with the body. The biodistribution of $^{111}\text{In}$-GSAO and $^{111}\text{In}$-GSCA was therefore investigated. Mice bearing a subcutaneous Lewis lung carcinoma were injected with $^{111}\text{In}$-GSAO or $^{111}\text{In}$-GSCA, and their organs harvested 5 hours later. Bound radioactivity in the tumour, blood, kidneys, liver and spleen were measured using a gamma counter.

The application of radio-labelled GSAO to whole body *in vivo* imaging was then explored. Mice bearing a subcutaneous Lewis lung carcinoma were co-injected with $^{111}\text{In}$-GSAO and $^{99m}\text{Tc}$-Annexin V and imaged at 5 hours using dual SPECT/CT. This technology combines the nuclear image from SPECT with detailed anatomical information from X-ray CT allowing greater localisation of the probe. Mice bearing a subcutaneous human colorectal carcinoma were also injected with $^{111}\text{In}$-GSAO and imaged by SPECT/CT.
Materials and Methods

The preparation of GSAO-DTPA

DTPA-bis(anhydride) (DTPAA) (4.0 g, 11.4 mmol) and sodium bicarbonate (2.6 g, 30.5 mmol) in 30 mL dimethylformamide were stirred under argon at room temperature. GSAO (0.20 g, 0.37 mmol) was dissolved in 2 mL of deaerated deionized water and added dropwise into the reaction mixture. The solution was stirred at room temperature overnight. The reaction mixture was acidified with conc hydrogen chloride (6 mL) and then evaporated. The crude product was dissolved with 1M hydrogen chloride (aq) and purified by C₁₈ flash chromatography (70 g column) in two portions with water-acetonitrile gradient as eluant (40 mL/min flow rate monitoring at 257 nm) to yield product (99.6 mg, 29.5%) upon lyophilization.

¹H NMR (300 MHz, D₂O) δ(ppm): 1.89 (m, 1H), 2.08 (m, 1H), 2.32 (t, 2H), 2.88 (m, 1H), 3.05 (m, 1H), 3.13 (t, 2H), 3.17 (t, 2H), 3.26 (t, 2H), 3.35 (t, 2H), 3.38 (s, 2H), 3.62 (s, 2H), 3.74 (s, 2H), 3.82 (s, 4H), 3.87 (s, 2H), 3.89 (s, 2H), 4.29 (m, 1H), 4.54 (m, 1H), 7.50 (t, 2H), 7.59 (d, 2H).

¹³C NMR (75.4 MHz, D₂O) δ(ppm): 26.3, 31.6, 33.8, 36.3, 41.2, 50.3, 50.8, 51.8, 51.9, 52.3, 52.8, 54.3, 56.2, 56.3, 121.1, 130.2, 139.2, 144.1, 168.4, 170.6, 170.7, 171.4, 172.3, 172.4, 172.9, 174.5, 174.8.

HRMS (negative ion mode) for C₃₂H₄₁N₇O₁₈S₈S: calculated 904.1658, found 904.1651.
Figure 46. Overview of the synthesis of GSAO-DTPA. Dimethylformamide (DMF). Sodium bicarbonate (NaHCO₃).

Figure 47. The structure of GSAO-DTPA.

The preparation of GSCA-DTPA

DTPAA (3.1g, 8.6 mmol) and disopropylethylamine (10 mL, 7.4 g, 57.4 mmol) in 40 mL dimethylformamide were stirred under argon at 60 °C until clear, then allowed to cool to room temperature. GSCA (0.21 g, 0.43 mmol) was dissolved in 3 mL of 0.5 M sodium bicarbonate solution and then added dropwise into the reaction mixture. The solution was stirred at room temperature overnight. The reaction mixture was evaporated and the residue
dissolved in hydrogen chloride (aq) to pH 1 (16 mL). The crude product solution was purified by C\textsubscript{18} flash chromatography (25 g column) in two portions with water-acetonitrile gradient as eluant (40 mL/min flow rate monitoring at 254 nm) to yield product (222.1 mg, 60.5%) upon lyophilization.

$^1$H NMR (300 MHz, D\textsubscript{2}O) δ(ppm): 1.79 (m, 1H), 1.96 (m, 1H), 2.24 (t, 2H), 2.82 (m, 1H), 3.00 (m, 1H), 3.06 (t, 2H), 3.11 (t, 2H), 3.22 (t, 2H), 3.33 (t, 2H), 3.34 (s, 2H), 3.54 (s, 2H), 3.69 (s, 2H), 3.78 (s, 4H), 3.82 (s, 2H), 3.84 (s, 2H), 4.18 (m, 1H), 4.45 (m, 1H), 7.42 (t, 2H), 7.81 (d, 2H).

$^{13}$C NMR (75.4 MHz, D\textsubscript{2}O) δ(ppm): 26.5, 31.8, 34.1, 36.7, 41.5, 50.4, 51.0, 52.2, 52.3, 52.5, 53.1, 54.6, 56.6, 56.7, 120.5, 126.0, 131.1, 141.9, 168.3, 169.9, 170.6, 171.1, 171.4, 172.5, 172.6, 173.0, 174.6, 175.0.

HRMS (negative ion mode) for C\textsubscript{33}H\textsubscript{45}N\textsubscript{7}O\textsubscript{18}S: calculated 858.2469, found 858.2448.

Figure 48. Overview of the synthesis of GSCA-DTPA. Dimethylformamide (DMF). Sodium bicarbonate (NaHCO\textsubscript{3}).
Figure 49. The structure of GSCA-DTPA.

The preparation of $^{111}$Indium labelled GSAO and GSCA

A stock solution of GSCA-DTPA or GSAO-DTPA was prepared in double-distilled water at 1 mg per mL and stored at 4°C. 9.2 µg of DTPA conjugate in 0.50 mM sodium acetate trihydrate, 0.25 mM ascorbic acid buffer was incubated with 1 mCi of $^{111}$In at room temperature for 10-20 minutes. Aliquots were stored frozen.

Cell culture

Jurkat A3 cells, CT26.WT cells (ATCC) were cultured in RPMI-1640 medium supplemented with 10% foetal bovine serum, 2 mM L-glutamine, and 1 µg per mL penicillin/streptomycin. Lewis lung carcinoma (LLC1) cells (ATCC) were cultured in DMEM medium supplemented with 10% foetal bovine serum, 2 mM L-glutamine, and 1 µg per mL penicillin/streptomycin.
The *in vitro* validation of $^{111}$Indium labelled GSAO and GSCA

$2 \times 10^5$ Jurkat A3 cells were treated with 500 nM staurosporine for 5 h. In one instance, cells were pre-treated with 50 µM of the caspase inhibitor, Z-VAD-FMK. Cells were then incubated with 5 µCi of $^{111}$In-GSCA or $^{111}$In-GSAO for 15 min and washed 3 times with ice cold PBS. Bound radioactivity was measured using a Wizard 1470 Automatic Gamma Counter (Perkin Elmer).

**Primary tumour growth**

7-9 week old mice C57BL/6 mice (Covidien Imaging Solutions) were held in groups of 3 at a 12 h day and night cycle and were given animal chow and water *ad libidum*. A suspension of $0.5 \times 10^6$ Lewis lung carcinoma (LLC1) cells in 0.1 mL of PBS was injected subcutaneously in the proximal midline (Yuan et al., 2006).

CT26.WT human colorectal carcinomas were established in Balb/c nude mice as described in Chapter 4.
The biodistribution of $^{111}\text{In}$-GSCA or $^{111}\text{In}$-GSAO in mice bearing Lewis lung carcinomas

Mice bearing 0.5 g subcutaneous Lewis lung carcinomas were injected intravenously with 350-750 µCi of $^{111}\text{In}$-GSCA or $^{111}\text{In}$-GSAO. 5 h later the blood, kidneys, livers, lungs, spleens and tumours were harvested and bound radioactivity measured using a Wizard 1470 Automatic Gamma Counter (Perkin Elmer). Results are shown as percentage of the injected dose per gram of tissue weight (%ID/g).

Dual SPECT/CT imaging of tumour cell death using $^{111}\text{In}$-GSAO and $^{99m}\text{Tc}$-Annexin V

C57BL/6 mice bearing Lewis lung carcinoma tumours were injected intravenously with 350-750 µCi of $^{111}\text{In}$-GSAO or $^{99m}\text{Tc}$-Annexin V (HYNIC-Annexin V, Theseus Imaging Corp). Balb/c nude mice bearing CT26.WT colorectal carcinomas were also injected with 750 µCi of $^{111}\text{In}$-GSAO. 5 h later dual images in separate energy windows were collected using a Nano SPECT/CT camera (Bioscan). The acquisition time was 28 min. Image intensities were calibrated to account for the difference in decay between $^{111}$Indium and $^{99m}$Technetium.
Results

The validation of radio-labelled GSAO and GSCA in in vitro models of cell death

In order to demonstrate the in vivo utility of radio-labelled GSAO, GSAO was conjugated to \(^{111}\)Indium via a DTPA linker. It was then validated in an in vitro model of cell death. Jurkat cells were treated with staurosporine in the presence or absence of the broad spectrum caspase inhibitor Z-VAD-FMK. In one condition cells were left untreated. Cells were then incubated with \(^{111}\)In-GSAO or \(^{111}\)In-GSCA and bound radioactivity measured using a gamma counter.

\(^{111}\)In-GSAO accumulated 6.1 fold more in staurosporine treated cells than untreated cells, and labelling was inhibited by the broad spectrum caspase inhibitor Z-VAD-FMK. There was no labelling of the cells with control \(^{111}\)In-GSCA (Figure 50). Radioisotope conjugates of GSAO and GSCA therefore behave the same way in vitro as their fluorophore counterparts.
Figure 50. The accumulation of $^{111}$In-GSAO or $^{111}$In-GSCA in an *in vitro* model of cell death.

Jurkat cells were treated with staurosporine in the presence or absence of the caspase inhibitor Z-VAD-FMK. In one condition cells were also left untreated. Cells were then incubated with either $^{111}$In-GSAO or $^{111}$In-GSCA and the bound radioactivity measured using a gamma counter. $^{111}$In-GSAO labelled staurosporine treated cells significantly more than untreated cells, and labelling was blocked by inhibition of caspases. $^{111}$In-GSCA did not label any cells. Results are the mean and standard deviation of samples in duplicate from three independent experiments.
The biodistribution of radio-labelled GSAO and GSCA in tumour bearing mice

Following *in vitro* validation, $^{111}$Indium conjugates were used to investigate the biodistribution of the compound *in vivo*. Mice bearing subcutaneous murine Lewis lung tumours were injected intravenously with $^{111}$In-GSCA or $^{111}$In-GSAO. 5 h later the blood, kidneys, livers, lungs, spleens and tumours were harvested and the radioactivity measured using a gamma counter. The data is presented as a percentage of the injected dose per gram of tissue weight (%ID/g). $^{111}$In-GSAO was mostly present in the kidneys, where the compound is excreted. Almost 1/5$^{th}$ of the total injected dose was observed in this organ (Table 2). Between 1-2% of the compound was also found in the liver, lungs, spleen and tumour. Very low levels of control $^{111}$In-GSCA were found in any of the organs (Figure 51). Like its near infra-red relative, this is probably because it is cleared from the body within the first couple of hours. Minimal uptake of $^{111}$In-GSAO by non target tissues such as the blood is likely to favour a high signal to noise ratio *in vivo*, however high retention in the kidneys will likely preclude resolution of cell death in this organ.
Figure 51. The biodistribution of $^{111}$In-GSAO or $^{111}$In-GSCA in mice bearing Lewis lung carcinomas. C57BL/6 mice bearing subcutaneous Lewis lung carcinomas were injected with either $^{111}$In-GSAO or $^{111}$In-GSCA. 5 h later various organs were harvested and measured for radioactivity using a gamma counter. $^{111}$In-GSAO predominantly accumulated in the kidneys, liver and tumour. Minimal amounts of $^{111}$In-GSCA could be detected in the organs. Results are the mean and standard deviation of 4-68 individual measurements.
Table 2. Quantitative biodistribution of $^{111}$In-GSCA or $^{111}$In-GSAO in C57BL/6 mice bearing Lewis lung carcinomas. C57BL/6 mice bearing subcutaneous Lewis lung carcinomas were injected with either $^{111}$In-GSAO or $^{111}$In-GSCA. 5 h later various organs and the tumour were harvested and measured for radioactivity using a gamma counter. The highest accumulation of $^{111}$In-GSAO was found in the kidneys, where $19.62 \pm 8.604 \%$ of the total injected dose was observed. This was followed by the tumour at $2.242 \pm 1.262 \%ID/g$ and the liver at $1.959 \pm 0.556 \%ID/g$. $^{111}$In-GSCA was barely detectable in any organ except for the kidneys. Results are the mean and standard deviation of 4-68 individual measurements.

The noninvasive imaging of tumour cell death in vivo using radio-labelled GSAO and SPECT/CT

$^{111}$Indium conjugates were then used to visualise tumour cell death in vivo by SPECT/CT imaging. Mice bearing subcutaneous murine Lewis lung tumours were co-injected with $^{111}$In-GSAO and $^{99m}$Tc-Annexin V. 5 h later dual SPECT/CT images were captured (Figure 52). Consistent with the biodistribution data above $^{111}$In-GSAO was observed in the kidneys, liver and tumour. A similar pattern was observed with $^{99m}$Tc-Annexin V, however uptake in the
kidneys and liver appeared a great deal higher. Accumulation of Annexin V in the liver is in accordance with its site of metabolism (Kartachova et al., 2004; Tait et al., 2005). $^{111}$In-GSCA could not be detected in the mice (results not shown).

Figure 52. SPECT/CT imaging of $^{111}$In-GSAO and $^{99m}$Tc-Annexin V in mice bearing Lewis lung carcinoma. Mice bearing a subcutaneous Lewis lung carcinoma were co-injected with $^{111}$In-GSAO and $^{99m}$Tc-Annexin V and imaged by SPECT/CT 5 h later. $^{111}$In-GSAO and $^{99m}$Tc-Annexin V was observed in the kidneys, liver and tumour. $^{111}$In-GSAO had less uptake in the kidneys and liver than $^{99m}$Tc-Annexin V. The position of the tumour is indicated.

$^{111}$In-GSAO was also injected in mice bearing subcutaneous CT26 colorectal carcinomas for SPECT/CT imaging. In accordance with previous murine tumour models, $^{111}$In-GSAO accumulated in the kidneys, liver and tumour (Figure 53). In both models, a punctuate distribution of $^{111}$In-GSAO in the tumour was observed, which is consistent with labelling of patches of dying or dead cells.
Figure 53. SPECT/CT imaging of $^{111}$In-GSAO in mice bearing CT26.WT colorectal carcinoma. Mice bearing a subcutaneous CT26.WT colorectal carcinoma were injected with $^{111}$In-GSAO and imaged by SPECT/CT 5 h later. $^{111}$In-GSAO was observed in the kidneys, liver and tumour, consistent with distribution of $^{111}$In-GSAO in the mouse tumour model above. The position of the tumour is indicated.
Discussion

In Chapter 5 we have explored the application of radio-labelled GSAO to imaging cell death. GSAO and GSCA were conjugated to $^{111}$Indium and the probes verified in an *in vitro* model of cell death. Accumulation of the compounds was measured by gamma counter. $^{111}$In-GSAO but not $^{111}$In-GSCA labelled dying cells *in vitro*.

The biodistribution of the compounds were then explored in mice. Mice bearing subcutaneous Lewis lung carcinomas were injected with $^{111}$In-GSAO or $^{111}$In-GSCA and their organs harvested 5 hours later. Bound radioactivity was measured by gamma counter. $^{111}$In-GSCA cleared rapidly from the body and was barely detectable in the tumour and organs investigated. $^{111}$In-GSAO also cleared rapidly from the majority of the body, however prolonged retention was observed in the kidneys where the compound is excreted. $^{111}$In-GSAO was also observed in the liver and tumour. The rapid clearance of $^{111}$In-GSAO from non target tissues is likely to contribute to a high signal to noise ratio *in vivo*, and thus greater resolution of cell death. High retention of the probe in the kidneys however is likely to preclude imaging in the renal system.

The quantitative biodistribution of the compound was then compared with whole body *in vivo* imaging using SPECT/CT. Mice bearing subcutaneous Lewis lung carcinomas were co-injected with $^{111}$In-GSAO and $^{99m}$Tc-Annexin V and imaged 5 hours later. Mice bearing a subcutaneous human colorectal carcinoma were also injected with $^{111}$In-GSAO and imaged by SPECT/CT. Consistent with the biodistribution data, the highest uptake of $^{111}$In-GSAO was observed in the kidneys, liver and tumour in both models. A similar pattern was observed for $^{99m}$Tc-Annexin V.
The studies so far are preliminary in nature. We are yet to verify that $^{111}$In-GSAO is labelling dying cells in the tumour. Certainly the sporadic distribution of $^{111}$In-GSAO is consistent with the patches of dying cells often observed histologically in tumours. This is particularly true of fast growing tumours where a lagging microcirculation leads to discrete areas of hypoxia throughout. Nonetheless future experiments will employ TUNEL/caspase staining alongside autoradiography of tumour sections to confirm co-localisation of $^{111}$In-GSAO with apoptotic cells. If $^{111}$In-GSAO is to be used to monitor treatment response in tumours clinically, it will also be necessary to demonstrate increased uptake of $^{111}$In-GSAO in response to chemotherapy. Murine models are currently being developed in the laboratory for this purpose.

Although further pre-clinical testing is required these preliminary studies indicate that radio-labelled GSAO could find utility in vivo. Certainly the biodistribution profile of $^{111}$In-GSAO favours a decent signal to noise ratio, and no signs of toxicity were observed. In comparison to $^{99m}$Tc-Annexin V, $^{111}$In-GSAO also exhibits less accumulation in the hepatic system permitting better imaging of the liver and liver proximal structures.
Chapter 6.

Discussion

General Discussion

This thesis describes a small synthetic organoarsenical GSAO, tagged with various reporter moieties for the noninvasive imaging of cell death.

Tagged GSAO labels dying cells \textit{in vitro}

GSAO was conjugated to fluorophores and applied to \textit{in vitro} models of cell death. Tagged GSAO labelled dead and dying cells, whilst the control compound GSCA, containing a chemically inert carboxylic acid in place of the reactive trivalent arsenic, did not. Labelling of dying cells with GSAO-conjugates was rapid, saturable and highly specific. GSAO-conjugates labelled dying cells independent of the cell death pathway activated or the reporter fluorophore attached.

Interestingly, multiple GSAO-fluorophore positive populations were observed with differing fluorescence intensities. For instance, in cells treated with Fas Ab, two distinct GSAO-OG$^+/PI^+$ subpopulations were observed with mean OG intensities of 7571 and 604. It’s possible this represents cells in different phases of death, and that those in the latter stages with increased membrane permeability, manifest greater GSAO-fluorophore accumulation and thus fluorescence. The converse is also possible. In the early stages of apoptosis water is
effluxed from the cytoplasm resulting in cellular shrinkage (Taylor et al., 2008). The dramatic reduction in cytosolic volume may limit the accumulation of GSAO-conjugates and reduce fluorescence intensity. As the cell progresses along the apoptotic pathway, membrane blebbing also gives rise to smaller apoptotic bodies and these may absorb even less GSAO-fluorophore.

All things being equal, pharmacological induction of apoptosis in culture would assumingly produce a homogenous population of cells uniformly progressing through the apoptotic pathway, rather than a heterogeneous mix undergoing different stages of cell death at different times. Indeed only a single Annexin V\(^+\)/PI\(^+\) population is observed at the end of staurosporine treatment. Nonetheless it is possible that Annexin V and PI are insensitive to subtle differences in the latter stages of cell death are thus unable to differentiate between the different populations. Other aspects of flow cytometry were therefore employed to investigate the contribution of smaller cells or apoptotic bodies to the GSAO-OG positive subpopulations.

In flow cytometry, a stream of single cells is passed through a laser and variations in light scattering used to determine characteristics of that cell. The forward scatter (FSC) of light is a robust measurement of cell size and the GSAO-OG positive subpopulations were back-gated against this parameter to determine whether differences in fluorescence intensity were related to variation in cell volume. Interestingly in both treated and untreated samples, each of the GSAO-OG positive subpopulations exhibited the same forward scatter and did not vary in size. A reduction in cell volume following apoptotic shrinkage or the shedding of apoptotic bodies is therefore unrelated to variations in GSAO-OG positivity.
It is also a possibility that variations in OG fluorescence intensity are related to different mechanisms of cell death occurring in vitro. Whilst staurosporine and Fas Ab directly activate intrinsic and extrinsic apoptotic pathways, long incubation times may also result in a nutrient deprived population of cells undergoing alternate cell death mechanisms. Multiple forms of cell death may thus be observed within one sample.

Nutrient deprivation has long been associated with the process of autophagy, whereby the cell undergoes partial self digestion in order to free up essential building blocks and extend cell survival. Large volumes of the cytoplasm including proteins and whole organelles are encapsulated in double membrane vesicles called autophagosomes. These in turn fuse with lysosomes to form autophagolysosomes in which the contents are degraded and the constituents recycled (Baehrecke, 2005; Vicencio et al., 2008). Autophagy is typically employed by cells in response to hypoxia and starvation and is thus a dominant feature of established tumours. The mechanism allows short term adaptation to the stressful tumour microenvironment until the delivery of nutrients is restored via angiogenesis (Mathew et al., 2007; Weiner and Lotze, 2012; Wirawan et al., 2012). Autophagy is also employed by tumour cells in response cellular damage and stress induced by therapeutic treatment (Dalby et al., 2010; Maycotte et al., 2010).

Because autophagy reflects situations of excessive cellular stress, it is not uncommon for it to be associated with cell death. Indeed the term ‘autophagic cell death’ is often used to describe tumour cell death in which the hallmarks of autophagy are observed. For example, cellular demise may be accompanied by formation of double membrane vesicles (autophagosomes or autophagolysosomes) or the redistribution of autophagy specific proteins such as microtubule-associated protein light chain 3 (LC3) into vesicle structures (Tsujimoto and Shimizu, 2005).
However there is very little evidence to suggest that autophagy actually precipitates cell death, that is, that cell death is executed by autophagy. Instead, it is now generally accepted that autophagy represents a cell’s attempt to prevent cell death, and that the cell dies with autophagy, rather than as a result of it (Green et al., 2009; Kroemer et al., 2005; Weiner and Lotze, 2012). Nonetheless, this population of cells is biochemically and morphologically distinct from cells undergoing canonical apoptotic pathways, and may thus contribute to the different GSAO-OG positive subpopulations observed.

Nutrient deprivation can also promote cellular necrosis, and necrotic cells may account for specific GSAO-OG positive subpopulations within treated and untreated samples. Unlike apoptotic cells which shrink following an efflux of cytoplasmic constituents, necrotic cells are characterised by excessive cellular oedema and are therefore likely to demonstrate a different capacity for GSAO-OG (Al-Rubeai and Fussenegger, 2004; Kroemer et al., 2005). This may result in varying degrees of GSAO-OG positivity.

There are two primary mechanisms through which nutrient deprivation promotes necrosis. Firstly, in direct contrast with apoptosis, necrosis is largely ‘passive’ or ATP independent. The withdrawal of ATP following glucose deprivation therefore limits the cells capacity for apoptosis and promotes a shift to necrotic death (Caro-Maldonado and Munoz-Pinedo, 2011). Secondly, starvation also deprives the cell of the metabolic intermediates required to produce antioxidants such as NADPH and glutathione. Nutrient deprivation therefore stimulates the production of reactive oxygen species (Ahmad et al., 2005). This is mechanism through which severe ischemia precipitates necrosis in cardiomyocytes (Bahi et al., 2006). This is also the mechanism through which nutrient deprivation and hypoxic stress induces a necrotic core in tumours, particularly in cancer cells that have acquired resistance to the mitochondrial
pathway of apoptosis (Caro-Maldonado and Munoz-Pinedo, 2011). If prolonged incubation times result in nutrient deprivation and so give rise to a heterogeneous mix of apoptotic, necrotic and post-autophagic cells, it is possible that these different populations may display different GSAO-OG fluorescence intensities.

Experiments were thus conducted in which cells were subjected to various starvation conditions. In the first instance cells were subjected to severe serum deprivation by culturing in the absence of foetal bovine serum. In the second instance cells were subjected to a gradual nutrient decline by culturing for extended periods of time without media replacement. Cells were stained with GSAO-OG/PI, analysed by flow cytometry and compared to samples treated with an apoptotic inducer (as displayed in Chapters 2 and 4).

In both starvation conditions, the proportion of cells contributing to the different GSAO-OG$^+$ subpopulations did not change. It is possible that these conditions were not sufficiently nutrient deprived and did not in fact push cells into autophagy or necrosis. Future studies will therefore subject cells to longer incubation times in the absence of serum or fresh media. Additional fluorescent probes (such as antibodies against LC3) will also be used to confirm induction of the respective pathways. Future studies will also explore the contribution of hypoxic cell death in vitro. This is particularly relevant to assessing the ability of tagged-GSAO in instances of tumour cell death, as both glucose starvation and oxygen deprivation are known to contribute to cell death in tumours (Caro-Maldonado and Munoz-Pinedo, 2011). Nonetheless, our preliminary data indicates that additional mechanisms of cell death do not account for differences in GSAO-OG positivity.
The mechanism of action of tagged GSAO has been elucidated

Tagged GSAO entered cells during the mid-late stages of cell death, coincident with a loss of plasma membrane integrity. The probe was retained in the cytosol predominantly by covalent reaction with the protein Hsp90. Hsp90 is the most abundant molecular chaperone of the eukaryotic cytoplasm and plays a role in a number of fundamental cellular pathways. Tagged GSAO cross links closely spaced thiols in the C-terminal end of Hsp90 (Figure 54).

Figure 54. An overview of the mechanism of action of GSAO-conjugates. The plasma membrane of viable cells is impermeable to GSAO-conjugates, however loss of plasma membrane integrity in late apoptotic or necrotic cells allows the probe to enter. GSAO-conjugates are retained in the cytosol by covalent reaction with dithiols in Hsp90.
Tagged GSAO can be used to noninvasively image cell death *in vivo*

Near infra-red conjugates of GSAO were applied to pre-clinical models of brain trauma and tumour cell death in mice. Tagged GSAO specifically labelled dying cells *in vivo*, whilst the control compound GSCA, containing a chemically inert carboxylic acid in place of the reactive trivalent arsenic, did not. Near infra-red GSAO was used to noninvasively image cell death in the body using small animal fluorescence imaging. GSAO was also conjugated to $^{111}$Indium and the biodistribution of the compound determined. $^{111}$In-GSAO was used to noninvasively image tumour cell death in mice using SPECT/CT imaging.
Features of ideal imaging agent

A number of criteria should be fulfilled for a cell death imaging agent to be successful and translatable. First and foremost it must demonstrate specificity for dying cells. Unlike phosphatidylserine binding agents that exhibit cross reactivity for activated platelets, macrophages, endothelial cells and aging erythrocytes, tagged GSAO appears to be selective for dead and dying cells because it only enters necrotic cells defined by loss of cell membrane integrity. Indeed, PS can be externalised in a reversible fashion in situations of physiological stress and PS targeted agents can therefore overestimate cell death.

Ideally, the agent should also be small, easily synthesized and demonstrate attractive pharmacokinetics. Large molecules are more likely to elicit an adverse immune response in the host and exhibit slow clearance/long blood half life. Extended residency in the body in turn leads to a high background signal and prolonged radiation exposure where radiotracers are involved. Large biomolecules are also likely to be preferentially taken up by the hepatic system precluding imaging of the liver and liver proximal organs (Kovar et al., 2007). This is certainly the case for Annexin V.

With a molecular weight of only 0.5 kDa (72 times less than Annexin V) tagged GSAO is rapidly cleared from the body resulting in low background and a favourable signal to noise ratio. The small size of the compound may also enhance vascular diffusion, optimising delivery to target tissues (Kovar et al., 2007).

The cellular target is also an important consideration for any imaging agent. Certainly PS binding agents are attractive because their ligand is readily available on the external surface
of the cell. Accumulation of the agent in dying cells however is limited both by the surface area of the plasma membrane and the abundance of externalised of PS. Tagged GSAO has the major advantage of cytosolic accumulation which represents a much larger volume. The main cytosolic target of tagged GSAO is also the most abundant molecular chaperone in the cell, Hsp90. High concentrations of tagged GSAO at target cells will increase the sensitivity of detection resulting in superior visualisation and resolution of cell death.

Tagged GSAO appears to satisfy many of the favoured criteria of a cell death imaging agent. It is selective for dying cells in vitro and in vivo, small, easily synthesised and displays favourable biodistribution. GSAO is also readily conjugated to a variety of reporter moieties, such as fluorophores for experimental animal models or radioisotopes for translation to a clinical setting. In view of the prevalence of cell death in normal physiology and disease, noninvasive imaging of this process is likely to have wide application in biological research, patient diagnosis and management. Tagged GSAO is poised to accommodate this niche, however if the agent is to progress to clinical trials further studies are required.
Future Directions

Optimisation of tagged GSAO in vivo

Future research will focus on optimisation of tagged GSAO in an \textit{in vivo} setting. Specifically tagged GSAO will be used to monitor tumour response to treatment in mice following chemotherapy.

In my preliminary studies I have employed subcutaneous xenograft models. This is the most widely used pre-clinical mouse model in cancer research because it is simple, inexpensive to establish and exhibits high reproducibility. It also enables easy quantification of tumour burden, progression and response to treatment. Its major disadvantage is that the tumour microenvironment is not accurately represented, especially with regards to perfusion (De Saint-Hubert et al., 2009; Huynh et al., 2011; Kerbel, 2003). This is particularly important in the context of imaging as the tracer is often administered intravenously and variation in tumour vascularity will affect dissemination of the probe. Future studies will therefore utilise orthotopic xenograft models in which tumour cells are implanted in the organ from which they originated. By replicating tumour-host interactions these models also recapitulate the pathways of metastasis, mimicking progress of the disease in humans. It is therefore the most clinically relevant system in which to test my probe.

Ideally, future studies will also incorporate a clinically relevant treatment regimen so that the level of tumour cell apoptosis in murine models reflects that observed in human patient data. Patient tumours typically exhibit no more that 5-15% apoptosis following treatment (Davis et al., 2003; Mohsin et al., 2005). In order to accurately demonstrate the sensitivity of the probe,
tumour cell death in experimental models should not exceed this value (Nguyen et al., 2009). The obvious difficulty with these studies is that a limited window of detection exists before dying tumour cells are engulfed by surrounding immune cells (Zhao, 2009). The timing of cell death imaging is therefore crucial, and likely to require optimisation in each experimental model.

Given that tagged GSAO targets Hsp90 in dying cells, future studies will also explore the capacity of the probe to image cells with a high Hsp90 content. As mentioned, tumours cells express up to 10 fold more Hsp90 than normal cells allowing them to adapt to a hostile micro-environment and tolerate otherwise fatal genetic lesions (Ferrarini et al., 1992; Whitesell and Lindquist, 2005). This bodes well for use of tagged GSAO in instances of tumour cell death as the abundance of Hsp90 may enhance accumulation of the probe in these cells.

Breast cancers in particular are renowned for high Hsp90 expression where it plays a crucial role stabilising mutated or over-expressed proteins such as the human epidermal growth factor receptor 2 (HER-2), the progesterone receptor (PR) and the estrogen receptor (ER) (Beliakoff and Whitesell, 2004; Pick et al., 2007). Future studies will thus incorporate in vitro and in vivo models of breast cancer cell death. For instance, flow cytometry will be used to observe GSAO-OG labelling of MCF-7 and SK-BR-3 breast cancer cell lines which over-express ER and HER-2, respectively. The mean fluorescence intensity of dying breast cancer cells will then be compared to a control CHO-K1 cell line. The effectiveness of an imaging agent is largely determined by how much of the agent accumulates at the target. A high concentration of Hsp90 in these tumours may lead to greater pooling of tagged GSAO in dying cells increasing the limits of detection and improving the resolution of cell death.
A second generation cell death imaging agent

We have developed an analogue of GSAO, 4-((S penicillaminylacetyl)amino)phenylarsonous acid (PENAO), in which the GSH moiety is replaced with D-penicillamine (Figure 55). I recently investigated the specificity of this second generation compound for dying cells and found it to be superior to that of tagged GSAO.

Figure 55. The Structure of PENAO. PENAO is an analogue of GSAO in which the GSH moiety is replaced with D-penicillamine (molecular weight 390).

PENAO was coupled to Fluorescein through the primary amine and successful conjugation confirmed via HPLC and infusion electron spray ionisation mass spectrometry (results not shown). The accumulation of PENAO-F was then compared to GSAO-F in Jurkat cells treated with Fas Ab for 24 hours. Annexin V-APC and PI labelling was used to ensure that the proportion of apoptotic cells did not vary between samples. The mean Fluorescein fluorescence of PI positive cells was used to indicate the relative accumulation of each compound.
PENAO-F and GSAO-F labelled the same proportion of dying cells (results not shown), however PENAO-F positive cells exhibited a 2.4 fold higher mean fluorescence than GSAO-F labelled cells (Figure 56).

Figure 56. PENAO-F accumulates twice as much as GSAO-F in apoptotic cells. Jurkat A3 cells were treated with Fas Ab for 24 h and incubated with Annexin V-APC, PI and either GSAO-F or PENAO-F. The mean Fluorescein fluorescence of the PI positive population is shown. PENAO-F labelled cells exhibited a significantly higher mean fluorescence value (p < 0.001).

The uptake or retention of PENAO-F in dying cells is superior to that of GSAO-F. This may translate to greater signal intensity in vivo and therefore superior detection and resolution of cell death. In future studies it will be interesting to investigate why PENAO-conjugates accumulate in dying cells more than GSAO-conjugates and to apply near infra-red and radio-labelled PENAO to mouse models of cell death.
Adapting GSAO and PENAO for PET imaging

Although our preliminary investigations have involved fluorescence and SPECT imaging, it would also be of interest to explore alternate imaging modalities such as PET. In PET imaging the patient is administered a positron emitting radionuclide. Upon collision with a neighbouring electron the positron is annihilated producing two photons or gamma rays. The photons are emitted in opposite directions and captured by opposing detectors creating a ‘line of response’. This allows the event to be localised with superb sensitivity and resolution up to three orders of magnitude more than SPECT. The line of response also permits the quantitative measurement of radiotracer distribution (Nguyen and Aboagye, 2010). Therein lay the main advantages of PET imaging.

One of the major challenges with PET is that isotopes with a short half life are employed. As such, radiotracers must be produced on site necessitating a cyclotron and radiochemistry production facilities. On the other hand, the use of short lived radioisotopes allows higher tracer dosing, and thus increases the sensitivity of detection, without contributing additional radiation. For example, if a PET radioisotope has a half life of only several minutes, high amounts can be administered as the overall radiation exposure remains the same as a SPECT isotope with an extended half life at lower concentrations. This not only increases the image sensitivity, but also reduces the total time required by the scan (Rahmim and Zaidi, 2008).

In the aim of increasing the detection and resolution of cell death, GSAO and PENAO will also be adapted for PET imaging. This will be achieved through conjugation to $^{18}$F with a half life of 110 min.
Potential difficulties imaging cell death with GSAO

The small size of GSAO lends itself to fast clearance and favourable bio-distribution as compared to large biomolecules like Annexin V. Certainly little evidence of hepatic uptake was observed for $^{111}$In-GSAO or GSAO-AF750 in mice. Nonetheless, renal clearance of the compound is likely to preclude imaging of the kidney and kidney proximal structures. This is not likely to have any baring on the primary indications of tagged-GSAO, such as stroke, neurodegenerative pathologies, ischemic heart disease and monitoring tumour therapy response.

More generally, GSAO is likely to encounter hurdles related to its clinical and pre-clinical development. Firstly, the success of any cell death imaging agent necessarily depends on the detection of cell death before the apoptotic or necrotic cells are cleared. The window of detection before dying cells are engulfed by surrounding phagocytes is likely to be small, and the specific timing of events will need to be optimised for any given tumour and its treatment. This is likely to be a challenge when establishing pre-clinical models in which to validate cell death imaging agents (Zhao, 2009).

It will also prove challenging to develop models that reflect typical clinical outcomes. For instance, patient tumours often display low baseline levels of apoptosis and exhibit no more than a 6 fold increase following treatment. Even after treatment, only 5-15% of the tumour is likely to be apoptotic at a particular time (Davis et al., 2003; Mohsin et al., 2005). The cell death imaging agent will need to be sensitive to this small proportion of cells and to distinguish the subtle change following treatment. Mouse models will also need to mimic this outcome to accurately predict the clinical relevance of the agent (Nguyen et al., 2009).
**Conclusion**

In conclusion, this thesis has described a small, synthetic organo-arsenical that when tagged with reporter moieties can be used to noninvasively image cell death. The mechanism of action has now been elucidated and the agent applied to pre-clinical mouse models. Although further *in vivo* studies are required, tagged GSAO certainly holds promise for the clinical diagnosis and management of disease.
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