IN VIVO OPTICAL IMAGING OF CELL DEATH USING FLUORESCENT
SYNTHETIC COORDINATION COMPLEXES

A Dissertation

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Structurally novel fluorescent molecular imaging probes were evaluated for ability to identify tissue containing dead and dying cells using whole-animal epi-fluorescence imaging and histological analysis. The near-infrared probe, PSS-794, with an appended Zn$_2$BDPA affinity ligand, was evaluated in both subcutaneous and spontaneous prostate and mammary tumors animal models. In vivo imaging showed that PSS-794 had selectively accumulated in all tumors and targeting ratios were twice that of two control fluorophores. Ex vivo biodistribution and histological analyses suggest that PSS-794 targeted the necrotic regions of the tumors, which is consistent with in vitro microscopy. Furthermore, PSS-794 detected an increase in tumor cell death due to successful anti-tumor treatment in animal models employing focal beam radiation and chemotherapy.

The kinetic and perfusion properties of PSS-794 were assessed in two additional models of a cell death: a thymus atrophy mouse model and acute tissue damage model model. Uptake of PSS-794 in the atrophic thymi was significantly higher than control
fluorophore at all time points, and the highest amount of PSS-794 accumulation occurred at 42 h after treatment. Fluorescent histological analyses of excised thymus tissue confirmed that PSS-794 targeted the dead and dying cells within the thymus. The tissue damage study used whole animal fluorescence imaging to visualize PSS-794 uptake at the site of injury, which was confirmed by ex vivo and histological analyses. Further, a comparative study with a mechanistic similar but larger, fluorescent protein conjugate, Annexin V, showed less intense uptake at the site of muscle damage.

A preclinical mouse model for traumatic brain injury was adapted to investigate a set of complementary fluorescent imaging probes. Using non-invasive whole-body fluorescence imaging, PSS-794 permitted visualization of the injury in a living animal. Ex vivo imaging and histological analysis confirmed PSS-794 localization to site of brain cell death. The non-targeted, deep-red Tracer-653 was validated as a tracer dye for monitoring blood-brain-barrier disruption, and a binary mixture of PSS-794 and Tracer-653 was employed for multicolor imaging of cell death and blood-brain-barrier permeability in a single animal. The imaging data indicated that at three days after brain cryoinjury the amount of cell death had decreased significantly, but the integrity of the blood-brain-barrier was still impaired; at seven days the blood-brain-barrier was still three times more permeable than before cryoinjury.

Finally, multivalent Zn₂BDPA were evaluated to determine the whether increasing the number of Zn₂BDPA ligands enhances cell death imaging. Imaging probes consisting of a fluorescent squaraine rotaxane scaffold with either two or four Zn₂BDPA ligands were developed and their targeting properties were assessed in etoposide-treated
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cells. Fluorescence microscopy showed that increasing the number Zn$_2$BDPA units enabled lower concentrations of probe to be used while retaining high selectivity for dead and dying cells. When tested in three animal models of cell death, the tetra Zn$_2$BDPA probe produced higher T/NT ratios than the analogous bis Zn$_2$BDPA probe and an untargeted control fluorophore. An in vivo cold block study using an excess of Zn$_2$BDPA inhibited accumulation of both the tetra and bis Zn$_2$BDPA probe at the site of cell death. Overall, the results in this dissertation indicate that synthetic fluorescent Zn$_2$BDPA conjugates are outstanding molecular probes for optical imaging of cell death.
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ABBREVIATIONS

NIR  near-infrared
ROS  reactive oxygen species
LMP  lysosomal membrane permeabilization
PS   phosphatidylserine
FRET fluorescence energy resonance transfer
BRET bioluminescence energy resonance transfer
PET  positron emission tomography
SPECT single photon computer tomography
MRI  magnetic resonance imaging
DTPA diethylene triamine pentaacetic acid
RIP-1 receptor-interacting protein-1
RIP-3 receptor-interacting protein-3
PC   phosphatidylcholine
PE   phosphatidylethanolamine
PI   phosphatidylinositol
PSS1 PS Synthase-1
PSS2 PS Synthase-2
TIM  T cell/transmembrane, immunoglobulin, and mucin
<table>
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<tr>
<th>Acronym</th>
<th>Description</th>
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<tr>
<td>MAMs</td>
<td>mitochondrial associated membranes</td>
</tr>
<tr>
<td>ITC</td>
<td>irradiated tumor cell</td>
</tr>
<tr>
<td>DAMPS</td>
<td>damage associated molecular patterns</td>
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<tr>
<td>Zn₂BDPA</td>
<td>zinc(II)-bis-dipicolylamine</td>
</tr>
<tr>
<td>ICG</td>
<td>indocyanine green</td>
</tr>
<tr>
<td>EPR</td>
<td>enhanced permeation-retention</td>
</tr>
<tr>
<td>TUNEL</td>
<td>terminal deoxynucleotidyl transferase</td>
</tr>
<tr>
<td>ROI</td>
<td>region of interest</td>
</tr>
<tr>
<td>MPI</td>
<td>mean pixel intensities</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>TBI</td>
<td>traumatic brain injury</td>
</tr>
<tr>
<td>CT</td>
<td>computer tomography</td>
</tr>
<tr>
<td>BBB</td>
<td>blood-brain-barrier</td>
</tr>
<tr>
<td>SR</td>
<td>squaraine rotaxane</td>
</tr>
<tr>
<td>POPC</td>
<td>1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>POPS</td>
<td>1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine</td>
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CHAPTER 1:
INTRODUCTION

1.1. Background

Cell death is an essential process that plays a vital physiological and pathological role within an organism. Cell death is necessary for the formation of organs and tissues during development and also functions to rid the body of cells that have been infected and damaged by pathogens. In our lifetime, over 99.9% of our cells will undergo cell death and be eliminated from the body allowing for cellular homeostasis and our bodies to be disease-free.\(^1\) Deregulation of this homeostasis either by excessive or deficient cell death causes is responsible for the development of many pathological conditions including cancer, neurodegenerative disorders, cardiovascular diseases, autoimmune diseases, etc.

Cell death is typically divided into two well-studied processes, apoptosis and necrosis; however there is increasing evidence that other cell death modes can occur.\(^2\) Apoptosis is usually characterized by cytoplasm shrinkage, cell detachment, chromatin condensation, nuclear fragmentation and the formation apoptotic bodies. In contrast, necrotic cells exhibit increased cytoplasmic vacuolation, organelle degeneration, condensation of chromatin into irregular patches, and an increase in cell volume that
results in irreversible rupture of the plasma membrane. Biochemically, apoptotic cell
death includes activation of caspases, mitochondrial outer membrane permeabilization,
DNA fragmentation, generation of reactive oxygen species (ROS), lysosomal membrane
permeabilization (LMP) and exposure of phosphatidylserine (PS) on the outer leaflet of
the plasma membrane. One must take caution interpreting cell death mechanisms by a
single biomarker since multiple processes can display the same biomarker. For example,
PS exposure, ROS generation, and LMP have been associated with not only apoptosis,
but also necrosis. There is also evidence that crosstalk can occur between multiple
processes and that multiple processes may occur at the same time. Thus, the cell death
community would be benefitted by 1) identification of biomarkers that are specific for
each cell death process and 2) the development of imaging agents that target these
biomarkers.

1.2. Imaging Modalities

The three major imaging modalities employed for cell death imaging are optical,
radioucler, and magnetic resonance imaging (MRI). These modalities have
complementary properties that make them suitable for different types of applications.
Optical imaging can be broken into two groups, fluorescence and bioluminescence.
Fluorescence is well-suited for diagnostic and microscopy studies of cells and tissue
sections. Bioluminescent methods utilize cells that have been genetically engineered to
convert chemical substrates into light. This modality does not require an external
excitation source, thus sensitivity and signal to noise ratios are typically greater than
fluorescence imaging. For in vivo studies of living animals, a major weakness with
optical methods is the poor penetration of visible light through skin and tissue. This
technical problem is mitigated by employing imaging probes or cells that emit deep red
or near-infrared light, but the light is still highly scattered and thus the resolution of whole-body, live animal optical images is quite poor. In comparison, radionuclear imaging involving positron emission tomography (PET) and single photon emission computer tomography (SPECT) modalities provides much deeper tissue penetration while retaining high sensitivity, and thus is more attractive for many types of clinical applications. However, the high cost, low throughput, and safety concerns associated with radionuclear imaging can be serious drawbacks. Magnetic resonance imaging does not employ ionizing radiation and can produce high resolution images of deep-tissue

![Figure 1.1](image-url)

**Figure 1.1.** Imaging modalities commonly used for imaging cell death in vivo. Each modality has particular advantages and disadvantages, thus researchers should be aware of these characteristics when choosing the appropriate imaging modality. Figure adapted from references 9 and 10.
sites, but the technique is relatively insensitive. Overall, optical imaging is often well-sited for preclinical research studies, whereas radionuclear and MRI seem to be the most promising modalities for clinical imaging.

1.3. Apoptosis Biomarkers

Since 1964 when the first document case of apoptosis was made, extensive research has lead to the discovery of specific biomarkers that have enabled researchers to visualize this process in real-time. Biomarkers for apoptosis can be divided into those that are sequestered within the intracellular milieu, which include biochemical and cell signaling events. Imaging cell death in vivo using probes that target these biomarkers tends to be technically difficult since physical barriers can impede cell penetration and prevent imaging these the biochemical events within the proper kinetic window. Other biomarkers are exposed on the cell surface, which are easier to access by exogenous probes in the bloodstream. The following sections will describe common biomarkers for apoptosis and the associated imaging agents used to monitor this process in vivo.

1.3.1. Caspases

Caspases are a family of intracellular cysteine proteases that play an important role in the initiation and execution of apoptosis. Caspases are typically divided into initiators (caspase-8, -9, and -10) that commence the apoptotic cascade and executioners (caspase-3, -6, and -7), which are activated by the initiators to execute apoptosis. Furthermore, initiators are divided into caspases participating in the extrinsic (caspase-8 and -10) or intrinsic (caspase-9) apoptotic pathway. Within the cell, caspases exist as zymogens that transition to an active form when initiated by a pro-apoptotic signal. Initiator caspases and executioner caspases are activated by different mechanisms with
initiator caspases homodimerizing to enter an active form. Executioner caspases require cleavage of a catalytic domain to become active, and an initiator caspase is typically responsible for the cleavage. Once executioner caspases are activated, a proteolytic cascade begins resulting in cell death.

Activation of caspases typically occurs by cleaving peptides after Asp residues. The specificity of executioner caspases for the DEVD sequence has allowed groups to non-invasively monitor apoptosis using numerous strategies. One strategy involves genetic modification of cells with caspase responsive constructs along with bioluminescence imaging.\textsuperscript{10a} Bioluminescence imaging utilizes the enzymatic reaction of luciferase proteins with a molecular substrate, which in turn produces light that can be captured by a sensitive camera. This modality does not require an external excitation source, thus sensitivity and signal to noise ratios are typically greater than fluorescence imaging. The two main strategies for designing bioluminescent apoptosis constructs include restoring luciferase activity through structural changes upon caspase activation and bioluminescence resonance energy transfer (BRET).

Another strategy combines fluorescence imaging with caspase activatable probes or “smart” probes, which are non-fluorescent until they are recognized by the target protease and subsequently turned on, to monitor cell death. Typical, fluorescence imaging agents are designed as an “always on” probe meaning that the probe consistently fluoresces. This type of probe can cause low signal-background ratios if the probe has a long blood circulation or is prone to accumulation in non-specific organs. Activatable probes lower the fluorescence background, increase specificity, and amplify the fluorescent signal allowing for higher detection sensitivity within a living animal. Several
groups have taken this strategy to develop near-infrared (NIR) apoptosis probes for deep tissue imaging in vivo. It is often difficult to get an adequate fluorescent signal from a caspase probe since the probe must pass through the cell membrane in the time window of caspase activation. To alleviate this problem, cell-penetrating peptides have been attached to caspase activatable probes. The probe, TcapQ_{647}, which consists of a cell permeable peptide attached to a caspase substrate sequence that was flanked by a deep-red fluorophore and a spectral quencher, was specifically activated by effector caspases and not initiator caspases both in cell culture and in amoeba-induced tumor cell death.\textsuperscript{14} This probe was also effective at imaging single-retinal ganglion cell apoptosis in an in vivo rat model of glaucoma.\textsuperscript{15}

Fluorescence resonance energy transfer (FRET) strategies using spectrally similar fluorescent proteins or fluorophores that are separated by the DEVD motif have been developed for fluorescence imaging of cell death. This strategy has proven quite useful for high throughput screening of potential drugs in cell systems\textsuperscript{16} and visualization of chemotherapy-induced apoptosis in disseminated tumors\textsuperscript{17}; however, in vivo utility is limited due to tissue absorption of emitted light by the fluorescent proteins and tissue autofluorescence at shorter wavelengths. Moreover, these strategies require cells to be genetically modified and introduced into a living animals, which limits their clinical translation.

While the DEVD peptide sequence has been targeted for the development of caspase responsive probes, other strategies exist for targeting caspases during apoptosis. Isatins are known inhibitors of activated caspase-3 and caspase-7 and bind by covalently binding to cysteine and histidines within their catalytic sites, making them attractive
ligands to attach to contrast agents for apoptosis imaging. Radiolabeled isatins have sub-nanomolar affinity for caspase-3 and are effective at monitoring response due to anti-tumor treatment in pre-clinical animal models. The promise of using isatins for cell death imaging is exemplified by ([18F]ICMT-11) being selected by the QuIC-ConCePT consortium for evaluation into humans as a candidate cell death imaging probe.

Edgington and coworkers developed acyloxymethyl ketone probes that irreversibly bound to active site of caspase 3/7 through covalent interactions. Fluorescent labeling of these probes allowed for direct, non-invasive in vivo imaging of apoptosis kinetics in multiple mouse models.

1.3.2. Other Apoptosis Biomarkers

Mitochondria play a key role in the apoptotic cascade and a loss of membrane potential across the inner mitochondrial membrane (Δψm) accompanies the induction of cell death. Lipophilic phosphonium cation based compounds permeate the membrane

Figure 1.2. Intracellular and extracellular biomarkers that are commonly targeted for imaging and measuring cell death.
lipid bilayer and accumulate into healthy mitochondrial based on the strongly negative transmembrane potential.\textsuperscript{24} During apoptosis, the electrochemical proton gradient across the inner mitochondrial membrane is lost causing a reduction in phosphonium cation tracer levels.\textsuperscript{25} A $^{18}$F-fluorobenzyl triphenylphosphonium cation ($^{18}$F-FBnTP) was able to detect alterations in mitochondrial membrane potential in vivo and in vitro. $^{18}$F-FBnTP was able to measure the pharmacodynamics of paclitaxel treatment in breast cancer cells, and the decrease in $^{18}$F-FBnTP uptake coincided with other apoptotic biomarkers such as Bax expression and cytochrome c release.\textsuperscript{26} In a pre-clinical orthotopic prostate tumor treatment study, $^{18}$F-FBnTP had a significantly lower tumor uptake compared to $^{18}$F-FDG by 48 h post-treatment.\textsuperscript{26} These studies required excision of tissues and ex vivo audioradioagraphy in order to obtain $^{18}$F-FBnTP quantification. Currently, it is not clear whether $^{18}$F-FBnTP can accurately measure the kinetics and extent of apoptosis in a living animal. Biodistribution studies in healthy animals showed high uptake in the kidneys, heart, and liver suggesting that these organs would provide a high enough background signal for detection of apoptotic-related $^{18}$F-FBnTP decreases.\textsuperscript{27}

Apoptotic cells are known to be a source of DNA fragments, nucleoprotein complexes, and histones that drive the production of autoantibodies in certain autoimmunity conditions such as lupus. Histones, are normally organized into nucleosomes (H2A, H2B, H3, H4) or located on the internucleosomal DNA (histone H1) in mammalian cells. During apoptosis, the histones are released from the cytoplasm and subsequently transport to the plasma membrane where they are accessible for recognition by professional phagocytes.\textsuperscript{28} The translocation of histone H1 from the nucleus to the cytoplasm and cell surface occurs in the early stages of apoptosis anis mediated by
caspases.\textsuperscript{29} With this in mind, Wang and coworkers utilized phage display to identify a six amino-acid CQRPPR peptide (Apopep-1), which bound to histone H1 exposed on the surface of apoptotic cells.\textsuperscript{30} Apopep-1 effectively labeled dead and dying cells within tumor tissue, and this homing was increased when tumors were treated with anti-cancer drugs allowing for monitoring of tumor cell death in response to therapy. The same Apopep-1 peptide was able to detect dying neurons in a preclinical mouse model of Parkinson’s disease.\textsuperscript{31}

The Aposense family of small molecules have proven to be effective detectors of apoptosis in living animals. One of the initial compounds, a \textit{N,N‘-didansyl-L-cystine} (DDC) was shown to accumulate in dead and dying cells upon intravenous injection into a rodent stroke model using fluorescence microscopy.\textsuperscript{32} Autoradiography of $^3$H-DDC corroborated results seen by fluorescence microscopy with the compound accumulating in the ischemic regions of the brain. Ex vivo fluorescence microscopy of histological samples showed accumulation of DDC in damaged tissue from traumatic brain injury and chemotherapy-induced enteropathy.\textsuperscript{33,34} The amphipatic DDC is believed to selectively accumulate into apoptotic cells due to plasma membrane depolarization and activation of scramblase proteins on the cell membrane although this mechanism has not been proven. Another limitation of this probe is that it is labeled with a dansyl fluorophore, which prevents non-invasive whole animal fluorescence imaging due to high background by endogenous fluorophores and limited tissue penetration. Another dansyl derivative, DFNSH, has also been shown to accumulate in the cytoplasm of apoptotic cancer cells.\textsuperscript{35} Radiolabeling of this dansylhydrazone with $^{18}$F enabled visualization of apoptotic neurons in ketamine-treated rat brains via PET imaging.\textsuperscript{36} Aposense developed another
low molecular weight detector of cell death based on the alkyl-malonate group from γ-carboxyglutamic acid. ML-10, a (2-(5-fluoro-pentyl)-2-methyl-malonic acid), showed increased intracellular uptake in apoptotic cells and this accumulation was caspase-dependent and in parallel with other molecular hallmarks of apoptosis. ML-10 did not accumulate in cells where the membrane had been disrupted thus the probe could be used identify cells in early apoptosis. The mechanism of ML-10 uptake has also not been proven, but is hypothesized to be the result of membrane depolarization and acidification during early apoptosis and is analogous to trans-membrane translocation of other hydrophobic anions. To facilitate PET imaging of apoptosis, Radiolabeling ML-10 with a $^{18}$F enabled visualization of cell death in the infarcted region using an experimental stroke mouse model. These results prompted a clinical study evaluating the dosimetry, biodistribution, stability, and safety profile of $^{18}$F-ML-10 in healthy humans. Administration of $^{18}$F-ML-10 proved to be safe and demonstrated high in vivo stability. Excretion was predominantly through the bladder and retention of the tracer was seen in the testes, which was due to binding normal apoptosis.

1.4. Necrosis Biomarkers

Necrosis has classically been thought of as an uncontrolled, accidental process that occurs as the result of physical or chemical insult. Recently, there has been a considerable amount of literature suggesting that a programmed form of necrosis, termed necroptosis, can occur under certain contexts. Whether accidental or programmed, these subroutines of cell death represent primary necrosis; however, in the absence of efficient cell removal by the immune system, apoptotic cells will undergo secondary necrosis.

During secondary necrosis, the plasma membrane is ruptured, albeit later than during
primary necrosis, and intracellular molecules are released into the extracellular environment. These released molecules, along with changes in intracellular metabolism, can be targeted for necrosis imaging, yet one must careful monitor their temporal appearance in order to differentiate primary and secondary necrosis from apoptosis.\textsuperscript{40}

A common biomarker for necrosis is the exposure of DNA due to plasma membrane disruption. Propidium iodide, a fluorescent DNA interchelating dye, has routinely been used in flow cytometry as a secondary marker for necrosis. This dye is impermeable to viable cells; however during necrosis, the dye is able to bind to exposed DNA causing the cell to fluoresce in the red region-of-light. Although no one has used this dye for whole-body in vivo necrosis imaging, other labs have taken a similar strategy to target necrosis in living animals. Dasari and co-workers developed a near-infrared necrosis probe by conjugating a NIR IR-786 dye to Hoechst, a biocompatible DNA binding agent.\textsuperscript{41} The Hoechst-IR probe proved to be effective at labeling cells that were permeabilized and not membrane-intact live cells. When evaluated in necrosis-inducing myocardial infraction and sepsis in vivo models, Hoechst-IR selectively accumulated in necrotic tissue allowing for visualization of necrosis through whole-body epifluorescence imaging. A multimodal DNA-binding agent was developed by appending a gadolinium chelating DTPA to TO-PRO-1, which binds to DNA though electrostatic interactions with the phosphate backbone.\textsuperscript{42} Similarly to Hoechst-IR, Gd-TO failed to accumulate in viable cells, but showed increased $T_1$ relaxivity and fluorescence intensity in camptothecin treated cells. The specificity of Gd-TO for necrosis in vivo was tested in a permanent myocardial infarction mouse model.\textsuperscript{43} Gd-TO was able to image acute necrosis as well as the progression and clearance of necrotic cells from the ischemic...
myocardium. Since the mechanism of binding and uptake of Gd-TO is well-understood, it is likely that this probe along with apoptosis-specific probes could be used for dual fluorochrome or multimodal imaging of cell death processes in vivo. Moreover, this general strategy of attaching contrast agents to DNA binding agents could be applied to other DNA binding agents though one must consider the possible toxicity of these compounds.44

A number of other compounds have been developed with high specificity for necrotic tissue. These necrosis avid agents have predominately been used for non-invasive imaging in ischemic myocardial injury, brain infarct, and assessment of radiofrequency ablation therapies. The most studied of these compounds is hypericin, which is a non-porphyrin photosynthesizer derived from the plant genus Hypericum. Radiolabeling a derivative of hypericin, mono$^{123}$I-iodohypericin, allowed for visualization of necrotic liver tissue after systematic injection.45 A less lipophilic version of mono$^{123}$I-iodohypericin containing a carboxylic acid, proved to have a high avidity for necrosis and faster blood clearance than mono$^{123}$I-iodohypericin, which suggests that this probe would be better for whole body imaging of necrosis.46 The inherent fluorescence properties of hypercine have also been exploited for microscopic necrosis imaging.47,48 Recently, a $^{64}$Cu-bis-DOTA-hypericin was used to assess anti-tumor therapeutic efficacy induced by photothermal ablation.49 SPECT and PET derivatives of pamoic acid and 3,3’-(benzylidene)-bis-(1H-indole-2-carbohydrazaide) also have high avidity for necrosis and can be used to delineate necrotic tissue from viable tissue in vivo and ex vivo.50,51,52
<table>
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<tr>
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<th>Targeting Group</th>
<th>Imaging Modalities</th>
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<td>Fluorescence</td>
<td>32-34</td>
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<td></td>
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<td>(ML-10)</td>
<td>PET</td>
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While these necrosis avidity compounds are effective at targeting necrosis tissue independent of the disease state, their mechanism of selectivity is not well-known. It is believed that they either bind to a protein exposed during necrosis or form a lipoprotein complex in the body and eventually, release from the complex allowing for accumulation into necrotic tissue.\textsuperscript{53} It is likely that the mechanism for necrosis avidity is specific for each compound’s structure, and not that one mechanism applies for the whole group. Thus, researchers should be careful about using these agents during mechanistic studies of cell death processes.

Much attention has been focused on developing biomarkers for necroptosis since it has been found to have an important role in numerous biological and pathophysiological settings.\textsuperscript{54} Necroptosis can be initiated by the same ligands as apoptosis; though its morphological features are characteristic of pathological necrosis and it is highly dependent on the activation of receptor-interacting protein-1 (RIP-1) and/or receptor-interacting protein-3 (RIP-3).\textsuperscript{2,55} Downstream events of RIP-1 and RIP-3 activation include cytosolic ATP depletion, reactive oxygen species (ROS) overproduction, activation of calpains and cathepsins, and lysosomal membrane permeabilization.\textsuperscript{54} Currently, only a few of these biomarkers have been investigated for

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<tr>
<td><strong>Biomarker</strong></td>
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<tr>
<td>DFNSH</td>
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<td>Pamoic Acid</td>
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<tr>
<td>3,3’-(benzylidene)-bis-((^{1}\text{H}-\text{indole}-2\text{-carboxyhydrazaide}))</td>
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imaging these cell death processes. Indeed, Vanden Berghe et al. utilized a commercially available ROS indicator (CM-H$_2$DCFDA), a mitochondrial transmembrane potential probe (TMRM), and a lysosome imaging agent (LysoTracker Red DND-99) to investigate the occurrence of necrosis signaling events via live-cell fluorescence imaging. An enzymatic inhibitor of RIP-1 kinase, necrostatin-1, has been discovered to protect against necroptotic cell death both in vitro and in vivo. This suggests that necrostatin-1 and other necrostatins may provide a novel scaffold for the development of imaging agents specific for necroptosis. The aforementioned molecular events can be found in other cell death processes such as apoptosis, thus the temporal occurrence of these biomarkers should be considered when differentiating cell death sub-routines.

1.5. Phosphatidylserine

Phosphatidylserine is a minor constituent of the plasma membrane: however, its relatively low abundance is outweighted by its biological significance. The proceeding sections will describe the structure, mammalian cell biosynthesis, and biological functions of this phospholipid. Moreover, it will highlight strategies used by biological receptors to selectively bind to phosphatidylserine that in turn can guide researchers in the development PS-targeting imaging agents and therapeutics.

1.5.1. Structure and Biosynthesis

Mammalian cell membranes contain more than 1000 different types of phospholipid molecules, which are important functions for maintaining cellular structure and initiating biological pathways. Among the phospholipids, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI) are the most abundant. Depending on the tissue, PC constitutes 40-50%, PE ranges from
20-45%, and PS accounts for 2-10% of the total phospholipid content.\textsuperscript{60} Moreover, the phospholipid composition and distribution varies among different organelle membranes. For example, the mitochondria contains approximately 1% PS whereas the plasma membrane contains 10-20% PS with essentially all of it in the inner leaflet.

Structurally, PS contains two acyl groups at the sn-1 and sn-2 positions of the glycerol, and a polar head group at the sn-3 position.\textsuperscript{60} The polar head group is made up of a neutral-charged serine causing PS to have an overall anionic charge in the plasma membrane. This differs from the PE and PC, which have a neutral charge at physiological pH. The sn-1 acyl chain is often comprised of saturated fatty acids while the unsaturated fatty acid are found on the sn-2 position.

In mammalian cells, two enzymes are responsible for producing PS from either PC or PE through a Ca\textsuperscript{2+}-dependent reaction that requires no additional source of energy. PS Synthase-1 (PSS1) synthesizes PS by exchanging the choline moiety on the PC head group with serine, whereas PS Synthase-2 (PSS2) catalyzes the exchange of ethanolamine from PE with serine to generate PS.\textsuperscript{61} In yeast and prokaryotes, PS is generated from a different pathway where serine is conjugated to CDP-diacylglycerol.\textsuperscript{61} PSS1 and PSS2 are mostly found in distinct domains within the ER called mitochondrial associated membranes (MAMs). While PS is generated in the ER, one would expect the ER to have a large concentration of PS; however, PS is mostly enriched in the plasma membrane and early endosomes with the ER and mitochondria having the lowest mole percent of PS. The exact mechanism of subcellular distribution of PS is not well-known but it is hypothesized that secretory vesicles may be responsible for transporting PS.\textsuperscript{60} PSS1 and PSS2 appear to have complementary roles as demonstrated in single knockout
studies in vitro and in vivo; however, differences in tissue distribution and regulation of phospholipid metabolism suggest each may have a specific function. Both PSS1 and PSS2 activity also appear to be regulated by a feed-back mechanism with the end-product, PS.

1.5.2. Biological Functions

PS is unique in that it can induce a sizable anionic charge on the membrane, which in turn can act as a recruitment mechanism for proteins and cations. Within the cell, PS is responsible for activating a number of signaling pathways through binding by protein kinase C, neural sphingomyelinase, kinase-associated domain 1 of MARK/PAR1 kinases, etc. As mentioned earlier, PS is almost exclusively maintained in the inner leaflet of the plasma membrane causing asymmetry within the lipid bilayer. This PS asymmetry is tightly regulated by the putative aminophospholipid flippase, which is responsible for translocating PS and to a less extent PE from the outer leaflet to the inner leaflet. It is believed that inhibition of the aminophospholipid flippase results in the externalization of PS on the plasma membrane. Other mechanisms of PS externalization have been proposed; however, there is no definitive explanation for this phenomena.

Cell surface PS has been extensively studied as an “eat-me” signal for the recognition and subsequent phagocytosis of apoptotic cells by the immune system. Dead and dying cells have approximately > 280 fold-increase in PS exposure compared to live cells, though it is believed that at least an 8 fold enhancement is needed for a cell to be targeted by phagocytes. This large increase in cell surface exposed PS is believed to responsible for selective targeting of dead and dying cells by the immune system. However, it is likely
that PS needs to be exposed with one or more other eat-me signals for phagocytosis, since PS alone does not induce macrophage-mediated phagocytosis.\textsuperscript{65}

Phosphatidylserine exposure on activated platelets plays a major role in the regulation of the coagulation cascade. Upon damage of the endothelium, exposed collagen along with other factors activate circulating platelets, which subsequently induces exposure of PS on the platelet-derived membrane surface. The exposure PS can regulate the generation of thrombin by binding to and altering the activity of factor Va, factor Xa, and prothrombin. Binding of these factors to negatively charged membrane surface is important for the assembly and activation of the tenase and prothrombinase complexes.\textsuperscript{66}

1.5.3. Biological PS Receptors

1.5.3.1. Metal-Dependent Binding Receptors

The biological functions of PS receptors are very diverse though many similarities are apparent in their PS recognition domains. Specifically, PS receptors can be divided
into two groups: those that require metals to bind to PS and those whose PS binding is
not dependent on a metal. Receptors requiring a metal cofactor typically use polybasic
regions to coordinate a Ca\textsuperscript{2+}, which electrostatically interacts with the anionic PS head
group. In this way, the Ca\textsuperscript{2+} ions act as a bridge between PS and the biological receptor.

The most studied Ca\textsuperscript{2+}-dependent PS binding protein is Annexin V. Annexin V is
a 36 kDa member of the annexin family of Ca\textsuperscript{2+} regulated intracellular proteins whose
cellular functions have been linked with trafficking, inhibition of coagulation, and signal
transduction. The structure of Annexin V consists of 4 tandem repeat domains, called the
annexin core, which cause the protein to have a convex shape. The domains are further
comprised of five \(\alpha\)-helices (A-E) that are connected through short loops. Membrane
binding is believed to be facilitated by the surface AB loop, which chelates two Ca\textsuperscript{2+} ions
that mediate association with PS. One Ca\textsuperscript{2+} ion coordinates to the phosphoryl oxygen and
the second Ca\textsuperscript{2+} ion to the carboxylate oxygen on the PS head group.\textsuperscript{67} The binding of PS
subsequently increases the affinity of Annexin V for Ca\textsuperscript{2+}. In addition, the Annexin V-PS
interaction is stabilized by insertion of hydrophobic side chains from surface loop amino
acids into the membrane core. The overall PS binding affinity arises from 1) a
collaboration between the Ca\textsuperscript{2+}-binding sites of the four domains with a dominant role for
domain 1 and 2) formation of trimers and oligomers upon binding to PS-containing
membrane surfaces.\textsuperscript{68}

The C2 domain is a common structural feature in many biological PS receptors
that bind to PS in a Ca\textsuperscript{2+}-dependent manner. C2 domains contain an 8-stranded
antiparallel \(\beta\)-sandwich with 3 key interstrand loops that are responsible for binding both
Ca\textsuperscript{2+} and membranes.\textsuperscript{69} Synapototagmin I is an intracellular protein that mediates
exocytosis of neurotransmitters through binding of the C2A domain to membranes. The C2A domain of Synaptotagmin coordinates Ca$^{2+}$ ions through multidentate aspartate carboxylates that are located in cup-like cavities between surface loops in the β-sandwich. These coordinated Ca$^{2+}$ ions are responsible for mediating membrane binding though electrostatic interactions with negatively charged phospholipids. Moreover, membrane binding is stabilized by hydrophobic interactions between methionine and phenyalanine exposed on the loop and the membrane. Similarly to Annexin V, the affinity of Synaptotagmin for Ca$^{2+}$ is increased in the presence of a membrane, which is likely due to coordination by the phospholipids.

The T cell/transmembrane, immunoglobulin, and mucin (TIM) family of proteins have recently been discovered as receptors for PS. TIM proteins are type I cell-surface glycoproteins found on immune cells, and are known to regulate autoimmune and allergic diseases. Structurally, All TIM family proteins are characterized by having an immunoglobulin variable (IgV) domain with six cysteines, a mucin-like domain, a transmembrane domain, and a cytoplasmic domain. The IgV domain comprises of two anti-parallel β-sheets, BED and GFC β-sheets, with the GFC β-sheet containing an upward folded loop, CC” loop, that is structurally distinct to all TIM IgV domains. The CC” loop and the neighboring FG loop form a conserved metal ion binding pocket. This pocket contains aspartic acid and asparagine amino acids that coordinate divalent metal ions, which in turn mediate binding to PS through electrostatic interactions. Upon binding, the PS head group is inserted into the cavity allowing for specific interaction between the aspartic acid and the PS amine group. In addition, the serine of PS interacts with the coordinated metal and a serine exposed on the CC” loop. Hydrophobic residues
exposed on the surface of the FG loop insert in the membrane and increase the binding of TIM proteins for PS.

1.5.3.2. Metal-Independent Binding Receptors

The discoidin C2 domains do not require metal ions for PS binding, but instead use basic amino acids on the loops of a β-barrel to form electrostatic interactions with PS. The best examples of proteins that bind to PS via discoidin C2 domains are lactadherin, factor Va, and factor VIIIa. Lactadherin is 46 kDa glycoprotein that mediates clearance of dead and dying cells by acting as a bridge between macrophages and apoptotic cells. The overall C2 domain structure of lactadherin is very similar to factors V and VIII, and is composed of a distorted β-barrel with three relatively large loops. Specifically, loops 1 and 3 contain four solvent exposed hydrophobic residues (tryptophan, leucine, and two phenylalanines) that appear to be important for membrane binding. Computational docking studies have proposed two PS binding sites on lactadherin that use either arginine or asparagine to specifically interact with PS. In factor Va, the three loops form a groove whose interior is lined by polar side chains that could interact with PS and exposed tryptophans and leucines that insert into the membrane. Like factor Va, the membrane binding site of factor VIIIa includes surface exposed hydrophobic residues (methionine, phenylalanine, leucine, and valine) and a belt of basic residues. These discoidin C2 domains have proven to exhibit a stereoselectivity for phosphatidyl-L-serine over its enantiomer or other anionic lipids and can bind to PS containing membranes in the nanomolar range.

1.5.4. PS Targeting for Cell Death Imaging
The appearance of PS on the cell surface occurs early in the apoptosis sequence, and appears to be a universal indicator of most types of cell death processes including apoptosis, necrosis, mitotic catastrophe, and senescence. Thus, cell surface exposed PS is a readily accessible, high abundance biomarker for cell death imaging. Extensive effort has been put into developing targeted imaging probes for exposed PS using appropriately labeled PS-binding proteins.\textsuperscript{80} Annexin V is the most-studied PS-binding protein and derivatives have been developed for SPECT,\textsuperscript{81} PET,\textsuperscript{82} MRI,\textsuperscript{83} ultrasound,\textsuperscript{84} and fluorescence\textsuperscript{85} imaging. Though results have been promising, the high cost of producing labeled Annexin V, in addition to its non-ideal bio-distribution profile, blood-half life, and questionable in vivo stability has prevented FDA approval. Other PS-binding proteins have been labeled with reporter groups to produce effective imaging agents of cell death. The C2A domain of Synaptotagmin-I has been labeled with $^{99m}$TC and $^{18}$F, enabling SPECT and PET imaging of cell death in animal models of myocardial infarction and cancer.\textsuperscript{86,87} Lactadherin has also been developed as a genetically encoded biosensor for PS.\textsuperscript{88}

Because of their relatively large molecular size, proteins and antibodies have the intrinsic benefit of high binding affinity and high target selectivity, but imaging performance can be limited by stability problems, undesired organ distribution profiles, slow diffusion rates, and slow bloodpool clearance rates. Compared to proteins, it is much easier to systematically fine-tune the pharmacokinetic properties of small molecules, and it is highly advantageous to develop small molecule imaging probes that exhibit the same targeting capabilities as proteins. With these thoughts in mind, investigators have started to search for low molecular weight molecules that exhibit high
and selective PS-binding affinity. Several research groups have independently employed phage panning technology to find different small peptides (hexamers to decamers) that bind exposed PS with nanomolar dissociation constants. Burtea and coworkers screened an M13 phage display library on PS-coated plates and identified a LIKKPF hexapeptide with high PS binding affinity but only modest PS/PC selectivity. The same group attached another PS-binding peptide (E3;TLVSSL peptide sequence) to ultrasmall superparamagnetic ion oxide particles (USPIO), which were able to detect increased apoptosis in irradiated tumors and this accumulation was significantly larger than a control MRI probe containing a scrambled peptide sequence. A second research group independently identified CLSYYPSY as a novel PS-interacting octapeptide that specifically homed to the tumor vasculature and apoptotic tumor cells in xenografts treated with camptothecin. Another group used rational design and SPR to identify a 14-mer PS-binding peptide (FNFRKLKAGAKIRFG) which was derived from a PS decarboxylase PS-binding site. When attached to a Lys[di(2-pyridinemethyl)]-CO₂H chelating group (SAAC), this probe showed significant uptake in a paclitaxel treated tumor mouse model. Using an alternative, rational design approach, Zheng and coworkers developed a new class of cyclic peptides that mimic the PS-binding domain of lactadherin. These peptides have micromolar association constants with membrane exposed PS allowing for effective labeling of apoptotic cells in culture.

1.5.5. Other Medical Applications for PS Targeting

In addition to imaging, PS represents a viable target for therapeutic treatments. Two strategies have been employed that involve targeting PS in order to induce cell death and inhibit tumor growth. The first strategy involves masking PS that is exposed on the
Figure 1.4. Biomedical applications of targeting phosphatidylserine.

surface of dead and dying tumor cells. Masking PS prevents efficient clearance of apoptotic cells by immune cells thus these cells will enter secondary necrosis. During secondary necrosis, the plasma membrane becomes permeable causing the release of damage associated molecular patterns (DAMPS) such as HMGB1, heat shock proteins, SAP130, etc.94 The release of DAMPS allow dendritic cells to take-up antigens from dead cells in addition to inducing a co-stimulatory effect towards these cells. These antigens can induce a specific immunity against apoptotic and necrotic cells thus it is advantageous to shift the clearance of dead and dying cells from macrophages to dendritic cells when developing treatments against cancer and other diseases. Munoz and coworkers used Annexin V to modulated the immunogenicity of apoptotic and necrotic
cells through the blockage of phagocytosis by monocyte-derived macrophages.\textsuperscript{95} When incubated with irradiated, apoptotic tumor cells (ITC), Annexin V promoted a pro-inflammatory environment and led to inhibition of tumor growth in 60\% of lymphoma-tumor bearing mice compared to 5\% with ITC alone. Interestingly, vaccination with Annexin V alone caused a reduction in tumor growth suggesting that targeting PS in and around the tumor may induce tumor cell death. Moreover, mutants of lactadherin, a bridging molecule that promotes phagocytosis by binding PS on apoptotic cells and integrins on phagocytes, have been shown to have immunomodulatory properties masking PS and preventing phagocytosis.\textsuperscript{96} Taken together, masking PS on stressed and apoptotic cells may be an effective strategy for initiating a pro-inflammatory milieu and suppressing tumor.

The second strategy uses PS targeting monoclonal antibodies to bind to PS exposed on the surface of endothelial cells in the tumor vasculature. This specific exposure on the tumor endothelium and not on normal endothelium is believed to be consequence of oxidative stress within in the tumor microenvironment.\textsuperscript{97} The Thorpe lab has developed multiple PS-binding monoclonal antibodies that can inhibit tumor growth, either alone or in combination with other cytotoxic agents, in a number of tumor mouse models.\textsuperscript{98,99,100} When used with other chemotherapeutics, there is an enhanced anti-tumor effect, which can be attributed to the ability of these chemotherapeutic drugs to increase PS exposure on tumor blood vessels thus amplifying the target for the antibody.\textsuperscript{101} Mechanistically, binding of these antibodies to PS is mediated through the dimerization of \(\beta\)-2-glycoprotein I, a 50-kDa glycoprotein that binds weakly to anionic phospholipids under physiological conditions.\textsuperscript{102} A single molecule of \(\beta\)-2-glycoprotein I binds to PS
with a dissociation constant of 4 μM; however, when dimerization is induced by antibody binding, avidity of this complex for PS can increase by 1000 fold.\textsuperscript{103,104} Once bound to PS, the Fc-region on the antibody can stimulate binding of monocytes and macrophages, thereby inducing antibody-dependent cell-mediated cytotoxicity on the tumor endothelial cells.\textsuperscript{98}

Targeting phosphatidylserine may also be an effective anti-pathogen strategy. Phosphatidylserine has been implicated in viral entry and is often exposed on the surface of infected host cells. Certain viruses such as the vaccinia virus, have membranes that are enriched in PS.\textsuperscript{105} Exposed PS on the vaccinia viral membrane is necessary for macropinocytosis by host cells and is likely inducing this response by mimicking apoptotic bodies.\textsuperscript{106} Moreover, PS is the only lipid required on the vaccinia viral membrane for infectivity. Vaccinia along with influenza A virus, HIV-1, and herpes simplex virus-1 can initiate PS exposure on the surface host cells undergoing virus-induced cell death.\textsuperscript{107} Soares and coworkers have shown that treating cytomegalovirus-infected mice and Pichinide virus-infected guinea pigs with a phosphatidylserine targeting antibody allowed for at least 50% of the animals to recover from infection.\textsuperscript{108} This highlights the promise of targeting exposed phosphatidyserine on virons and infected cells as an antiviral strategy. It is likely that this strategy could be applied to Leishamania diseases, whose associated pathogens are known to expose PS and use apoptotic mimicry to evade the immune system and infect host cells.\textsuperscript{109}

1.6. Zinc(II)-Bis-Dipicolylamine Coordination Complexes

Metal coordination complexes make attractive ligands especially for anion recognition impart to their charge and open coordination sites for binding.\textsuperscript{110} Both Cu(II)
and Zn(II) complexes have been extensively studied as biosensors and therapeutics that target phosphorylation events.\textsuperscript{111} Zn(II) complexes are especially appealing for molecular imaging because 1) they are typically non-quenching when attached to fluorescent dyes and 2) Zn(II) is abundant in the blood thus the risk of cytotoxicity is lower than for other metals.\textsuperscript{111} The Smith group discovered that synthetic Zn(II)-bis-2,2-dipicolyamine (Zn\textsubscript{2}BDPA) coordination complexes have selective affinity for anionic cell membranes. In vitro liposome studies have shown that Zn\textsubscript{2}BDPA complexes have high PS/PC selectivity. When incubated with camptothecin-treated Jurkat cells, a fluorescein labeled Zn\textsubscript{2}BDPA targeted the membranes of dead and dying cells and not healthy cells in culture.\textsuperscript{112} The membrane association process utilizes a three-component assembly process where Zn\textsuperscript{2+} ions mediate cooperative association of the DPA molecule to the anionic head groups of the membrane-bound PS. Functionally, this is similar to the way that Ca\textsuperscript{2+} ions promote membrane association of Annexin V.

In this dissertation, we evaluated the imaging and targeting properties of fluorescent Zn\textsubscript{2}BDPA probes for cell death in living animal. The following chapters will highlight the versatility and effectiveness of fluorescent Zn\textsubscript{2}BDPAs as in vivo cell death imaging probes. Chapter 2 evaluates a synthetic fluorescent near-infrared probe, called PSS-794, for localization to tumor cell death in animal models of prostate cancer, breast cancer, and anti-tumor treatment efficacy. Chapter 3 uses the same probe PSS-794 and tests it in pre-clinical animal models of thymus atrophy and acute tissue damage to further understand the kinetic and perfusion properties of PSS-794 in vivo. Chapter 4 simultaneously monitors cell death and blood-brain barrier disruption in an experimental model of traumatic brain injury using multicolor fluorescence imaging. Chapter 5
evaluates fluorescent multivalent Zn$_2$BDPA probes in cell culture and in animal models of cell death to understand how increasing the number of Zn$_2$BDPAs affects imaging and pharmacokinetic properties in vitro and in vivo. Another chapter that tests the substrate specificity of the aminophospholipid flippase for fluorescent phosphatidylserine and phosphatidylcholine analogs is included in the appendix.
CHAPTER 2:
EVALUATING PSS-794 IN PRE-CLINICAL ANIMAL MODELS OF CELL DEATH: ENDogenous AND TREATMENT-INDUCED CELL DEATH WITHIN TUMORS

2.1. Background

There is a major ongoing research effort to identify oligonucleotide and protein biomarkers of malignant disease.\textsuperscript{113} Phospholipid biomarkers are less common, however, there is increasing evidence that the membrane surfaces of certain cells and particles of biomedical significance, such as apoptotic cells,\textsuperscript{114} activated cells,\textsuperscript{115} tumor vasculature,\textsuperscript{97} microvesicles,\textsuperscript{116} bacteria,\textsuperscript{117} and viruses,\textsuperscript{118} expose unusually high levels of negatively charged phospholipids. Proteins and antibodies that can selectively target these anionic membrane surfaces and distinguish them from the near-neutral membrane surfaces of normal human cells have promising potential as imaging probes,\textsuperscript{119} drug delivery agents,\textsuperscript{120} and targeted molecular therapeutics.\textsuperscript{98} Notable examples are the protein, Annexin V, which is under clinical investigation as an imaging probe for dead/dying tissue,\textsuperscript{121} and the antibody, Bavituximab, which targets viruses and tumor vasculature.\textsuperscript{108,122} It is often challenging to optimize the formulation and pharmaceutical properties of proteins, thus, there is a need to develop small molecule mimics of these proteins that exhibit the same targeting capabilities.\textsuperscript{123}
Figure 2.1. Chemical structures of PSS-794, 794 Control, and ICG. The fluorophores are blue and the affinity ligand is black. From Reprinted with permission from Smith, B. A. et al. (2010) Optical Imaging of Mammary and Prostate Tumors in Living Animals using a Synthetic Near Infrared Zinc(II)-Dipicolylamine Probe for Anionic Cell Surfaces. J. Am. Chem. Soc. 132, 67-69. Copyright 2010 American Chemical Society.

Synthetic zinc(II)-bis-dipicolylamine (Zn$_2$BDPA) coordination complexes are known to associate with multianionic phosphorylated biomolecules, and we have discovered that they can be converted into optical imaging probes that target the outer surfaces of anionic vesicle and cell membranes. Fluorescent Zn$_2$BDPA probes can
distinguish dead and dying mammalian cells from healthy cells in a cell culture,\textsuperscript{112,126} and also selectively target bacteria in heterogeneous biological media.\textsuperscript{127} Moreover, we have recently demonstrated that the near-infrared fluorescent probe PSS-794 can be used to image bacterial infections in living mice,\textsuperscript{128} indicating that PSS-794 has a notable ability to selectively target anionic cells over other anionic sites in the bloodstream and extracellular matrix. Here, we greatly expand the animal imaging capability of PSS-794 by showing that it can also target the anionic dead and dying cells within subcutaneous and autochthonous tumors in rat and mouse models. The structure of PSS-794 includes a near-infrared carbocyanine fluorophore whose absorption and emission wavelengths of 794 and 810 nm, respectively, are within the optimal window for maximum penetration through skin and tissue.\textsuperscript{129} The high tumor selectivity of PSS-794 is demonstrated by comparison to the less-selective imaging that is achieved by using control near-infrared fluorophores and indocyanine green (ICG) whose structures do not have Zn\textsubscript{2}BDPA targeting ligands. ICG is approved for use in humans as a vascular imaging agent.\textsuperscript{130} It associates strongly with plasma proteins, and is rapidly cleared by the liver. ICG and non-targeted ICG-conjugates typically exhibit modest tumor targeting ability (T/NT < 2) due to enhanced permeation-retention (EPR) effects.\textsuperscript{131} Furthermore, we provide evidence that PSS-794 can detect an increase in tumor cell death due to successful treatment by focal beam radiation and chemotherapy.

2.2. Results and Discussion

The expected ability of PSS-794 to selectively target dead and dying cells with exposed anionic phosphatidylserine was confirmed with in vitro fluorescence microscopy studies of mammalian cells treated with a cytotoxic agent. Specifically, treatment of
Jurkat cells (T lymphocytes) with camptothecin induced significant amounts of cell death, and as shown in Figure 2.2, the near-infrared PSS-794 stained the same cells as fluorescently labeled Annexin V.

Using procedures that were approved by the appropriate institutional animal care and use committee, two tumor bearing animal models were selected for in vivo imaging: 1) immunocompetent Lobund Wistar rats with PAIII prostate tumors, and 2) athymic nude mice containing EMT-6 mammary tumors. These two tumor models were chosen, in part, because they develop foci of necrotic cells, especially in the tumor cores.
Figure 2.3. X-ray and fluorescence overlay image of a rat prostate tumor model at 24 h post-injection of PSS-794 (A). The image was acquired at a 190 mm field of view. Bar graph showing ex vivo tissue distribution of PSS-794, 794 Control, and ICG (B). The values represent the mean (n=3), ± standard error of the mean. *P<0.0005. This imaging data is representative of four replicate studies using independent cohorts. Reprinted with permission from Smith, B. A. et al. (2010) Optical Imaging of Mammary and Prostate Tumors in Living Animals using a Synthetic Near Infrared Zinc(II)-Dipicolylamine Probe for Anionic Cell Surfaces. J. Am. Chem. Soc. 132, 67-69. Copyright 2010 American Chemical Society.

A typical imaging study of the rat prostate tumor model employed three cohorts of rats with $1 \times 10^6$ PAIII cells injected subcutaneously in the right flank. The tumors grew over approximately 14 days followed by intravenous injection of either PSS-794, 794 Control, or ICG (3.0 mg/kg). The rats were anesthetized and placed in a whole-body, small animal imaging station that was configured for epifluorescence imaging. Each animal was
illuminated with filtered light at 750 ± 10 nm and the emission intensity was collected at 830 ± 20 nm. In addition, a co-registered X-ray image was acquired. Clearance of the probes from the blood streams of the living animals was monitored by imaging at regular 3-hour intervals. In the case of PSS-794, there was clear evidence for selective accumulation in the tumor after 24 hrs (Figure 2.3). A region of interest analysis compared the tumor signal intensity with the signal from the same area of skin on the opposite side of the rat, to give a target to non-target ratio (T/NT). At the 24 hr time point, the average T/NT for the cohort treated with PSS-794 was 2.2 and about twice that for control fluorophores, 794 Control and ICG. The rats were then sacrificed and their tissues harvested for ex vivo analysis of probe biodistribution. Fluorescence intensity images of the excised tissues confirmed the relatively high tumor selectivity of PSS-794 (Figure 2.4). The bar graph in Figure 2.3 shows that average tumor targeting with

![Figure 2.4](image_url). Representative fluorescent images of tissues and tumors dissected from Lobund Wistar rats at 24 hours post-injection of PSS-794 (A), 794 Control (B), or ICG (C). Tissues are the following: liver (Lvr), kidney (Kdn), lungs (Lng), heart (Hrt), spleen (Spl), blood (Bld), PAIII tumor (Tmr), muscle (Msc), and skin (Skn). Images were acquired at a 190 mm field of view. Fluorescence intensity scale bar applies for all images. Reprinted with permission from Smith, B. A. et al. (2010) Optical Imaging of Mammary and Prostate Tumors in Living Animals using a Synthetic Near Infrared Zinc(II)-Dipicolylamine Probe for Anionic Cell Surfaces. *J. Am. Chem. Soc.* 132, 67-69. Copyright 2010 American Chemical Society.
Figure 2.5. Ex vivo analysis of PSS-794 localization in a rat prostate tumor. Excised PAIII prostate tumors were sliced along the longest axis, and a 30 mm field of view generated the representative near-IR fluorescence intensity image (A). Representative co-registered micrographs of a 5 µm histological slice of tumor core; the brightfield image (B) shows necrotic cells as darker regions that colocalize with near-IR fluorescence intensity image from PSS-794 (C). Scale bar = 100 µm. Reprinted with permission from Smith, B. A. et al. (2010) Optical Imaging of Mammary and Prostate Tumors in Living Animals using a Synthetic Near Infrared Zinc(II)-Dipicolylamine Probe for Anionic Cell Surfaces. *J. Am. Chem. Soc.* 132, 67-69. Copyright 2010 American Chemical Society.

PSS-794 was 36-fold higher than 794 Control and also much higher than ICG. Direct comparison of ex vivo mean pixel intensities for PSS-794 and 794 Control is possible because they have essentially identical brightness and the same fraction of light absorption/scattering by the excised tissue. The average ex vivo tumor intensity from rat dosed with PSS-794 is 16-fold higher than rat dosed with ICG, however, this is only a qualitative measure of relative tumor selectivity because the brightness of ICG in tumor tissue is somewhat uncertain. As expected, the ex vivo imaging with PSS-794 shows
higher tumor selectivity than the in vivo imaging (T/NT) because fluorescence signal from the implanted tumor is attenuated by the surrounding skin and tissue. The spatial distribution of PSS-794 within the resected tumors was determined by slicing them in half along the longest axis. Fluorescence imaging of the interior-facing surfaces of these tumor halves revealed that PSS-794 was not distributed uniformly, with the highest fluorescence intensities coming from the core of the tumors (Figure 2.5). Additional microscopic imaging of histological slices showed that the near-infrared fluorescence from PSS-794 colocalized with the tumor’s necrotic regions (compare Figures 2.5 B and 2.5 C).

PSS-794 was further evaluated in an induced autochthonous prostate tumor model, which is phenotypically more similar to human prostate cancer than the subcutaneous model. In short, a cohort of Lobund-Wistar rats were intravenously

![Figure 2.6. Representative fluorescence image of PSS-794 targeting a spontaneous prostate tumor in a Lobund-Wistar rat (A). The image was acquired 24 h post-injection and the skin overlying the pelvic-abdominal region was removed to enable visualization of the prostate tumor. The tumor is located within the dashed circle. Bar graph showing ex vivo tissue distribution of PSS-794 (B). The values represent the mean (n=3), ± standard error of the mean.](image-url)
injected with N-methyl-N-nitrosourea (MNU, 30 mg/kg) followed by subcutaneous implantation of testosterone propionate (25 mg) pellets at 7 d, 2 mo, and 4 mo post MNU injection. Once palpable prostate tumors had formed, the rats were injected with PSS-794 (3 mg/kg). Twenty-four hours post-probe injection, the rats were anesthetized, sacrificed, and the skin over the pelvic-abdominal region was removed to facilitate visualization of PSS-794 via epi-fluorescence imaging. As shown in Figure 2.6, PSS-794 accumulated within the spontaneous prostate tumor and these levels were similar to those seen in the subcutaneous prostate tumor model.

The ability of PSS-794 to target and identify tumors in vivo was also tested using a mouse EMT-6 mammary tumor model. In a typical study, two cohorts of NCRNU nude mice were subcutaneously injected with $1 \times 10^5$ EMT-6 mammary carcinoma cells in the right shoulder. After 10 days, the tumors were palpable, and PSS-794 or 794 Control (3.0 mg/kg) was injected intravenously into the mice via the tail vein. As before, fluorescence imaging was used to monitor clearance of the probes from the blood streams of the living animals. The in vivo image in Figure 2.7 shows a typical tumor bearing mouse at 24 hrs post-administration of PSS-794. The average T/NT ratio for the cohort treated with probe PSS-794 was 70% greater than T/NT for 794 Control. As with the rat prostate tumor model, ex vivo imaging of the excised mouse tissues showed that average tumor selectivity for PSS-794 was almost 18-fold higher than 794 Control. Again, the tumors were sliced in half and fluorescence imaging showed that the probe localized towards the interior of the tumors. Confirmation that the tumor cores contained significant regions of dead/dying tissue was gained by TUNEL analysis of histological slices.
Figure 2.7. Representative overlay image of a nude mouse with an EMT-6 mammary tumor. Brightfield and fluorescence intensity images were acquired 24 h following injection of PSS-794 (A). The fluorescence intensity is plotted along the z-axis of this 3D surface diagram. Images were taken at an 80 mm field of view. Bar graph showing ex vivo tissue distribution of PSS-794 and 794 Control (B). The values represent the mean (n=3), ± standard error of the mean. *P<0.005. Reprinted with permission from Smith, B. A. et al. (2010) Optical Imaging of Mammary and Prostate Tumors in Living Animals using a Synthetic Near Infrared Zinc(II)-Dipicolylamine Probe for Anionic Cell Surfaces. J. Am. Chem. Soc. 132, 67-69. Copyright 2010 American Chemical Society.

A more clinically relevant animal model to evaluate the performance of PSS-794 used focal beam radiation to induce anti-tumor cell death. A rat model was generated having two subcutaneously implanted, syngenic prostate tumors on each flank - one tumor was treated with beam radiation while the other tumor served as a non-treated control. The main advantage gained by having the experimental and control tumors on the same animal is a reduction in animal-to-animal variability. In short, PAIII prostate
adenocarcinoma cells, taken from the same parent culture, were injected subcutaneously into both rear flanks of a cohort of syngeneic Lobund-Wistar rats. After six days, the implanted cells had developed into two palpable tumors of similar size. With each animal, the right side tumor was treated with 20 Gy of focal beam radiation while the other tumor served as the non-treated control. Seventeen hours after radiation, each rat was injected intravenously with either PSS-794 or 794-Control and 24 h later it was anesthetized and imaged using a planar, whole body epifluorescence scanner. Immediately after live animal imaging, each rat was euthanized and imaged again with the skin removed from the lower region. The live animal images showed qualitatively that there was enhanced PSS-794 uptake in the radiation-treated tumor (Figure 2.8). However, unambiguous interpretation of these images was not possible because, (a) the tumor signals were broad and diffuse due to scattering of the light by surrounding skin and tissue, an effect that varies substantially with tumor tissue depth, and (b) PSS-794 also accumulated in the dead skin cells overlaying the tumor. These obfuscating factors were eliminated by imaging the rats post-mortem with the skin peeled away from the tumors. As shown by the images in Figure 2.8. B, there was significantly higher uptake of PSS-794 in the radiation-treated tumor relative to the non-treated tumor. In contrast, an identical study using 794 Control showed only accumulation in the kidneys and no uptake in either tumor (Figure 2.8. D). To evaluate the biodistribution of PSS-794, the internal organs and tissues were removed and imaged using the same near-infrared filter set. The ex vivo fluorescence imaging data in Figure 2.9. A confirms the high selectivity of PSS-794 for the radiation-treated tumor compared to the non-treated tumor. MPIs were quantified by taking ROI pixel intensity measurements for each tissue, and shown in
Figure 2.8. Representative in vivo (A, C) and ex vivo (B, D) fluorescence images of rats bearing two subcutaneous PAIII prostate tumors and dosed with PSS-794 (A, B) or 794 Control (C, D) after radiation therapy. The right flank tumor (arrow) received focal beam radiation therapy, and the left flank tumor (arrow head) was not treated. At 17 h after radiation, each rat was injected intravenously with PSS-794 or 794 Control (3.0 mg/kg), and 24 h later, a planar, whole body epifluorescence image was acquired. The rats were then sacrificed and subjected to ex vivo imaging with the lower body skin removed. The 794 Control does not target the tumors but accumulates in the kidneys. N = 5. Springer and Smith, B. A. et al. (2011) In Vivo Targeting of Cell Death using a Synthetic Fluorescent Molecular Probe. Apoptosis 16, 722-731, figure 6 is given to the publication in which the material was originally published, by adding; with kind permission from Springer Science and Business Media.

Figure 2.9. B is a bar graph comparing PSS-794 fluorescence MPI in each tissue relative to the heart, which serves as an anatomical control. The amount of PSS-794 in the radiation-treated tumor was almost twice that in the non-treated tumor (11.3 ± 0.2 vs. 6.5 ± 0.8; MPI ± SEM) and approximately 8 fold higher than the amount of 794-Control in a
Figure 2.9. Probe biodistribution in PAIII tumor bearing rats that received anti-tumor focal beam radiation. Representative ex vivo fluorescence images of tissues excised from tumor-bearing rats treated with radiation therapy, dosed with PSS-794 (3.0 mg/kg) and sacrificed 24 h later (A). Tissues are the following: liver (Lvr), kidney (Kdn), lungs (Lng), heart (Hrt), spleen (Spl), blood (Bld), non-treated tumor (NT Tmr), radiation-treated tumor (Trt Tmr), muscle (Msc), and skin (Skn). Bar graph showing ex vivo tissue distribution of PSS-794 (black bars) and 794 Control (white bars) (B). A region of interest was drawn around each tissue and the mean pixel intensity (MPI) was recorded. The MPI from the tissues were normalized to the MPI from the heart tissue. MPI ± SEM. N = 5. *P< 0.01, **P< 0.0001. Springer and Smith, B. A. et al. (2011) In Vivo Targeting of Cell Death using a Synthetic Fluorescent Molecular Probe. *Apoptosis* 16, 722-731, figure 7 is given to the publication in which the material was originally published, by adding; with kind permission from Springer Science and Business Media.

Treated tumor (11.3 ± 0.2 vs. 1.4 ± 0.2; MPI ± SEM). Furthermore, uptake of 794 Control in the radiation-treated tumor was the same as the non-treated tumor (1.4 ± 0.2 vs. 1.4 ± 0.1; MPI ± SEM). PSS-794 also accumulated in the liver, kidneys, and lungs, as seen in tumor bearing rats that were not treated with radiation. Probe signal in the liver and
kidneys is not surprising since they are the clearance organs, but we presently do not have an explanation for the moderate levels of PSS-794 in the lungs. The relatively high accumulation in the skin is attributed to the localized cell death since the skin sample was taken from the area receiving focal radiation.

We further tested the generality of PSS-794 to measure anti-tumor treatment efficacy by using an orthotopic breast cancer mouse model that was treated with gambogic acid. Gambogic acid is a xanthone based natural product derived from the resin of *Garcinia hanburyi*, a plant that predominately found in Southeast Asia.\(^{137}\) Gambogic acid can induce cell death and inhibit the growth of different types of cancer including melanoma, hepatocellular carcinoma, breast cancer, etc.\(^{138}\) Two cohorts of athymic nude mice were orthotopically injected with 4T1 breast cancer cells in the mammary fat pad. Once tumors reached approximately 3 mm in diameter, one cohort received two treatments of gambogic acid (40 mg/kg) that were spaced two days apart while the other cohort were dosed with a vehicle control. Each mouse then received an intracardiac injection of PSS-794 (3.0 mg/kg) 24 h after the last gambogic acid treatment. Non-invasive whole-body, epifluorescence imaging showed significant accumulation of PSS-794 in the gambogic acid treated tumor while a negligible signal was seen in the non-treated control tumor (Figure 2.10. A). Quantification of the in vivo images showed that treated breast tumors had 42 % higher PSS-794 signal than non-treated tumors (treated = 20.23 ± 2.83, non-treated = 8.49 ± 3.96) (Figure 2.10. D). Excised tumors were cut into histological sections and placed on a highly sensitive, near-infrared scanner to determine intratumoral PSS-794 localization. Most of the PSS-794 appeared to be located at the periphery of the gambogic-acid treated tumors, while the non-treated tumors exhibited
Figure 2.10. Representative fluorescence image of mice bearing orthotopic breast tumors that were either not treated or treated with gambogic acid and injected with PSS-794 (A). Image was acquired 24 h post-probe injection. Following the 24 h timepoint, the mice were sacrificed, and the tumors were excised, flash frozen, and cut in histological slices. Near-infrared images were acquired from the non-treated (B) and gambogic acid-treated (C) tumor slices. In vivo quantification of PSS-794 localization to treated and non-treated breast tumors (D). Bar graph showing ex vivo tissue distribution of PSS-794 and 794 Control (E). *P< 0.01.

nearly homogenous staining of PSS-794 (Figure 2.10. B, C). This is not surprising as physical barriers within the tumor such as high interstitial pressure and irregular vascular prevent chemotherapeutics from entering the core of the tumor causing the drug to be concentrated at the tumor periphery. Quantification of ex vivo imaging corroborated with the in vivo results suggesting that PSS-794 can measure successful anti-tumor chemotherapeutic treatment in a living mouse (Figure 2.10. E).

Monitoring PS externalization appears to be an objective way of assessing the level of therapy-induced tumor cell death; thus, a reliable and robust molecular probe
for exposed PS is likely to expedite anticancer research and improve the clinical decision-making process. Our results indicate that Zn$_2$BDPA probes have great promise as targeting agents for detecting increased tumor cell death due to successful therapy. However, this study also demonstrates some practical limitations with planar fluorescence imaging using PSS-794. Although the probe has an appended near-infrared fluorophore for maximum penetration of light through skin and tissue, it is important to realize that the penetration depth is only a few millimeters and the signal intensity is highly surface weighted. Even though the tumors in this study were subcutaneous, it was not possible to generate quantitative images of the living rats showing enhanced probe uptake due to the beam radiation. Quantitative imaging could only be achieved after postmortem removal of the overlaying skin, which directly exposed the tumor. While it is possible to envision useful clinical applications of PSS-794 as a fluorescent probe for cell death, especially in the emerging field of fluorescence guided surgery, it is clear that broader impact will be gained by creating imaging probes that detect deep tissue targets.

2.3. Conclusions

In summary, the synthetic fluorescent near-infrared imaging probe, PSS-794, with an appended Zn$_2$BDPA affinity ligand, can selectively accumulate in prostate and mammary tumors in both subcutaneous and spontaneous animal models. Ex vivo biodistribution and histological analyses suggest that PSS-794 targets the necrotic regions of the tumors, which is consistent with in vitro microscopy showing selective targeting of the anionic membrane surfaces of dead and dying cells. Imaging probes that can determine, non-invasively, the amount and type of cell death in tumors may have utility
in clinical prognosis of tumor pathogenesis. PSS-794 may also become a useful optical probe for preclinical research efforts that aim to discover and assess new methods of anticancer treatment. It may even be possible to develop clinically useful early detection and intra-operative methods to assess cell death using surface or endoscopic optical imaging technologies.

2.4. Methods and Materials

2.4.1. Synthesis

PSS-794 and 794 Control were synthesized as previously reported. They have the following photophysical properties: PSS-794 in water, absorption $\lambda_{\text{max}} = 794$ nm, emission $\lambda_{\text{max}} = 810$ nm, $\epsilon = 1.1 \times 10^5$ M$^{-1}$ cm$^{-1}$, quantum yield = 0.14. Control fluorophore 794 Control in water, absorption $\lambda_{\text{max}} = 787$ nm, $\epsilon = 1.5 \times 10^5$ M$^{-1}$ cm$^{-1}$, emission $\lambda_{\text{max}} = 810$ nm, quantum yield = 0.12. Control fluorophore ICG was purchased (Sigma; St. Louis, MO) and used without further purification. Its photophysical properties change with environment and concentration. In pure water, ICG is aggregated and strongly quenched. In plasma, absorption $\lambda_{\text{max}} = 803$ nm, $\epsilon = 2.15 \times 10^5$ M$^{-1}$ cm$^{-1}$, emission $\lambda_{\text{max}} = 830$ nm, quantum yield = 0.03.

2.4.2. Cell Staining and Fluorescence Microscopy

Annexin V- Alexa Fluor 488 was purchased from Invitrogen (Eugene, OR), and 7AAD was purchased from BD Bioscience (San José, CA). Jurkat cells were grown to a density of approximately $1.0 \times 10^6$ mL$^{-1}$ in RPMI 1640, 10% FBS at 37 °C, 5% CO$_2$. A 5 mL volume of cells was treated with camptothecin (10 µM final concentration) in growth medium for 3.5 h at 37 °C, 5% CO$_2$. Cells were spun down and resuspended in 1X annexin binding buffer (10 mM HEPES sodium salt, 2.5 mM CaCl$_2$, 140 mM NaCl, pH
7.4). Aliquots (0.5 mL) of the treated cells were then treated with the indicated staining reagents at the indicated concentrations. Annexin V-Alexa Fluor 488 was used according to the manufacturer’s protocol (Invitrogen). All reagents were added simultaneously. The cell suspensions were mixed thoroughly by repeated inversion and then incubated 15 min at 37 °C. Cells were then centrifuged, resuspended, and washed twice in TES (5 mM), NaCl (145 mM), pH 7.4 buffer. At this point, 150 µL of the suspension was transferred to an 8-well chamber slide for microscopy.

Fluorescence microscopy was performed on a Nikon TE-2000U epifluorescence microscope equipped with filters that allowed detection of each probe. Filter sets were obtained from Nikon and included UV-2E/C filter (ex: 340/80, em: 435/85), EN GFP HQ filter (ex: 450/90, em: 500/50), Cy3 filter (ex: 535/50 nm, em: 610/75), Cy5 filter (ex: 620/60, em: 700/75), and Cy7 filter (ex: 710/75, em: 810/90). Fluorescence images were captured using Metamorph software (Universal) and analyzed using ImageJ.

2.4.3. Rodent Tumor Models and Protocol

All animal procedures were approved by either the University of Notre Dame or the Washington University Animal Study Committees. Eight-week old Lobund Wistar rats (Freimann Life Science Center; 250 g) were injected subcutaneously into the right flank with 1×10^6 Prostate Adenocarcinoma III (PAIII) cells, suspended in 300 µL of DMEM medium. Tumors grew for 14 days prior to injection of the probe. Lobund Wistar rats were anesthetized (1.5% isoflurane inhalation) and injected intravenously via the tail vein with 3.0 mg/kg of either PSS-794 (n = 3) or 794 Control (n = 3) in 1% DMSO/H_2O or ICG (n = 3) in H_2O. Fluorescence images were acquired at 0 hrs, 3 hrs, 6 hrs, 12 hrs, and 24 hrs post-injection by a Carestream Health In-Vivo Multispectral Imaging System.
FX (Rochester, NY) using a 750 ± 10 nm excitation filter and 830 ± 20 nm emission filter (f-stop = 2.51, FOV = 190 mm, binning = 4, 60 second image acquisition). Following the 24 hr time point, an X-ray image was acquired for each rat (f-stop = 2.86, filter = 0.4 mm, FOV= 190 mm, no bin, 180 second image acquisition).

A cohort of 3-4 month old Lobund-Wistar rats (n = 3) were injected intravenously with N-methyl-N-nitrosourea (MNU, 30 mg/kg) followed by subcutaneous implantation of testosterone propionate (25 mg) pellets at 7 d, 2 mo, and 4 mo post MNU injection. Once prostate tumors were palpable (typically by 10-12 months of age), the rats were injected intravenously via the tail vein with 3.0 mg/kg of PSS-794. Twenty-four hours post-injection, the rats were euthanized by CO₂ aphyxiation and the skin overlying the pelvic-abdominal region was removed. The rats were then placed inside a Carestream Health In-Vivo Multispectral Imaging System FX (Rochester, NY) and subjected to epifluorescence imaging using a 750 ± 10 nm excitation filter and 830 ± 20 nm emission filter. Selected tissues were then excised and placed on an imaging tray for ex vivo imaging and biodistribution studies.

Four-week old NCRNU mice (25 g; Taconic; Hudson, NY) were injected subcutaneously into the right shoulder with 1 x 10⁵ EMT-6 mammary carcinoma cells. Once palpable tumors appeared, about 10 days post inoculation, the mice were anesthetized by isoflurane inhalation (2% v/v in 100% O₂) and injected via tail vein with 3.0 mg/kg of either PSS-794 (n = 5) or 794 Control (n = 3) in 1% DMSO/H₂O. In vivo imaging occurred using a Carestream Health 4000mm Imaging Station (Rochester, NY) equipped with a 755 ± 20 nm excitation/830 ± 20 nm emission filter set. Fluorescence images were acquired with 60 s exposure (f-stop = 2.8, FOV = 80 mm, binning = 2) at
various time points post-injection of the probe.

After the 24 hr time point, the animals were euthanized by either CO₂ asphyxiation or cervical dislocation under anesthesia. The selected tissues were excised, placed on a transparent imaging tray, and optically imaged as described above. The tumors, from each animal, were then separated into halves by slicing along the longest axis. The tumor halves were placed onto a transparent imaging tray so that the inner cores faced the CCD. Fluorescence images were acquired for 3 minutes at a 30 mm field of view (f-stop = 2.51, no bin).

2.4.4. Radiation-Treated Prostate Tumor Rat Model

The right and left rear flanks of eight-week old male Lobund Wistar rats (Freimann Life Science Center, 250 g, n =5) were injected subcutaneously with 1x10⁶ rat Prostate Adenocarcinoma III (PAIII) cells, suspended in 300 µL of Dulbecco’s Modified Eagle Medium. Six days later the tumor-bearing rats were anesthetized with an intraperitoneal injection of a ketamine/xylazine (75/10 mg/kg) cocktail. The rats were then placed on their left side, and the right flank tumor irradiated with a 3.0 cm diameter beam (20 Gy dose of 9 MeV electrons) produced by a clinical Varian 21 EX accelerator (Varian Medical Systems Inc., Palo Alto, CA). The rats were injected intravenously via the tail vein with PSS-794 (3.0 mg/kg) or 794-Control (3.0 mg/kg) at 17 h post-radiation treatment, and 24 hours later the live rats were anesthetized and subjected to whole-body, epifluorescence imaging. Immediately after live animal imaging, each rat was euthanized and imaged again with the skin removed from the lower region to improve signal intensity at the tumors.

For radiation-treated rats, selected rat tissues were excised after sacrifice and imaging, placed on a transparent imaging tray, and imaged using a Carestream Health In-
Vivo Multispectral Imaging System FX equipped with a 750 ± 10 nm excitation filter and 830 ± 20 nm emission filter (30 s time acquisition, bin = 2 x 2, f-stop = 2.51, field of view = 190 mm).

2.4.5. Gambogic-Acid Treated Breast Cancer Mouse Model

NCRNU mice (n = 8) were injected with 250,000 4T1 mammary carcinoma cells in the lower mammary fat pad. Once the tumors reached 3 mm, one cohort of 4 mice received two treatments of gambogic acid (40 mg/kg, 70 μL in EtOH, i.p. injection) that were separated by two days. Twenty-four hours after the second treatment, all mice received an intracardiac injection of PSS-794 (3.0 mg/kg, 1 % DMSO/H_2O). Whole-body, near-infrared fluorescence images were acquired immediately after injection and 3 h, 6 h, and 24 h post-injection of the probe using an IVIS Spectrum (Caliper Life Sciences, Hopkinton, MA) equipped with a 710 nm excitation filter and 820 nm emission filter. After the 24 h time-point, the mice were anesthetized, sacrificed, and selected tissues were excised for ex vivo imaging. Tumors from all mice were flash frozen in OCT and cryosectioned for histological analysis.

2.4.6. Fluorescence Image Analysis

Images were analyzed using ImageJ. The 16-bit images were imported, opened in sequential order, and converted to an image stack. Background subtraction was applied to the images using the rolling ball algorithm (radius = 200 pixels). The stack was then converted to a montage and pseudocolored as “Thai” (under the “Lookup Tables” menu). Region of interest (ROI) analysis was performed on each in vivo image by drawing an identical area around the tumor (Target, T) and the same anatomical location on the contralateral side of the animal (Non-Target, NT). The mean pixel intensities for the
target and non-target regions were measured and recorded for each animal. For selected studies, the signal to noise (S/N) ratio was calculated by the following equation.

\[
S/N = \frac{(\text{MPI of Target}) - (\text{MPI of Non-Target})}{(\text{Standard Deviation of Non-Target})}
\]

The resulting ROI values were plotted using Graphpad Prism 4. For ex vivo biodistribution images, a ROI was manually drawn to outline each tissue and the mean pixel intensity was recorded.

2.4.7. Histology

Tumors were excised 24 hrs post-injection of the probe and snap-frozen, then embedded in OCT media. The tumors were sliced at 5 µm thickness. The Terminal dUTP-mediated nick end labeling (TUNEL) staining was performed using a commercial kit (In Situ Cell Death Detection Kit, POD; Roche Applied Science, Indianapolis, IN) according to manufacturer’s protocol and 3,3’-diaminobenzidine (DAB) to visualize. For fluorescence imaging, slices were fixed in 10% neutral buffered formalin and blocked with PBS containing bovine serum albumin. Bright field and fluorescence images of the slices were acquired using a Nikon TE-2000U epifluorescence microscope equipped with the appropriate Cy7 filter (ex: 710/75, em: 810/90). Images were captured using Metamorph software (Universal) and analyzed using ImageJ.

For gambogic acid treated and non-treated mice, the tumors were excised and flash frozen in OCT media. The tumors were then cut to an 8 µm thickness and placed onto the stage of a LI-COR Odyssey (LI-COR Biosciences, Lincoln, NE) near-infrared scanner. Near-infrared images were acquired (resolution = 42 µm, focus = 1.0 mm, intensity = 3.5) and exported as 8-bit TIFF images for analysis in ImageJ.
2.4.8. Statistical Analysis

All values are depicted as mean ± standard error of the mean (SEM). Statistical analysis was performed using a Student’s t-test.
CHAPTER 3:
EVALUATING PSS-794 IN PRE-CLINICAL ANIMAL MODELS OF CELL DEATH:
TISSUE DAMAGE AND THYMUS ATROPHY

3.1. Background

In the previous chapter, we evaluated the targeting properties of a near-infrared Zn$_2$BDPA probe, PSS-794, for cell death within a tumor and as the result of successful anti-tumor treatment. In the tumor-bearing animal models tested, cell death is relatively static thus these models do not give us a good read-out about the kinetic and perfusion properties of PSS-794. Within most tissues, cell death is a highly time dependent process and a high contrast image must be achieved during a specific window in time that in turn depends on the specifics of the cell death phenomenon under study and the probe’s bioavailability. Here, we assessed the kinetic and perfusion properties in two additional animal models of cell death: a thymus atrophy mouse model and an acute tissue damage mouse model. The thymus atrophy model is well-established with cell death kinetic parameters that are highly defined, thus this model served as a good system for understanding PSS-794’s pharmacokinetics. The acute tissue damage model directly tested the perfusion properties of PSS-794. Larger proteins are known to have limited perfusion in certain tissues, which can prevent accurate measurement of cell death.$^{144}$
Knowing this, we directly compared PSS-794 to a mechanistically similar, albeit larger, near-infrared Annexin V in the acute tissue damage model.

3.2. Results and Discussion

This study examined two in vivo models of cell death that operate by distinctly different mechanisms and in different anatomical locations, thus testing the generality of the fluorescent molecular probe, PSS-794, to identify cell death in a living animal.

Targeting performance was assessed by direct comparison to the untargeted fluorophore, 794-Control, that lacks a Zn$_2$BDPA affinity unit. The first animal model treated

**Figure 3.1.** Mean pixel intensities (MPI) of excised thymi from rats that were treated with dexamethasone (50 mg/kg) and dosed with either PSS-794 or 794 Control (3.0 mg/kg) (A). The times indicate the period from dexamethasone treatment to sacrifice and include injection of the fluorescent probe at 24 h before sacrifice. Rats not treated with dexamethasone (No Dex) were dosed with either PSS-794 or 794 Control (3.0 mg/kg) and sacrificed 24 h later. In each case, the MPI for the excised thymus was normalized to the MPI for the heart tissue. Mean ± SEM. N =4. *P< 0.01, ** P< 0.0001. Accumulation of PSS-794 in the excised thymi depends on the dexamethasone dose size (B). Ex vivo fluorescence images and quantification of MPI were performed 42 h after treatment with dexamethasone. Mean ± SEM. N =4. *P<0.05, **P<0.005, ***P< 0.001. Springer and Smith, B. A. et al. (2011) In Vivo Targeting of Cell Death using a Synthetic Fluorescent Molecular Probe. *Apoptosis* 16, 722-731, figure 2 is given to the publication in which the material was originally published, by adding; with kind permission from Springer Science and Business Media.
Lobund-Wistar rats with the glucocorticosteroid dexamethasone, which induces extensive thymocyte cell death. This is a classic experimental model that is technically straightforward to conduct and has high animal throughput. Furthermore, the kinetics for thymocyte apoptosis are well characterized and the model has been used to validate other cell death imaging probes including Annexin V. Separate cohorts of rats (n=4) were treated with intraperitoneal injections of dexamethasone (50 mg/kg) to induce thymocyte cell death. The time from dexamethasone treatment to sacrifice for each cohort (and its control cohort) was either 30 h, 42 h, or 48 h, with injection of fluorescent probe (3.0 mg/kg of PSS-794 or 794-Control) at 24 h before sacrifice. The moderately high probe dose (3.0 mg/kg) promoted maximum uptake at the sites of cell death, and the relatively long waiting period after probe injection (24 h) ensured complete wash out of any transient, non-targeted, tissue retention effect. The excised internal organs and tissues were imaged using a planar, whole body epifluorescence scanner with a near-infrared filter set (ex. 750 ± 10 nm, em. 830 ± 20 nm). Region of interest (ROI) analysis produced a pixel intensity map of each excised thymus and allowed calculation of mean pixel intensity (MPI). The chromophores in PSS-794 and 794-Control have essentially the same near-infrared absorption/emission wavelengths, so the relative amount of probe in each thymus could be determined by direct comparison of the MPIs. As seen in Figure 3.1 A, uptake of PSS-794 in the dexamethasone-treated thymi was significantly higher than 794-Control at all time points, and the highest amount of PSS-794 accumulation occurred at 42 h after dexamethasone treatment. Uptake of PSS-794 was also higher than 794-Control in the thymi taken from control rats that were not treated with dexamethasone, which is attributed to PSS-794 targeting the basal cell death in the organ.
Figure 3.2. Fluorescence microscopy of thymus histological sections from rats that were treated or not treated with dexamethasone. Rats were treated with dexamethasone (50 mg/kg) and sacrificed 42 h later, with a dose of either PSS-794 (A) or 794 Control (B) delivered at 24 h before sacrifice. Rats not treated with dexamethasone (No Dex) were dosed with either PSS-794 or 794 Control (3.0 mg/kg) and sacrificed 24 h later. The 10 μm thick sections were counterstained with DAPI and anti-caspase-3 antibody. The near-infrared (NIR) image in A shows PSS-794 targeting regions of tissue containing high levels of caspase-3 due to dexamethasone treatment, whereas, the NIR image in B shows negligible accumulation of 794 Control. Scale bar = 200 μm. Springer and Smith, B. A. et al. (2011) In Vivo Targeting of Cell Death using a Synthetic Fluorescent Molecular Probe. Apoptosis 16, 722-731, figure 3 is given to the publication in which the material was originally published, by adding; with kind permission from Springer Science and Business Media.

Increasing the dexamethasone dosage produced concomitantly higher PSS-794 accumulation in the thymus (Figure 3.1 B), providing further evidence that PSS-794 is targeting cell death.
Independent confirmation that PSS-794 targets the dead and dying cells within the thymus, was gained from fluorescent histological analyses of excised thymus tissue. Each thymus was flash frozen in the tissue embedding material OCT, sliced into 10 μm thick sections and stained with an anti-caspase-3 antibody and DAPI. Thymus sections from

**Figure 3.3.** Histological quantification of caspase-3 levels in rat thymus sections. (A) Rats were treated with dexamethasone (50 mg/kg) for the indicated times then sacrificed. (B) Rats treated with dexamethasone (50 mg/kg) for the indicated times and dosed with PSS-794 (3.0 mg/kg). The times in B indicate the period from dexamethasone treatment to sacrifice and include dosing of PSS-794 24 h before sacrifice. Rats not treated with dexamethasone (No Dex) in B were dosed with PSS-794 and sacrificed 24 h after probe injection. *P < 0.01, **P < 0.0001. Springer and Smith, B. A. et al. (2011) In Vivo Targeting of Cell Death using a Synthetic Fluorescent Molecular Probe. *Apoptosis* 16, 722-731, figure 4 is given to the publication in which the material was originally published, by adding; with kind permission from Springer Science and Business Media.
rats that were treated with dexamethasone showed a much stronger caspase-3 signal than sections from non-treated rats, indicating increased amounts of cell death (Figure 3.2). Near-infrared fluorescence microscopy of the same tissues showed that PSS-794 stained only the regions of tissue that were high in caspase-3 expression (Figure 3.2 A); however, the colocalization is not perfect. This is expected since the timing of caspase-3 expression during apoptosis does not coincide exactly with appearance of the PS that is targeted by PSS-794. In comparison, there was very little uptake of 794 Control in essentially identical thymus tissue sections with high apoptotic index (Figure 3.2 B). The PSS-794 targeting data in Figure 3.3 A suggests that the number of dead and dying thymocytes reached a maximum at 42 h after dexamethasone treatment. This conclusion is consistent

![Graph](image)

**Figure 3.4.** Comparison of caspase-3 levels in healthy thymus tissues taken from rats either dosed or not dosed with PSS-794 (3.0 mg/kg). Two cohorts of rats were intravenously injected or not injected with PSS-794 then sacrificed after 24 h. The thymus tissues were excised, flash frozen, and cut into 10 μm thick sections that were stained with anti-caspase-3 antibody. *P < 0.05. Springer and Smith, B. A. et al. (2011) In Vivo Targeting of Cell Death using a Synthetic Fluorescent Molecular Probe. *Apoptosis* 16, 722-731, figure 5 is given to the publication in which the material was originally published, by adding; with kind permission from Springer Science and Business Media.
with the histology data in Figure 3.3 B showing that the expression of caspase-3 in thymi from rats treated with both dexamethasone and PSS-794 also peaked at the same time point. The caspase-3 histology data in Figure 3.3 A corresponds to an experiment that treated a cohort of rats with dexamethasone but did not dose with PSS-794. In this case, the number of dead and dying thymocytes reached a maximum at 12 h after dexamethasone treatment, an earlier time point that agrees with previous studies of this thymocyte cell death model. Figure 3.4 shows a comparison of caspase-3 histology data for two cohorts of rats that were not treated with dexamethasone but one cohort was dosed with PSS-794. The amount of caspase-3 was 50% higher in the thymus tissues from the rats dosed with PSS-794. The data in Figures 3.3 and 3.4 indicate that the presence of PSS-794 is either inducing an increase in the amount of thymocyte cell death or decreasing the rate of dead cell clearance. Our previous studies of Zn$_2$BDPA probes have produced no evidence for cell toxicity, so it seems more likely that PSS-794 is inhibiting cell clearance. It is well-known that the appearance of PS on the cell surface is a biomarker that triggers recognition and subsequent cell clearance by phagocytes. Furthermore, it is reported that the presence of PS-binding Annexin V can inhibit phagocytosis in vivo. Therefore, PSS-794 may be acting like Annexin V and inhibiting dead cell clearance from the thymus by binding to PS and preventing recognition by phagocytes. Future studies will attempt to confirm this explanation and address its implications. For example, the value of a diagnostic imaging probe may be limited if it elicits a physiological response, but this effect can be attenuated by employing lower probe doses that only occupy a small fraction of the target site.
The second animal model compared the performance of PSS-794, 794 Control, and a commercially available, near-infrared Annexin V (Annexin-Vivo 750) to target acute and localized tissue damage achieved by injecting a dose of one of three chemicals that cause cell death (ionophore, ethanol, or ketamine) into the rear leg muscle of anesthetized mice. Although this tissue death model has limited clinical relevance, it acts as a highly reproducible, in vivo experimental system for quantitative comparison of fluorescence imaging probes. Cell death occurs very quickly at the site of injection. The rear leg muscle is a relatively shallow anatomical location that allows ready acquisition of target fluorescence signals that are distant from non-target background signals emanating from the major internal organs.

Initial experiments compared the ability of PSS-794 and 794 Control to target and image damaged muscle tissue in immunocompromised mice. First, three cohorts of eight animals were anesthetized and given an intramuscular injection in the right rear leg muscle with either ionophore, ethanol, or ketamine. Next, each animal was given an injection of saline solution in the opposite leg to serve as an injection control. After the animals were dosed with cytotoxin, they were returned to their cages for two hours. Each injection group of eight mice was then separated into two cohorts of four mice. One cohort was injected intravenously with a solution of PSS-794, and a second cohort was treated with an identical intravenous injection of 794 Control. Each animal was then imaged over time using a planar, whole-body fluorescence scanner with a near-infrared filter set (ex. $750 \pm 10$ nm, em. $830 \pm 20$ nm). The reflected fluorescence images were overlaid on X-ray images to facilitate anatomical co-registration. Figure 3.5 shows a comparison of images acquired 24 hours after the injection of imaging probes. PSS-794
Figure 3.5. X-ray and fluorescence overlay images of mice treated with either synthetic ionophore, ethanol, or ketamine and dosed with PSS-794 (A-C) or 794 Control (D-F). Images were acquired 24 h post-injection of the probes. The calibration bar shows the fold increase in fluorescence counts from the minimum fluorescence counts. Reprinted with permission from Smith, B. A. et al. (2011) In Vivo Optical Imaging of Acute Cell Death Using a Near-Infrared Fluorescent Zinc−Dipicolylamine Probe. Mol. Pharmaceutics 8, 583-590. Copyright 2011 American Chemical Society.

had localized to the sites of tissue damage in the rear leg, while 794 Control could only be seen in the liver and intestines. Histological analysis of the cytotoxin-treated tissues was performed to confirm molecular targeting of PSS-794 to dead and dying cells. The tissues were flash frozen in OCT, and subjected to cryosectioning and microscopic imaging with and without H&E staining. The leg muscle injected with ionophore was clearly damaged as judged by the degradation of cell structure (Figure 3.6.). Near-infrared fluorescence microscopy of the same cells showed PSS-794 localization in the
periphery of the damaged cells. This result was expected given the probe’s affinity for the anionic PS exposed in these membranes. PSS-794 showed very little uptake in sections from healthy, control tissue (Figure 3.6.). Similar histology and fluorescence microscopy data were obtained for tissue sections taken from the ethanol and ketamine injected cohorts (Figure 3.7.).

**Figure 3.6.** Representative histological sections from damaged leg muscle due to injection of ionophore (A-C) or undamaged leg muscle that was only injected with saline (D-F). The micrographs were subjected either to H&E staining (A, D) or left unstained. Unstained micrographs were viewed using the brightfield (B, E) and NIR (C, F) filter sets. Scale bar = 200 μm for panels A and D. Scale bar = 50 μm for panels B, C, E, F. Reprinted with permission from Smith, B. A. et al. (2011) In Vivo Optical Imaging of Acute Cell Death Using a Near-Infrared Fluorescent Zinc–Dipicolylamine Probe. *Mol. Pharmaceutics* 8, 583-590. Copyright 2011 American Chemical Society.
As a second comparison, we conducted an imaging study using Annexin-Vivo 750, a commercially available, near-infrared fluorescent conjugate of Annexin V that can be monitored using the same near-infrared fluorescence filter set as above. Two cohorts of four mice were treated with either ionophore or ethanol to induce tissue damage. Subsequently, the mice were dosed with the Annexin-Vivo 750 and imaged periodically over 48 hours. A comparison of the time course images for PSS-794 and Annexin-Vivo

Figure 3.7. Histological micrographs of PSS-794 accumulation in tissue sections from damaged leg muscles treated with either ethanol (A-C) or ketamine (D-F). Micrographs were stained with hematoxylin and eosin (A, D) or left unstained. Unstained micrographs were imaged in the brightfield (B, E) and NIR (C, F) filter sets. Scale bar = 200 μm for panels A and D. Scale bar = 50 μm for panels B, C, E, F. Reprinted with permission from Smith, B. A. et al. (2011) In Vivo Optical Imaging of Acute Cell Death Using a Near-Infrared Fluorescent Zinc–Dipicolylamine Probe. Mol. Pharmaceutics 8, 583-590. Copyright 2011 American Chemical Society.
Figure 3.8. Representative near-infrared fluorescence images of a mouse treated with ionophore in the hind leg and injected with either PSS-794 (top row) or Annexin-Vivo 750 (bottom row) via the tail vein. Both cohorts of mice were injected with ionophore in the right hind leg muscle and saline in the left hind leg muscle. The mice were dosed with either PSS-794 or Annexin-Vivo 750 two hours post-treatment. Images were acquired at the indicated time points after probe injection. The calibration bar applies to all images. Reprinted with permission from Smith, B. A. et al. (2011) In Vivo Optical Imaging of Acute Cell Death Using a Near-Infrared Fluorescent Zinc–Dipicolylamine Probe. Mol. Pharmaceutics 8, 583-590. Copyright 2011 American Chemical Society.

750 (Figure 3.8) shows that PSS-794 clears more rapidly from the bloodstream and through the liver, where the major clearance pathway for the Annexin-Vivo 750 is through the kidneys and bladder. Although the vendor for Annexin-Vivo 750 recommends imaging 2-4 hours after intravenous dosing, the images in Figure 3.8 show that it took more than 12 hours for the signal at the site of tissue damage to be clearly delineated.
A quantitative, in vivo image analysis was performed on each cohort of living mice. In each case, a region of interest (ROI) was drawn around the target (T) site of damaged tissue in the leg that received the cytotoxin and also around a non-target (NT) site of equivalent size on the opposite leg of the mouse that received the saline injection. Each ROI provided a mean pixel intensity, which was used to calculate an average T/NT ratio for each cohort. Since all three probes have essentially the same photophysical properties, and thus the same amount of signal attenuation with tissue depth, the T/NT ratios can be treated as quantitative measures of probe uptake at the tissue damage site. Animals injected with PSS-794 showed significantly higher T/NT ratios than either 794 Control or Annexin-Vivo 750. By the 24 h time point, PSS-794 yielded T/NT values of 5.97 ± 0.42 for tissue damage caused by ionophore, 4.15 ± 0.25 with ethanol, and 2.83 ± 0.09 with ketamine. As expected, 794 Control showed very little uptake at the site of tissue damage. In the case of Annexin Vivo 750, the T/NT ratios were relatively low due to slow clearance of background signal from the non-target site and steadily increased over time to reach values of 2.5 after 48 hours.

The size and molecular weight difference between PSS-794 and Annexin-Vivo 750 is probably why PSS-794 exhibits faster exchange with the site of tissue damage and why there is a higher accumulated signal intensity. PSS-794 is about 30 times smaller than Annexin V (MW ~36 kDa), thus diffusion of PSS-794 between the blood pool and the site of leg muscle damage is expected to be faster. It is known that each Annexin V protein covers approximately 50 phospholipid molecules on the membrane surface. It is possible that, at target site saturation, the smaller PSS-794 can produce a higher loading of fluorophores per membrane surface area. There also may be a size-dependent
difference in the ability of the probes to permeate into the cytosol of dying cells. It is estimated that early apoptotic monocytes have approximately $10^9$ available binding sites for Annexin V, and after these cells become necrotic, the number of binding sites increases 2.5 fold. It is possible that the smaller PSS-794 may diffuse through the plasma membranes of early/middle stage apoptotic cells that are comparatively impermeable to Annexin V and thus gain access to a higher number of anionic binding sites. Finally, the two probes may have different effects on the ability of the innate immune system to clear probe-labeled dead and dying cells. For example, it is known that Annexin V inhibits phagocytosis,\textsuperscript{152} by promoting internalization of crucial membrane proteins. Slow clearance of Annexin-bound apoptotic cells may explain why the site of tissue damage

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.9}
\caption{Bar graph showing ex vivo muscle and skin tissue distribution of PSS-794, 794 Control, and Annexin-Vivo 750 in ionophore treated mice. Tissues are abbreviated as the following: Msc (muscle) and Skn (skin). Error bars represent the standard error of the mean. N = 4. Reprinted with permission from Smith, B. A. et al. (2011) In Vivo Optical Imaging of Acute Cell Death Using a Near-Infrared Fluorescent Zinc−Dipicolylamine Probe. \textit{Mol. Pharmaceutics} 8, 583-590. Copyright 2011 American Chemical Society.}
\end{figure}
still shows residual Annexin V signal at 48 hours after intravenous injection.

The in vivo imaging results were confirmed by performing quantitative image analyses on the excised organs. After the final time point, the animals from each cohort were anesthetized and euthanized by cervical dislocation. The internal organs and tissues were removed and imaged using the fluorescence scanner and filter set described above. ROI analysis of the images provided mean pixel intensities. Figure 3.9 shows a graph of mean pixel intensities for a select group of muscle and tissue samples from mice that were treated with ionophore. The highest staining of damaged muscle tissue was obtained with PSS-794, and as expected, the same trend was observed with the animal groups that were treated with ethanol and ketamine. There was also significant staining of the skin that covered the site of tissue damage indicating local, limited diffusion of the cytotoxic chemical from the injection site. As expected for the other two probes, there was minimal tissue uptake of the 794 Control, but there was clear evidence that Annexin-Vivo 750 selectively targeted the damaged muscle and skin (Figure 3.9). Taken together, the in vivo images in Figure 3.5, the ex vivo data given in Figure 3.9, and the histological results presented in Figure 3.6 demonstrate that intravenous dosing of PSS-794 leads to rapid and selective accumulation at the site of tissue damage.

3.3. Conclusions

In summary, the results of this study add to the growing evidence that the synthetic fluorescent molecular probe, PSS-794, is able to target dead and dying cells in various animal models of cell death. The importance of the Zn$_2$BDPA unit as a targeting group for exposed PS on the surface of the dead and dying cells is highlighted by the lack of measurable uptake with the control dye, 794 Control. When compared to the
mechanistically similar Annexin-Vivo 750, PSS-794 produced a higher targeting ratio and faster kinetics, in part because there was a high bladder background signal with the Annexin V probe. The different clearance pathways for PSS-794 (liver/intestines) and Annexin-Vivo 750 (kidney/bladder) suggests that they could have value as a complementary pair of fluorescent probes for imaging cell death in different anatomical locations.

3.4. Methods and Materials

3.4.1. Synthesis

The synthesis of fluorescent probes, PSS-794 and 794-Control, have been reported previously.\textsuperscript{128a} Ionophore was prepared as previously reported in detail.\textsuperscript{153} Annexin-Vivo 750\textsuperscript{®} (\(\lambda_{ex}: 755\) nm, \(\lambda_{em}: 772\) nm) was purchased from PerkinElmer (Waltham, MA).

3.4.2. Dexamethasone-Induced Thymocyte Cell Death Rat Model

All animal procedures were approved by the University of Notre Dame Institutional Animal Care and Use Committee. Six cohorts of 8-week old male Lobund-Wistar rats (Freimann Life Science Center, 250 g) (n = 4) were given intraperitoneal injections (50 mg/kg) of water soluble dexamethasone (Sigma Aldrich, St. Louis, MO) dissolved in 400 \(\mu\)L of distilled H\(_2\)O. The time from dexamethasone dosing to sacrifice of the cohort (and its related control cohort) was either 30, 42, or 48 h, with injection of fluorescent probe at 24 h before sacrifice. The fluorescent probe (either PSS-794 in 1% DMSO/H\(_2\)O or 794 Control in H\(_2\)O) was injected intravenously via the tail vein and produced a dosage of 3.0 mg/kg. Two additional cohorts of rats (n = 4) were not treated with dexamethasone, but were injected with fluorescent probe (either PSS-794 in 1%
DMSO/H₂O or 794 Control in H₂O). To evaluate the ability of PSS-794 to detect different levels of thymocyte cell death, three cohorts of rats (n = 4) were treated with 10 mg/kg, 25 mg/kg, or 50 mg/kg of dexamethasone. Eighteen hours later, the rats were injected intravenously with PSS-794 (3.0 mg/kg) and sacrificed after another 24 h. Two cohorts of rats (n = 4) were used to measure the effect of PSS-794 on the basal level of thymocyte cell death. The rats were not treated with dexamethasone but one cohort was injected intravenously with PSS-794 (3.0 mg/kg). Twenty-four hours later both cohorts were sacrificed and the excised thymus from each animal was evaluated by a caspase-3 histology assay. All animals were euthanized by either CO₂ asphyxiation or cervical dislocation under anesthesia.

After sacrifice, selected rat tissues were excised, placed on a transparent imaging tray, and imaged using a Carestream Health In-Vivo Multispectral Imaging System FX equipped with a 750 ± 10 nm excitation filter and 830 ± 20 nm emission filter (30 s time acquisition, bin = 2 x 2, f-stop = 2.51, field of view = 190 mm).

3.4.3. Acute Tissue Damage Mouse Model

Athymic nude mice (strain nu/nu, Taconic Inc., New York) were anesthetized (1.5% isoflurane inhalation) and injected intramuscularly in the rear leg muscle with 50 µL of either ionophore (0.5 mg in 50 µL ethanol), ethanol (100 %, 50 µL), or ketamine (5.0 mg in 50 µL H₂O). The same location on the opposite leg was injected with 50 µL of saline, which served as a vehicle control. Two hours after treatment, each mouse was intravenously injected via the tail vein with either PSS-794 (3.0 mg/kg in 100 µL 1% DMSO/ H₂O) or 794 Control (3.0 mg/kg in 100 µL H₂O), or Annexin-Vivo 750 in accordance with manufacturer protocol.
For whole body, epifluorescence imaging, nude mice were anesthetized (1.5% isoflurane inhalation) and placed inside a Carestream Health In Vivo Multispectral Imaging Station FX (Rochester, NY) configured for epi-illumination. The animals were irradiated with filtered light of wavelength 750 ± 10 nm, and an image of emission intensity at 830 ± 20 nm was collected by a CCD camera during a 30 s acquisition period (bin = 2 x 2, f-stop = 2.51, field of view = 120 mm). Mice were imaged before and immediately after injection of PSS-794 or 794 Control, and at 3, 6, 12 and 24 h time points. Following the 24 h time point, an x-ray image was acquired for each mouse (f-stop = 2.86, filter = 0.4 mm, field of view = 120 mm, bin = 0, image acquisition time = 180 second).

After the 24 h time point, the animals were euthanized by cervical dislocation under anesthesia. The selected tissues were excised, placed on a transparent imaging tray, and imaged using the 750 ± 10 nm excitation filter and 830 ± 20 nm emission filter (30 s time acquisition, bin = 2 x 2, f-stop = 2.51, field of view = 120 mm). The excised organs were not examined for signs of chemical induced toxicity.

3.4.4. Image Analysis

Images were analyzed using ImageJ. The 16-bit images were imported, opened in sequential order, and converted to an image stack. Background subtraction was applied to the images using the rolling ball algorithm (radius = 1000 pixels). The stack was then converted to a montage and pseudocolored as “Thai” (under the “Lookup Tables” menu). Region of interest (ROI) analysis was performed on each ex vivo image by manually drawing an ROI to outline each tissue. The mean pixel intensities for each tissue were measured and recorded. The resulting ROI values were plotted using Graphpad Prism 4.
3.4.5. Histology

For the dexamethasone rat model studies, the thymus was excised, flash frozen in OCT compound and sliced at 10 μm thickness. The sections were incubated overnight with a rabbit anti-human polyclonal caspase-3 antibody (Abcam Inc.; 1:50). The sections were incubated with goat anti-rabbit IgG conjugated to Alexa-Fluor 488 (Invitrogen; 1:500) for 15 min, and then counterstained with DAPI. Fluorescence images of the sections were acquired using a Nikon TE-2000U epifluorescence microscope equipped with the appropriate UV (ex. 340/80 nm, em. 435/85 nm), GFP (ex. 450/90 nm, em. 500/50 nm), and Cy7 filters (ex. 710/75 nm, em. 810/90 nm). Images were captured using Metamorph software (Universal) and analyzed using ImageJ. The caspase-3 levels in Figures 3.3. and 3.4. were determined by averaging the integrated fluorescence in the GFP channel for eight fields of view chosen at random.

For the acute tissue damage mouse model studies, samples of skeletal muscle from injection sites were harvested, fixed for 24 h in 10% neutral buffered formalin, and placed in 70% ethanol before embedding in paraffin. The tissues were then sectioned at 4 μm, stained with hematoxylin and eosin and examined by light microscopy for histological changes. Unstained sections were imaged using a Nikon Eclipse TE-2000U epifluorescence microscope (Nikon Corporation, Tokyo, Japan) equipped with the appropriate Cy7 filter (ex: 710/75, em: 810/90). Images were captured using Metamorph software (Universal) and analyzed using ImageJ.

3.4.6. Statistical Analysis

All values are depicted as mean ± standard error of the mean (SEM). Statistical analysis was performed using a Student’s t-test.
4.1. Background

Traumatic brain injury (TBI) is a major public health concern in the United States with 1.7 million people sustaining a TBI annually.\textsuperscript{154} Over 2% of the US population is believed to experience TBI-associated disabilities, accounting for approximately $60 billion annually in direct and indirect costs.\textsuperscript{155} TBI is a highly heterogeneous disorder that can manifest different pathophysiological changes depending on the type, severity, and location of the brain injury. TBI is typically characterized in two stages: 1) the primary injury at the site of impact which results in tissue damage and hemorrhaging, and 2) the delayed secondary insult that represents non-mechanical damage due to continuous pathological processes.\textsuperscript{156} These pathological processes include blood-brain barrier disruption, edema, oxidative stress, inflammation and cell death. Clinical presentation of the secondary insult is usually delayed and believed to be sensitive to therapeutic intervention. Thus, the secondary pathological processes may be viable options as therapeutic and imaging targets for treatment and diagnosis of TBI.
Brain imaging is routinely used in the clinic to diagnose and manage TBI. Non-contrast computer tomography (CT) is the standard modality for patients with an acute brain injury as it is very accurate at identifying changes in cerebral morphology. However, the lack of sensitivity has prevented CT images from offering accurate prognosis of TBI. Although magnetic resonance imaging (MRI) is a more sensitive imaging alternative, technical difficulties and problems with quantification methods deter its use in the clinic. Both modalities rely on morphological changes, which occur later in the disease process thus TBI diagnosis and prognosis would be better served using molecular imaging techniques that target early-stage biochemical changes.

Fluorescence optical imaging is an attractive option due to its inherent safety and high sensitivity, but very few optical imaging probes have been evaluated in animal models of TBI. The cell death probe, N,N′-didansyl-L-cystine (DDC), was found to accumulate in apoptotic cells in the brain; however, poor tissue penetration of the visible light associated with this fluorophore prevented non-invasive imaging of living animals. Thus, there is a need for improved deep-red and near-infrared fluorescent probes that allow deeper penetration of the light for improved in vivo imaging of TBI. An attractive concept is the possibility of multicolor imaging using multiple probes in a single animal; each probe with its distinctive wavelength and ability to report on different biomolecular processes. At present, there is a small but growing number of literature examples of simultaneous multicolor in vivo optical imaging. In the case of TBI, imaging studies would be greatly facilitated by the development of protocols that employ a mixture of targeted probe for cell death and non-targeted tracer for blood-brain-barrier (BBB) permeability. This requires two sets of technical advances: 1) development and
validation of deep-red and near-infrared fluorescent imaging and tracer probes that enable multicolor in vivo imaging in living animals, and 2) straightforward pre-clinical TBI models that are amenable to optical imaging. Here we address both needs by adapting a preclinical mouse model for TBI and investigating a set of complementary fluorescent imaging probes. We demonstrate the ability of PSS-794 to non-invasively detect cell
death in a brain cryolesion TBI mouse model using whole-body, epi-fluorescence imaging. Specifically, we compare cell death imaging performance of PSS-794 and fluorescently labeled Annexin V, a well-known protein-based probe for cell death imaging.\textsuperscript{164} We also describe the non-targeted, deep-red dye, Tracer-653, for monitoring blood-brain-barrier disruption, and a binary mixture of PSS-794 and Tracer-653 for multicolor imaging of cell death and blood-brain-barrier permeability in a single animal.

4.2. Results and Discussion

The study examined four fluorescent imaging probes. Two of the probes, the synthetic zinc(II)-bis-dipicolylamine complex, PSS-794, and the dye-labeled protein, Annexin-Vivo 750, are known to selectively target the anionic membranes of dead and dying cells. The other two probes, 794 Control and Tracer-653, are non-targeted fluorophores that diffuse through the blood pool, and thus serve to measure permeability of the BBB.\textsuperscript{165,166} Tracer-653 is a deep-red dye with a narrow emission band that can be monitored simultaneously with near-infrared PSS-794 in the same animal using different imaging filter sets (filter sets for PSS-794 ex: 705-780 nm, em: 810-885 nm; filter sets for Tracer-653 ex: 615-665 nm, em: 695-770 nm). The specific targeting of the four fluorescent probes was tested in an adapted brain cryolesion mouse model that induces rapid breakdown of the BBB causing vasogenic brain edema and tissue damage.\textsuperscript{167} This model is a technically simple, high-throughput version of a previously reported model that contacts a pre-cooled rod with the surgically exposed skull of mice.\textsuperscript{168,169,170} We chose to not remove the skin around the skull because preliminary studies indicated that the cell death probes target the dead skin cells generated during the surgery, thus complicating the in vivo imaging. We find that the lesions caused by this adapted
Figure 4.2. Representative in vivo near-infrared fluorescence montages of PSS-794, 794 Control, and Annexin-Vivo 750 accumulation in a brain cryoinjury mouse model. A pre-cooled metal cylinder was applied to the head of each mouse for 60 s followed by intravenous injection of either PSS-794 (3.0 mg/kg), 794 Control (3.0 mg/kg), or Annexin-Vivo 750. Images were acquired at the indicated time points after probe injection. N = 5. Reprinted with permission from Smith, B. A. et al. (2012) Multicolor Fluorescence Imaging of Traumatic Brain Injury. ACS Chem. Neurosci. DOI: 10.1021/cn3000197. Copyright 2012 American Chemical Society.

cryoinjury model are highly reproducible in size and location and can be clearly delineated from the rest of the brain. However, this model only conditionally mimics human TBI and lacks the diffusion axonal injuries that complicate human head injuries. Other TBI mouse models such as the controlled cortical impact and fluid percussion injury models can mimic the whole spectrum of focal-type brain injuries and produce axonal injuries. But these models have technical drawbacks including the need for specialized equipment and the requirement to perform animal craniotomy.
In short, two separate cohorts of athymic mice (n = 5) were anesthetized by isoflurane inhalation. A metal cylinder with a 3 mm diameter was pre-cooled in liquid nitrogen and applied to each mouse’s head for 60 s. Individual mice then received an intracardiac injection of either PSS-794 (3.0 mg/kg), 794 Control (3.0 mg/kg), or Annexin-Vivo 750 and imaged in an IVIS Spectrum at 1 h, 3 h, 6 h and 24 h post-probe injection. Figure 4.2 shows representative images at the different timepoints. Immediately after probe injection, there is apparent accumulation of 794 Control at the site of cryolesion but the fluorophore soon clears from the animal. At 3 h post-probe injection, cell death probes PSS-794 and Annexin-Vivo 750 both produce intense signals at the site of cryoinjury. As expected, the Annexin-Vivo 750 also showed extensive accumulation.
Figure 4.4. Comparison of probe localization at the cryoinjury site in different tissues. A pre-cooled metal cylinder was applied to the heads of mice for 60 s, and each mouse was dosed with either PSS-794, 794 Control, or Annexin-Vivo 750. The mice were imaged 24 h later, sacrificed and imaged again with the skin removed (no skin), and with both the skin and skull removed (no skull). With each image, a region of interest (ROI) was drawn around the cryoinjury site; the mean pixel intensity (MPI) was recorded and normalized to the mean pixel intensity of the in vivo (normal) image. Normalized MPI ± standard error of the mean. N = 5. Reprinted with permission from Smith, B. A. et al. (2012) Multicolor Fluorescence Imaging of Traumatic Brain Injury. ACS Chem. Neurosci. DOI: 10.1021/cn3000197. Copyright 2012 American Chemical Society.

in the kidneys. Target to non-target (T/NT) quantification of in vivo images was performed by region of interest (ROI) analysis that compared the mean pixel intensities (MPI) at the cryoinjury site (T) to MPI at a non-target site (NT) on the lower back (Figure 4.3). PSS-794 exhibited the highest T/NT at the 1 h timepoint (7.74 ± 0.59; T/NT ± SEM), and then decreased. In contrast, the T/NT for Annexin-Vivo 750 increased incrementally to a value of 4.87 ± 0.54 at the 24 h endpoint, reflecting a slower rate of clearance from the non-target site. At 24 h after probe injection, the mice were sacrificed and subjected to ex vivo imaging and histological analysis. Ex vivo whole-body images
Figure 4.5. Absolute mean pixel intensities used to create the data in Figure 4.4. Error bars are ± standard error of the mean. * P < 0.03, ** P < 0.001, *** P < 0.0005. N = 5. Reprinted with permission from Smith, B. A. et al. (2012) Multicolor Fluorescence Imaging of Traumatic Brain Injury. ACS Chem. Neurosci. DOI: 10.1021/cn3000197. Copyright 2012 American Chemical Society.

were acquired with 1) the skin removed from the head, which exposed the tissue over the skull, and 2) both the skin and the skull removed, which exposed the brain. ROI analysis compared the cryoinjury site in vivo just before animal sacrifice (labeled as Normal), to the deceased animal with skin removed (labeled as No Skin), and with both the skin and skull removed (labeled as No Skull). In each case, the MPI were recorded and normalized to the in vivo values. The normalized MPI for PSS-794 and Annexin-Vivo 750 decreased with each layer of tissue removed from the head (Figure 4.4). This is unusual because MPI at a deep-tissue site typically increases as the intervening skin and tissue is removed. It appears that PSS-794 and Annexin-Vivo 750 target the cryolesion-induced cell death that is occurring on the skin, the pericranium, and on the brain. The normalized MPI for 794 Control images exhibited a different trend, and increased with removal of
the skin followed by a decrease in MPI with removal of the skull (Figure 4.4). But the absolute MPI for the 794 Control images were substantially lower than the values for the PSS-794 images, reflecting the much greater clearance of tracer dye from the cryoinjury (Figure 4.5). These spatial and temporal differences in probe localization indicate that the targeted cell death probe PSS-794 and non-targeted 794 Control accumulate in the brain.

**Figure 4.6.** Representative histological micrographs from cryoinjured mouse brains. The micrographs were subjected either to H&E staining (A) or counterstained with an anti-caspase-3 antibody and DAPI (B). Images in B are from the region of the brain cryoinjury. Scale bar in A = 500 μM. Scale bar in B = 200 μM. Reprinted with permission from Smith, B. A. et al. (2012) Multicolor Fluorescence Imaging of Traumatic Brain Injury. *ACS Chem. Neurosci.* DOI: 10.1021/cn3000197. Copyright 2012 American Chemical Society.
cryoinjury by different mechanisms.

H&E micrographs of sectioned cryoinjured brains from mice sacrificed at 24 h after probe injection, showed a focal region of cell death that was surrounded by healthy brain tissue (Figure 4.6 A). Cryoinjured brains were also fixed in 4% formaldehyde, cut into 10 μM paraffin sections and imaged on a LI-COR Odyssey fluorescence scanner to determine probe distribution throughout the brain. There was high accumulation of PSS-794 at the cryolesion site, while only negligible amounts of 794 Control were in the cryoinjured brain (Figure 4.7). To further confirm that PSS-794 was targeting sites of brain cell death, immunohistochemistry was performed on the cryoinjured brains using an

![Figure 4.7](image)

**Figure 4.7.** Ex vivo NIR fluorescence images of cryoinjured brain sections containing PSS-794 (A) or 794 Control (B). A pre-cooled metal cylinder was applied to the heads of mice for 60 s, and each mouse was dosed with either PSS-794 (3.0 mg/kg) or 794 Control (3.0 mg/kg). After an additional 24 h, the mice were sacrificed and the brains were excised. Brains were flash frozen in OCT and cryosectioned at 10 μM thickness. The images show PSS-794 around the site of cryolesion, while a negligible amount of 794 Control remains in the brain after 24 h. Reprinted with permission from Smith, B. A. et al. (2012) Multicolor Fluorescence Imaging of Traumatic Brain Injury. *ACS Chem. Neurosci.* DOI: 10.1021/cn3000197. Copyright 2012 American Chemical Society.
antibody specific for activated caspase-3. Fluorescence microscopy showed extensive staining of activated caspase-3 around the cryolesion, and also strong near-infrared fluorescence signal from the PSS-794 (Figure 4.6 B). Caspase-3 and PSS-794 staining could not be visualized in healthy regions of the brain. Fluorescence from 794 Control could not be detected in the same regions as caspase-3 in the cryoinjured brains.

To determine if PSS-794 could measure TBI severity in vivo, the cryolesion experiment was repeated, with the time for contacting the pre-cooled metal cylinder to the mouse’s head reduced to 20 s. The animals were subsequently dosed with either PSS-794 or 794 Control and then imaged over time (Figure 4.8). As shown in Figure 4.9,

![Figure 4.8. Representative in vivo near-infrared fluorescence montage of mice with a 20 s brain cryoinjury. Both cohorts of mice were anesthetized and a pre-cooled metal cylinder was applied to each mouse’s head for 20 s. The mice were dosed with either PSS-794 or 794 Control immediately after the cryoinjury. Images were acquired at the indicated time points after probe injection. The calibration bar applies to all images. N = 5. Reprinted with permission from Smith, B. A. et al. (2012) Multicolor Fluorescence Imaging of Traumatic Brain Injury. ACS Chem. Neurosci. DOI: 10.1021/cn3000197. Copyright 2012 American Chemical Society.](image-url)
Figure 4.9. Comparison of PSS-794 (blue) and 794 Control (red) accumulation in the 20 s (dashed line) and 60 s (solid line) brain cryoinjury mouse models. T/NT ratios were calculated by ROI analysis. T/NT ± SEM. N = 5. Reprinted with permission from Smith, B. A. et al. (2012) Multicolor Fluorescence Imaging of Traumatic Brain Injury. ACS Chem. Neurosci. DOI: 10.1021/cn3000197. Copyright 2012 American Chemical Society.

T/NT values for PSS-794 and 794 Control were lower in the 20 s cryolesion mouse model indicating less tissue damage compared to the 60 s cryolesion. Quantification of the ex vivo images with skin or skull removed showed similar PSS-794 and 794 Control staining patterns as with the 60 s cryolesion. These results suggest that for pre-clinical studies measuring TBI severity in mouse models, optical imaging using PSS-794 appears to be a complementary alternate to classical methods that monitor changes in lesion volume.\textsuperscript{175}

Evans Blue is routinely used to monitor BBB disruption in animal models;\textsuperscript{176,177} thus, it served as a positive control for Tracer-653 in this mouse TBI model. Separate cohorts of cryoinjured mice were administered Evans Blue and Tracer-653. Evans Blue extravasation is typically quantified using histology; however, we utilized its weak red fluorescence emission to perform ex vivo imaging of the brain sections.\textsuperscript{178} The ex vivo
images of cryoinjured brains clearly showed accumulation of Evans Blue at Day 0 and less so at Day 3 post-injury (Figure 4.10). When compared to Tracer-653, the area of Evans Blue staining was more localized at the site of cryoinjury. In vivo, Evans Blue binds to albumin proteins causing it to have a significantly higher molecular weight compared to Tracer-653 (67 kDa vs. 2 kDa). The smaller effective size of Tracer-653 likely allows it to permeate into areas that are not accessible by the Evans Blue-albumin

**Figure 4.10.** Comparison between Evans Blue and Tracer-653 accumulation into cryoinjured mouse brains. Ex vivo fluorescence images of Evans Blue and Tracer-653 in brains excised from mice that were dosed with probe either 6 h (Day 0) or 3 days (Day 3) after cryoinjury (A). Bar graph of Evans Blue and Tracer-653 mean pixel intensities at the site of the cryoinjury (B). A region of interest was drawn around the area of the cryoinjury on the brain, and the mean pixel intensity was recorded. Mean pixel intensity ± standard error of the mean. N = 3-4. Reprinted with permission from Smith, B. A. et al. (2012) Multicolor Fluorescence Imaging of Traumatic Brain Injury. *ACS Chem. Neurosci.* DOI: 10.1021/cn3000197. Copyright 2012 American Chemical Society.
complex thus increasing the area of tissue that is stained by Tracer-653.\textsuperscript{180,181} Taken together, the results indicate that Tracer-653 is an effective substitute for Evans Blue as a tracer probe for monitoring BBB disruption in TBI. Compared to Evans Blue, Tracer-653 exhibits a much brighter and more narrow, deep-red emission band, and thus is more amenable to multicolor optical imaging. Multicolor fluorescence imaging is a promising new method to simultaneously monitor different physiological processes,\textsuperscript{182,183} but has previously not been exploited to monitor TBI progression in mice. To determine if we

\textbf{Figure 4.11.} Multicolor fluorescence imaging of cell death and blood-brain barrier disruption in cryoinjured brains. Three cohorts of hairless mice were given a 60 s brain cryoinjury. Mice were then injected with a single dose of PSS-794 either immediately following cryoinjury (Day 0), 3 days post-cryoinjury (Day 3), or 7 days post-cryoinjury (Day 7). Each mouse was also injected with Tracer-653 at five hours post-PSS-794 injection. One hour after Tracer-653 injection, the mice were anesthetized and sacrificed. The brains were excised and placed in an epi-fluorescence imaging station for ex vivo imaging. Reprinted with permission from Smith, B. A. et al. (2012) Multicolor Fluorescence Imaging of Traumatic Brain Injury. \textit{ACS Chem. Neurosci.} DOI: 10.1021/cn3000197. Copyright 2012 American Chemical Society.
could simultaneously follow cranial cell death and BBB disruption in a single animal, we injected near-infrared PSS-794 and deep-red, Tracer-653 into mice that had received cryolesions. In short, three cohorts of hairless mice were administered 60 s brain cryolesions, and then injected with PSS-794 either immediately following cryoinjury, at 3 days post-cryoinjury, or at 7 days post-cryoinjury. Each mouse was also injected with Tracer-653 at 5 h post-PSS-794 injection (the injection lag time accounts for the difference in probe clearance rates). At one hour after Tracer-653 injection, the mice were anesthetized and sacrificed. The brains were excised and placed in an epifluorescence imaging station for ex vivo imaging. As shown in Figure 4.11, the amount of PSS-794 accumulation in these cryoinjured brain sections decreased greatly with the

![Image of bar graph]

**Figure 4.12.** Comparison of PSS-794 and Tracer-653 in brain sections taken from mice that were dosed with probe at the following timepoints after cryoinjury, Day 0 (black bar), Day 3 (dashed bar), and Day 7 (white bar). A region of interest was drawn around the area of the cryoinjury on the brain, and the mean pixel intensity was recorded. Mean pixel intensity ± standard error of the mean. N = 3-4. Reprinted with permission from Smith, B. A. et al. (2012) Multicolor Fluorescence Imaging of Traumatic Brain Injury. ACS Chem. Neurosci. DOI: 10.1021/cn3000197. Copyright 2012 American Chemical Society.
age of the injury. For example, the MPI for a cryolesion section on Day 3 was six-fold lower than the equivalent section on Day 0 (Figure 4.12), indicating a substantial and relatively rapid decrease in the number dead and dying cells, presumably due to efficient dead cell clearance by the animal’s innate immune system. In comparison the Tracer-653 images indicate that healing and repair of the BBB was a much slower process. For example, the MPI for a cryolesion section on Day 7 was only about two-fold lower than the equivalent section on Day 0 (Figure 4.12). Furthermore, the amount of Tracer-653 in a cryoinjured brain on Day 7 (as judged by comparing MPI) was three times higher than an equivalent brain taken from a healthy control mouse treated with Tracer-653. Taken together, the Tracer-653 data consistently indicates that there is a gradual but incomplete improvement in BBB integrity over the seven day post-cryolesion period. This timeframe for BBB disruption is consistent with previous literature observations.

4.3. Conclusions

In summary, we report that the synthetic near-infrared fluorescent probe, PSS-794, can be used to visualize cell death in an adapted cryolesion mouse model of TBI. The optical images with PSS-794 produced higher cryolesion signal contrast (higher T/NT ratio) than the mechanistically similar protein probe, Annexin-Vivo 750. Tracer-653 was validated as a low molecular weight, deep-red tracer dye for monitoring BBB disruption, and a binary mixture of PSS-794 and Tracer-653 was employed for multicolor imaging of cell death and BBB permeability in a single animal. The imaging data indicates that at three days after brain cryoinjury the amount of cell death had decreased significantly, but the integrity of the BBB was still impaired; at seven days the BBB was still substantially more permeable than before cryoinjury. The pathophysiological
outcomes of TBI are highly heterogeneous in terms of severity and rate of progression. The time between physical trauma and the onset of secondary processes such as BBB breakdown is a potential window for therapeutic treatment.\textsuperscript{185} It should be possible to develop this adapted cryoinjury mouse model into a high throughput, optical imaging screen of experimental therapeutics for TBI.

4.4. Methods and Materials

4.4.1. Synthesis

The synthesis and properties of 794 Control, PSS-794, and Tracer-653 ($\lambda_{\text{ex}}$: 653 nm, $\lambda_{\text{em}}$: 673 nm) have been reported previously.\textsuperscript{128a,186} PSS-794 is commercially available as PSVue\textsuperscript{®} 794 (Molecular Targeting Technologies Inc., West Chester, PA). Annexin-Vivo 750\textsuperscript{®} was purchased from PerkinElmer (Waltham, MA).

4.4.2. Traumatic Brain Injury Mouse Models

Two cohorts of 4-6 week old athymic mice (male, ~25 g, $nu/nu$) (n = 5) were anesthetized by 2-3 % isoflurane inhalation. A metal cylinder, with a 3 mm diameter, was pre-cooled in liquid nitrogen and applied to the parietal region of each mouse’s head for either 20 s or 60 s. The mice then received an intracardiac injection of either PSS-794 (3.0 mg/kg, 100 $\mu$L in 1% DMSO/H$_2$O) or 794 Control (3.0 mg/kg, 100 $\mu$L in H$_2$O). A cohort of athymic mice (n = 5) were subjected to a 60 s cryoinjury and received an intracardiac injection of Annexin-Vivo 750.

To investigate the progression of blood-brain disruption and cell death in traumatic brain injury, three cohorts of immunocompetent hairless mice (male, 25 g, $SKH1-E$) (n = 4-5) were anesthetized by 2-3 % isoflurane inhalation. A metal cylinder, with a 3 mm diameter, was pre-cooled in liquid nitrogen and applied to the parietal region
of each mouse’s head for 60 s. The mice then received a retro-orbital injection of PSS-794 (3.0 mg/kg, 100 μL in 1% DMSO/H2O) and placed back into their cages. Five hours later, the mice received a retro-orbital injection of Tracer-653 (2.0 mg/kg, 100 μL in H2O). One hour later, the mice were anesthetized by isoflurane inhalation and placed inside an IVIS Lumina (Caliper Life Sciences, Hopkinton, MA) configured for whole-body, epi-fluorescence. After imaging, one cohort was sacrificed and ex vivo fluorescence imaging was performed on the excised brain. The other cohorts were placed back into their cages. Cohorts were subjected to the same PSS-794 and Tracer-653 injection and imaging procedures, either three or seven days post-injury.

Evans Blue (Sigma, St. Louis, MO) (25 mg/kg, 100 μL in H2O) was injected intravenously into hairless mice following 60 s brain cryolesion. The probe was allowed to circulate for one hour, then the mice were anesthetized and sacrificed. The brains were excised and placed inside an IVIS Lumina for ex vivo epi-fluorescence imaging. Another cohort of hairless mice were subjected to the same injection and imaging procedure three days post-injury.

4.4.3. In Vivo Near-Infrared Fluorescence Imaging

Athymic mice were anesthetized by 2-3 % isoflurane inhalation and placed inside an IVIS Spectrum (Caliper Life Sciences, Hopkinton, MA) configured for whole-body epi-fluorescence imaging. For mice injected with PSS-794 or 794 Control, images were acquired immediately after probe injection and at 1, 3, 6, and 24 h time points (excitation filter: 710 nm, emission filter: 820 nm, exposure time: 1 s, bin: 8, f/stop: 2, field of view: 6.6 cm). For mice injected with Annexin-Vivo 750, images were acquired for 1 s using a
710 nm excitation filter and a 780 nm emission filter (bin: 4, f/stop: 2, field of view: 6.6 cm). After each time point, the mice were returned to their cages and fed *ad libitum*.

Acquired images were exported as 16 bit tiff files and region of interest (ROI) analysis was performed using *ImageJ*. In short, a ROI was drawn around the cryoinjury site (T) and an equal sized ROI was drawn on the lower back (NT) of each mouse. The mean pixel intensity of the T and NT was measured and recorded for each mouse. The T/NT ratios were then calculated, and statistical analysis was performed to acquire the average of each ratio (n = 5) with the standard error of the mean (SEM). The resulting ROI values were plotted using *Graphpad Prism 4*.

4.4.4. Ex Vivo Near-Infrared Fluorescence Imaging

Following the 24 h time point, mice were sacrificed by cervical dislocation. *Ex vivo* images were acquired with the skin from the head removed and both the skin and the skull removed to facilitate epi-fluorescence imaging with an IVIS Spectrum (excitation filter: 710 nm, emission filter: 820 nm, exposure time: 1 s, bin: 8, f/stop: 2, field of view: 6.6 cm for PSS-794 and 794 Control) (excitation filter: 710 nm, emission filter: 780 nm, exposure time: 1 s, bin: 4, f/stop: 2, field of view: 6.6 cm for Annexin-Vivo 750).

Immunocompetent hairless mice were sacrificed, the brains excised and placed inside an IVIS Lumina for multicolor epi-fluorescence imaging (excitation filter: 705-780 nm, emission filter: 810-885 nm, exposure time: 5 s, bin: 2, f/stop: 2, field of view: 5 cm for PSS-794) (excitation filter: 615-665 nm, emission filter: 695-770 nm, exposure time: 8 s, bin: 2, f/stop: 2, field of view: 5 cm for Tracer-653).
ROI analysis was performed by drawing an ROI around the cryoinjury site and recording the mean pixel intensity. The resulting mean pixel intensities were plotted using Graphpad Prism 4.

4.4.5. Histology

Brains were flash frozen in OCT and cut into 10 μm sections. Tissue sections were mounted onto slides and imaged using a LI-COR Odyssey scanner equipped with a 785 nm diode laser. Slides were also subjected to hematoxylin and eosin (H&E) staining to determine the extent of cell death and cellular morphological changes in the cerebral cortex. Selected brain sections were incubated overnight with a rabbit anti-human polyclonal caspase-3 antibody (Abcam Inc.; 1:50). The sections were then incubated with goat anti-rabbit IgG conjugated to Alexa-Fluor 488 (Invitrogen; 1:500) for 15 min, and counterstained with DAPI. Fluorescence images of the sections were acquired using a Nikon TE-2000 U epi-fluorescence microscope equipped with the appropriate UV (ex. 340/80 nm, em. 435/85 nm), GFP (ex. 450/90 nm, em. 500/50 nm), and near-infrared filters (ex. 710/75 nm, em. 810/90 nm). Fluorescence images were captured using Metamorph software (Universal) and analyzed using ImageJ.

4.4.6. Statistical Analysis

Results are depicted as mean ± standard error of the mean (SEM). Statistical analysis was performed using a Student’s t-test.
5.1. Background

Multivalency is characterized as the interaction of multiple recognition elements on a single scaffold with receptors on a separate system. Multivalent interactions and biological systems are found extensively in nature and are especially important for recognition and attachment of pathogens to host cells. Moreover, multivalent interactions can induce unique properties that are qualitatively different than the monovalent interactions with the same ligand-receptor pair. Researchers have taken advantage of these properties to develop novel small molecule effectors and inhibitors that can module cell signaling responses through different receptor binding mechanisms.

One major advantage of multivalent systems is the enhanced binding affinity compared to an analogous monovalent system. Our current generation of fluorescent Zn$_2$BDPA probes is monovalent, and while these probes have been successful for in vivo imaging of cell death, they only have a modest affinity for surface exposed phosphatidylserine (low micromolar dissociation constants). Our Zn$_2$BDPA probes mechanistically mimic Annexin V, which uses multivalency to gain nanomolar affinity.
Figure 5.1. Chemical structures of Tetra-$\text{Zn}_2\text{BDPA}$-SR, Bis-$\text{Zn}_2\text{BDPA}$-SR, Tracer-653, and $\text{Zn}_2\text{BDPA}$.

for exposed PS. Thus we believed that appending more than one $\text{Zn}_2\text{BDPA}$ would increase binding to PS. Squaraine rotaxanes are a class of highly stable and extremely bright fluorescent dyes that emit in deep-red wavelengths (650 nm-750 nm), which can be used for whole-body, epi-fluorescence imaging. Their architecture is amendable to conjugation with more than one $\text{Zn}_2\text{BDPA}$ allowing for the development of multivalent $\text{Zn}_2\text{BDPA}$ probes for in vivo imaging. Indeed, a bivalent $\text{Zn}_2\text{BDPA}$ probe on a squaraine rotaxane scaffold enabled non-invasive visualization of a bacterial infection in a living mouse. Here, we evaluate the ability squaraine rotaxanes with either two or four $\text{Zn}_2\text{BDPA}$ recognition elements to target and detect cell death in cell culture and in three animal models of cell death. Increasing the number of $\text{Zn}_2\text{BDPA}$ on a squaraine rotaxane (SR) enabled imaging of cell death in vitro at lower probe concentrations and produced higher targeting ratios in all cell death animal models tested suggesting that multivalent $\text{Zn}_2\text{BDPA}$ fluorescent probes improve cell death imaging.

5.2. Results and Discussion
Figure 5.2. Fluorescence microscopy of dead and dying MDA-MB-231 cells stained with either Tetra-Zn$_2$BDPA-SR, Bis-Zn$_2$BDPA-SR, or Tracer-653. MDA-MB-231 cells were treated with etoposide (15 μM) for 11 hr then stained with 10 μM of the indicated probe. The cells were incubated at 37 °C for 30 min then washed with HEPES buffer. Scale bar = 30 μm.

This study employed three squaraine rotaxane-based fluorescent probes to evaluate cell death imaging performance: a SR with four Zn$_2$BDPA (Tetra-Zn$_2$BDPA-SR), a SR with two Zn$_2$BDPA s (Bis-Zn$_2$BDPA-SR), and a control SR that has negligible affinity for either proteins or cells (Tracer-653). All probes have similar scaffolds and similar optical characteristics, thus their targeting properties can be compared through fluorescence imaging. Initial in vitro studies used MDA-MB-231 breast cancer cells that had been treated with etoposide in culture to induce cell death. Etoposide is a topoisomerase inhibitor that is commonly used to induce cell death in a number of different cell lines including MDA-MB-231. After treatment for 11 h, the cells were
**Figure 5.3.** Fluorescence microscopy of dead and dying MDA-MB-231 cells stained with different concentrations of Tetra-Zn$_2$BDPA-SR (top row) or Bis-Zn$_2$BDPA-SR (bottom row). MDA-MB-231 cells were treated with etoposide (15 μM) for 11 hr then stained with the indicated concentrations of Tetra-Zn$_2$BDPA-SRR or Bis-Zn$_2$BDPA-SR. The cells were incubated at 37 °C for 30 min then washed with HEPES buffer. Scale bar = 30 μm.

subsequently stained with either Tetra-Zn$_2$BDPA-SQR, Bis-Zn$_2$BDPA-SR, or Tracer-653 and subjected to fluorescence microscopy. As shown in Figure 5.2, both Tetra-Zn$_2$BDPA-SQR and Bis-Zn$_2$BDPA-SR localized to the plasma membranes of treated cells enabling identification dead and dying cells, whereas negligible staining was apparent with Tracer-653. This result suggests that the Zn$_2$BDPA recognition element is vital for identification of cell death. To determine if additional Zn$_2$BDPA ligands increase localization to anionic membranes on dead and dying cells, MDA-MB-231 cells were treated with etoposide for 11 hr then stained with different concentrations of either Tetra-Zn$_2$BDPA-SQR or Bis-Zn$_2$BDPA-SR. Tetra-Zn$_2$BDPA-SQR was able to target dead and dying MDA-MB-231 cells down to a concentration of 500 nM. Bis-Zn$_2$BDPA-SR also
localized to treated MDA-MB-231 cells at a concentration of 500 nM, though the fluorescence signal was weak (Figure 5.3). Both probes were able to detect cell death at a significantly lower concentration than that typically used for monovalent Zn\(_2\)BDPA fluorescent probes (10 μM). A large background signal is apparent in the panels with

![Image](image-url)

**Figure 5.4.** Cold block of Tetra-Zn\(_2\)BDPA-SR binding to treated MDA-MB-231 cells using POPC and POPC:POPS (50:50) liposomes. Fluorescence microscopy of etoposide-treated MDA-MB-231 cells stained with either Tetra-Zn\(_2\)BDPA-SR alone, Tetra-Zn\(_2\)BDPA-SR with POPC liposomes, or Tetra-Zn\(_2\)BDPA-SR with POPC:POPS liposomes (A). The liposome concentration is in 100-fold excess compared to the concentration of Tetra-Zn\(_2\)BDPA-SR. Scale bar = 30 μm. Quantification of fluorescence images from A (B). The total fluorescence was measured from 6 random fields of view. * P < 0.0002, ** P < 0.002.
1 μM and 500 nM Bis-Zn$_2$BDPA-SR but not with the same concentrations of Tetra-Zn$_2$BDPA-SQR. This high background signal seen with lower concentrations of Bis-Zn$_2$BDPA-SR may be the result of Bis-Zn$_2$BDPA-SR releasing from the anionic membrane thus increasing the concentration of free Bis-Zn$_2$BDPA-SR in solution. If this is the case, then the lack of a high background in the Tetra-Zn$_2$BDPA-SQR images suggests that Tetra-Zn$_2$BDPA-SQR likely has a higher affinity for dead and dying cells than the Bis-Zn$_2$BDPA-SR.

To prove that the multivalent Zn$_2$BDPA probes are targeting exposed PS on dead and dying cells, we incubated etoposide-treated MDA-MB-231 cells with Tetra-Zn$_2$BDPA-SQR and a 100 fold excess of either POPC or POPC:POPS (50:50) liposomes. The POPC:POPS liposomes are a model system for the plasma membrane of a cell undergoing the cell death program, thus these liposomes should prevent binding of Tetra-Zn$_2$BDPA-SQR to treated MDA-MB-231 cells compared to the control POPC liposomes. As seen in Figure 5.4, POPC and POPC:POPS liposomes inhibited binding of Tetra-Zn$_2$BDPA-SR to dead and dying cells; however, POPC:POPS prevented less binding than the control POPC liposomes. Quantification of Tetra-Zn$_2$BDPA-SR fluorescence confirmed that the POPC:POPS liposomes significantly inhibited Tetra-Zn$_2$BDPA-SR to cell death and this inhibition was binding better than POPC liposomes (Figure 5.4 B). The apparent affinity of Tetra-Zn$_2$BDPA-SR for the control POPC liposomes could be attributed to the lipophilicity of the squaraine rotaxane scaffold. Taken together, these results suggest that multivalent Zn$_2$BDPAs effectively target exposed anionic phosphatidylserine allowing for visualization of cell death in cell culture.
Figure 5.5. Representative in vivo fluorescence montages of Tetra-Zn$_2$BDPA-SR (top row), Bis-Zn$_2$BDPA-SR (middle row), and Tracer-653 (bottom row) accumulation in a brain cryoinjury mouse model. A pre-cooled metal cylinder was applied to the head of each mouse for 60 s followed by intravenous injection of either Tetra-Zn$_2$BDPA-SR, Bis-Zn$_2$BDPA-SR, or Tracer-653. Images were acquired at the indicated time points after probe injection. N = 4.

The idea that increasing the number of Zn$_2$BDPAs enhances cell death imaging was further tested in vivo using three animal models of cell death. The first model employed a cryolesion traumatic brain injury model that induces rapid breakdown of the blood-brain barrier and tissue damage. In short, three separate cohorts of immunocompetent mice (n = 4) were anesthetized by isoflurane inhalation. A metal cylinder with a 3 mm diameter was pre-cooled in liquid nitrogen and applied to each mouse’s head for 60 s. The mice were immediately injected with either Tetra-Zn$_2$BDPA-SR, Bis-Zn$_2$BDPA-SR, or Tracer-653 and subjected to non-invasive epi-fluorescence 0 h, 3 h, 6 h, and 24 h post-injection. Figure 5.5 shows a montage of representative
fluorescence images of mice that received a cryolesion and were injected with a SR probe. Both Tetra-Zn$_2$BDPA-SR and Bis-Zn$_2$BDPA-SR enabled demarcation of the cryolesion from the rest of the head by the 0 h timepoint, whereas Tracer-653 localized to the margin of the cryolesion at the initial timepoint. By 3 h and 6 h post-probe injection, a significant amount of both multivalent Zn$_2$BDPA probes had remained at the injury site, while Tracer-653 had cleared from the head and body to approximately background fluorescence levels. A clear difference between Tetra-Zn$_2$BDPA-SR and Bis-Zn$_2$BDPA-SR is apparent at the 24 h timepoint with Tetra-Zn$_2$BDPA-SR still localized at the injury site and Bis-Zn$_2$BDPA-SR cleared from the body. Quantification of the in vivo images from Figure 5.6 A confirm that Tetra-Zn$_2$BDPA-SR and Bis-Zn$_2$BDPA-SR exhibit approximately the same targeting ratios at the earliest timepoints with maximum T/NT value at the 3 h timepoint for each probe. However, at the 24 h timepoint, Tetra-Zn$_2$BDPA-SR had a T/NT value of 4.78 ± 0.33, which was significantly higher than Bis-Zn$_2$BDPA-SR (2.12 ± 0.12). When looking at I/I$_0$ (a ratio of mean pixel intensities at the

**Figure 5.6.** Quantification of Tetra-Zn$_2$BDPA-SR, Bis-Zn$_2$BDPA-SR, and Tracer-653 from in vivo fluorescence images. T/NT (A) and I/I$_0$ (B) ratios were calculated by ROI analysis. N = 4.
Figure 5.7. Ex vivo fluorescence images (A) and quantification (B) of Tetra-Zn$_2$BDPA-SR, Bis-Zn$_2$BDPA-SR, and Tracer-653 accumulation in cryoinjured brains. Brains were excised and imaged 24 h post-probe injection. T/NT ratios were calculated by ROI analysis. * P < 0.03, ** P < 0.001.

cryolesion site at each timpoint compared to the mean pixel intensities at the initial
timepoint), Tetra-Zn$_2$BDPA-SR had higher values than both Bis-Zn$_2$BDPA-SR and
Tracer-653 at all timepoints (Figure 5.6 B). Interestingly, by the 24 h timepoint, Tetra-
Zn$_2$BDPA-SR had not cleared from the injury site like Bis-Zn$_2$BDPA-SR and Tracer-653,
but remained at the site at a higher concentration than the initial timepoint ($I/I_0$ at 24 h =
$1.31 \pm 0.33$). Masking of PS on dead and dying cells can prevent clearance by the
immune system causing buildup of these cells in the body. Tetra-Zn$_2$BDPA-SR may be
preventing clearance of dead cells by the immune system, which could explain why the
24 h timpoint has higher intensities than the initial timepoint. Bis-Zn$_2$BDPA-SR does not
exhibit a similar pattern of staining impart to the lower affinity for cell death.
**Figure 5.8.** In vivo cold block of Tetra-Zn$_2$BDPA-SR and Bis-Zn$_2$BDPA-SR accumulation in cryoinjured brains using Zn$_2$BDPA. Representative fluorescence images of cryoinjured brains dosed with either a vehicle control (Alone) or a 15-fold excess of Zn$_2$BDPA (Cold Block) then injected with either Tetra-Zn$_2$BDPA-SR or Bis-Zn$_2$BDPA-SR (A). Images were acquired 24 h post-probe injection. Quantification of Tetra-Zn$_2$BDPA-SR and Bis-Zn$_2$BDPA-SR mean pixel intensities from ex vivo images. Mean pixel intensities were measured at the site of the cryolesion for both Tetra-Zn$_2$BDPA-SR and Bis-Zn$_2$BDPA-SR associated images. * P < 0.02, ** P < 0.005.

After the 24 h timpoint, the mice were anesthetized, sacrificed, and the brains were excised for ex vivo imaging. Tetra-Zn$_2$BDPA-SR and Bis-Zn$_2$BDPA-SR localized predominately to the injury site with minor accumulation in other regions of the brain (Figure 5.7. A). Tracer-653 homogenously stained the brain, showing no affinity for injured cranial tissue. Region of interests were drawn around the injured site (Target) and
a contralateral position on the brain (Non-target) and the mean pixel intensities were measured. Tetra-Zn$_2$BDPA-SR had a significantly higher T/NT value (3.66 ± 0.29) than both Bis-Zn$_2$BDPA-SR (2.43 ± 0.07) and Tracer-653 (1.10 ± 0.06), which corroborates with the in vivo data (Figure 5.7 B).

To further confirm selectivity for cell death in vivo, we performed a cold block study using the cryolesion mouse model and an excess of the Zn$_2$BDPA ligand. The cryolesion experiment was repeated and a 15-fold excess of Zn$_2$BDPA (compared to the concentration of Zn$_2$BDPAs on Tetra-Zn$_2$BDPA-SR and Bis-Zn$_2$BDPA-SR) or a vehicle control was injected retro-orbitally immediately after injury. Thirty minutes was allowed to elapse followed by an intravenous injection of Tetra-Zn$_2$BDPA-SR or Bis-Zn$_2$BDPA-SR. Figure 5.8 A shows representative ex vivo brain images that were acquired 24 h post-SR probe injections. When comparing the multivalent Zn$_2$BDPA probe staining with the vehicle control (Alone) to the excess Zn$_2$BDPA ligand (Cold Block), each probe had significantly higher mean pixel intensities with the vehicle control (Figure 5.8 B). The cold block inhibited Tetra-Zn$_2$BDPA-SR and Bis-Zn$_2$BDPA-SR targeting by approximately 50 %. This study confirms that multivalent Zn$_2$BDPAs probes are binding to the same biological moiety as monovalent Zn$_2$BDPAs. Moreover, this is the first in vivo cold block study performed using any Zn$_2$BDPA system.

Next, we utilized a subcutaneous PAIII prostate tumor rat model that is well-established in our lab. In short, PAIII prostate cancer cells were injected into the flanks of Lobund-Wistar rats and allowed to grow for 14 days to ensure that necrotic foci had developed inside the tumor. Rats were then injected with either Tetra-Zn$_2$BDPA-SR, Bis-Zn$_2$BDPA-SR, or Tracer-653 and 24 h elapsed before imaging. Fluorescence from SRs is
Figure 5.9. Representative fluorescence images of Tetra-Zn$_2$BDPA-SR, Bis-Zn$_2$BDPA-SR, and Tracer-653 tumor accumulation in a subcutaneous prostate tumor rat model (A). Twenty-four hours post-probe administration, the tumor-bearing rats were anesthetized, sacrificed, and the skin from their flanks were removed for epi-fluorescence imaging. Comparison of Tetra-Zn$_2$BDPA-SR (black bar), Bis-Zn$_2$BDPA-SR (dashed bar), and Tracer-653 (white bar) targeting to prostate tumors. T/NT values were calculated by ROI analysis. * $P < 0.05$, ** $P < 0.02$, *** $P < 0.003$.

subject to light scattering even though they emit at the deep-red wavelengths thus the skin from the flanks of the rats were removed for imaging. As seen in Figure 5.9 A, tumors can be easily visualized in animals injected with Tetra-Zn$_2$BDPA-SR and Bis-Zn$_2$BDPA-SR while Tracer-653 showed minor accumulation into the tumor. Interestingly, the mean pixel intensities of tumors with Tetra-Zn$_2$BDPA-SR were lower than Bis-Zn$_2$BDPA-SR tumor MPIs, even though Tetra-Zn$_2$BDPA-SR had a significantly higher T/NT value (Figure 5.9 B). Ex vivo imaging revealed that a large amount of Tetra-Zn$_2$BDPA-SR
Figure 5.10. Mean pixel intensities (MPI) of excised thymi from rats that were treated with dexamethasone (50 mg/kg) and dosed with either Tetra-Zn\textsubscript{2}BDPA-SR, Bis-Zn\textsubscript{2}BDPA-SR, or Tracer-653. Images were acquired 24 h after probe administration. N = 4.

accumulated in the liver, lungs, and tumor whereas Bis-Zn\textsubscript{2}BDPA-SR was mostly in the liver, kidneys, and tumor. Other members in my lab have shown that Tetra-Zn\textsubscript{2}BDPA-SR can crosslink highly anionic species such as bacteria causing micrometer aggregates to form. Zn\textsubscript{2}BDPAs are known to have high affinity for phosphorylated species like ATP thus it is likely that Tetra-Zn\textsubscript{2}BDPA-SR is crosslinking phosphorylated species once it is introduced into the blood stream. This crosslinking will initiate the formation of large aggregates, which are getting trapped in the lungs. The remaining Tetra-Zn\textsubscript{2}BDPA-SR that did not aggregate can circulate throughout the entire animal and accumulate inside the tumor. The spatial distribution of multivalent Zn\textsubscript{2}BDPA probes within the resected tumors was determined by slicing them in half along the longest axis. Fluorescence imaging of the interior-facing surfaces of these tumor halves revealed that Tetra-
Zn$_2$BDPA-SR and Bis-Zn$_2$BDPA-SR showed similar patterns of staining, with the highest fluorescence intensities coming from the core of the tumors.

To further test the generality of multivalent Zn$_2$BDPA probes to target and image cell death, immunocompetent mice were injected intraperitoneally with dexamethasone to induce extensive thymocyte cell death. The model is technically straightforward to conduct, has high animal throughput, and works by a different mechanism than the other animal models tested. Tetra-Zn$_2$BDPA-SR, Bis-Zn$_2$BDPA-SR, and Tracer-653 were intravenously injected into mice 18 h post-dexamethasone or vehicle control treatment and then sacrificed for ex vivo imaging 24 h post-probe injection. The mean pixel intensities were measured for all thymi and plotted as seen in Figure 5.10. Tetra-Zn$_2$BDPA-SR exhibited the highest mean pixel intensities for treated thymi and these intensities were approximately 2.5 and 5.5 fold greater Bis-Zn$_2$BDPA-SR and Tracer-653 mean pixel intensities, respectively. Tetra-Zn$_2$BDPA-SR also had the highest intensities in the control thymi, which could be attributed to a build up of endogenous cell death. Masking PS can prevent efficient clearance of dead and dying cells by the immune system causing build up of these cells in the tissue. Chapter 2 showed that PSS-794, a monovalent Zn$_2$BDPA, can cause induce a build up of dead thymocytes without dexamethasone treatment, thus it is highly likely that Tetra-Zn$_2$BDPA-SR is acting in an analogous fashion. Similarly to the other animal models tested, increasing the number of Zn$_2$BDPAs on a SR scaffold enhanced the ability to target and visualize cell death.

5.3. Conclusions

In summary, multivalent Zn$_2$BDPA probes attached to a deep-red emitting squaraine rotaxane scaffold enabled visualization of cell death in cell culture and in three
animal models of cell death. Multivalent Zn$_2$BDPA ligands allowed for lower concentrations of probe to be used and increased targeting ratios when compared to monovalent Zn$_2$BDPA imaging agents in similar models. Furthermore, increasing the number of Zn$_2$BDPAs from two to four appeared to increase affinity for dead and dying cells both in vitro and in vivo. While increasing the multivalency of Zn$_2$BDPA ligands on a squaraine rotaxane may enhance cell death imaging, the propensity of these probes to aggregate in blood will deter clinical translation. Future studies will need to look how the architecture of the multivalent Zn$_2$BDPA probes affects aggregation and cell death imaging. An alternative strategy is to append lipid penetrating functionalities (like tryptophan) to the end of a Zn$_2$BDPA to increase affinity for exposed PS, mimics how Annexin V and other PS-binding proteins achieve high affinity for anionic membranes during cell death.

5.4. Materials and Methods

5.4.1. Materials

Culture media and bovine serum albumin (BSA) were purchased from Sigma Aldrich (St. Louis, MO). MDA-MB-231 (ATCC: HB-26) cells were certified and obtained from the ATCC. POPC and POPS lipids were purchased from Avanti Polar Lipids (Alabaster, AL).

5.4.2. Synthesis

The synthesis for Bis-Zn$_2$BDPA-SR and Tracer-653 have been previously reported.$^{186}$

5.4.2.1. Zn$_2$BDPA
To a solution of α,α'-Dibromo-m-xylene (104.1 mg, 0.389 mmol) in 2 mL CHCl₃ was added DIPEA (0.17 mL, 1.00 mmol), and 2,2’-Dipicolylamine (0.15 mL, 0.856 mmol). The reaction mixture was allowed to stir at room temperature for 4 hours. The resulting solution was washed once with water and was dried over MgSO₄. The crude residue was purified by column chromatography with 0-15% MeOH:EtOAc as the eluent to yield Zn₂BDPA (59.9 mg, 30%) as a brown oil.

¹H NMR (500 MHz, CDCl₃) δ 3.67 (s, 4H), 3.79 (s, 8H), 7.11 (m, 4H), 7.29 (m, 4H), 7.48 (s, 1H), 7.60 (m, 7H), 8.51 (d, J = 4.5 Hz, 4H) ppm; ¹³C NMR (500 MHz, CDCl₃) δ 58.5, 59.9, 122.0, 122.8, 127.7, 128.3, 129.3, 136.5, 138.8, 148.9, 159.4 ppm; HRMS (ESI, MeCN): m/z = 501.2757 ([M+H]+).

5.4.2.2. Tetra-Zn₂BDPA-SR

Rotaxane-squaraine dye (32 mg, 0.0209 mmol) and DPA-NCS (105.5 mg, 0.1675 mmol) were dissolved in ethanol (2.5 mL). After the addition of TEA (146 µL, 1.0465 mmol), the mixture was refluxed for four hours, and then the solvents were removed. After purification by column, a blue solid was obtained (40%).

¹H NMR (CDCl₃) δ 10.03 (t, J = 5.25 Hz, 4H), 8.45 (m, 20H), 8.12 (t, J = 7.80 Hz, 2H), 8.04 (d, J = 8.70 Hz, 4H), 7.71 (s, 4H), 7.58 (m, 32H), 7.10 (t, J = 6 Hz, 16H), 7.02 (s, 4H), 6.78 (s, 8H), 6.55 (s, 8H), 6.21 (d, J = 8.40 Hz, 4H), 4.53 (br, 16H), 4.40 (t, J = 6.45 Hz, 8H), 3.92 (t, J = 5.55 Hz, 8H), 3.77 (s, 32H), 3.62 (br, 64H), 2.17 (m, 8H).

¹³C NMR (CDCl₃) δ 185.0, 184.6, 183.0, 163.6, 159.5, 158.9, 154.0, 149.3, 148.9, 144.4, 140.3, 139.0, 136.5, 133.4, 128.8, 125.2, 123.2, 122.8, 122.0, 121.6, 119.2, 113.6, 112.0 (23 peaks), 67.6, 67.3, 64.4, 60.0, 58.5, 51.5, 47.7, 44.0, 43.4, 41.2, 29.8, 26.6, 25.9 (13 peaks). ESI-MS: m/z: 1350.1 [M+3H]3⁺, 1012.9 [M+4H]⁴⁺, 810.6 [M+5H]⁵⁺.
5.4.3. Cell Culture

Monolayer cultures of MDA-MB-231 cells were grown in RPMI media supplemented with 10% fetal bovine serum (FBS), penicillin, streptomycin, and 2.25 mM L-glutamine. Cells were grown in plastic culture flasks at 37°C in 5% CO\textsubscript{2} atmosphere, passed in 1:5 dilutions and seeded at 1:4 dilutions on chambered coverslips for microscopy studies.

5.4.5. Liposome Preparation

An appropriate mixture of phospholipid was dried as a film in vacuo for 1 h. A stock solution of vesicles (10 mm phospholipid) was made by rehydration at room temperature with the appropriate buffer. Multilamellar vesicles were extruded to form unilamellar vesicles with a Basic LiposoFast device purchased from Avestin, Inc. (Ottawa, Canada). The vesicles were extruded 11 times through a 19 mm polycarbonate Nucleopore filter with 200 nm diameter pores.

5.4.4. Cell Microscopy

Brightfield and fluorescence microscopy was performed on a Nikon TE-2000U epifluorescence microscope equipped with filters that allowed detection of each probe. Filter sets were obtained from Nikon and included UV-2E/C filter (ex: 340/80, em: 435/85), EN GFP HQ filter (ex: 450/90, em: 500/50), Cy3 filter (ex: 535/50 nm, em: 610/75), and Cy5 filter (ex: 620/60, em: 700/75). Fluorescence images were captured using Metamorph software (Universal) and analyzed using ImageJ.

MDA-MB-231 cells were seeded onto a chambered coverslip system. Once the cells reached 80% confluency, etoposide (15 μM) was added to the cells and allowed to incubate at 37 °C for 11 h. The media was then removed, HEPES buffer was added to the
wells, and different concentrations of Tetra-ZnDPA-SR, Bis-ZnDPA-SR, and Tracer-653 were added to the cells. The cells were incubated at 37 °C for 30 min then washed three times with HEPES buffer.

For the cold block study, MDA-MB-231 cells were seeded onto a chambered coverslip system and treated with etoposide (15 μM) at 37 °C for 11 h once 80 % confluency was reached. The media was removed and replaced with HEPES buffer. Tetra-ZnDPA-SR (1 μM) was added to the cells along with 100 fold excess of either POPC:POPS (50:50) or POPC liposomes. The cells were then incubated at 37 °C for 30 min followed by a washing step with HEPES buffer.

5.4.5. Traumatic Brain Injury Mouse Models

Three cohorts of 4-6 week old immunocompetent hairless mice (male, ~25 g, SKH-1) (n = 5) were anesthetized by 2-3 % isoflurane inhalation. A metal cylinder, with a 3 mm diameter, was pre-cooled in liquid nitrogen and applied to the parietal region of each mouse’s head for either 20 s or 60 s. The mice then received an intravenous injection of either Tetra-Zn$_2$BDPA-SR (2.0 mg/kg, 100 μL in H$_2$O), Bis-Zn$_2$BDPA-SR (2.0 mg/kg, 100 μL in H$_2$O), or Tracer-653 (2.0 mg/kg, 100 μL in H$_2$O).

For cold block studies, immunocompetent hairless mice (SKH-1, ~25 g, n = 4) were anesthetized by 2-3 % isoflurane inhalation. A metal cylinder, with a 3 mm diameter, was pre-cooled in liquid nitrogen and applied to the parietal region of each mouse’s head for either 60 s. Immediately after cryoinjury, mice were administered either Zn$_2$BDPA (100 μL in 10% DMSO/H$_2$O) or a vehicle control (100 μL of 10% DMSO/H$_2$O). Thirty minutes was allowed to elapse then the mice were injected retro-orbitally with either Tetra-Zn$_2$BDPA-SR (2.0 mg/kg, 100 μL in H$_2$O) or Bis-Zn$_2$BDPA-
SR (2.0 mg/kg, 100 μL in H₂O). The concentration of Zn₂BDPA was in 15 fold excess compared to the effective concentration of Zn₂BDPA ligands on Tetra-Zn₂BDPA-SR and Bis-Zn₂BDPA-SR. Following twenty-four hours, the mice were anesthetized, sacrificed, and the brains were excised for ex vivo imaging.

Mice were anesthetized by 2-3 % isoflurane inhalation and placed inside an Carestream Health In-Vivo Multispectral Imaging System FX configured for whole-body epi-fluorescence imaging. Images were acquired immediately after probe injection and at 1, 3, 6, and 24 h time points.

5.4.6. Dexamethasone-Induced Thymocyte Cell Death Mouse Model

All animal procedures were approved by the University of Notre Dame Institutional Animal Care and Use Committee. Six cohorts of 8-week old male immunocompetent hairless mice (SKH-1, Freimann Life Science Center, ~ 25 g) (n = 4) were given intraperitoneal injections (50 mg/kg) of water soluble dexamethasone (Sigma Aldrich, St. Louis, MO) dissolved in 100 μL of distilled H₂O. The time from dexamethasone dosing to sacrifice of the cohort (and its related control cohort) was either 42 h with injection of fluorescent probe at 24 h before sacrifice. The fluorescent probe (either Tetra-Zn₂BDPA-SR, Bis-Zn₂BDPA-SR, or Tracer-653 in H₂O) was injected intravenously via the tail vein and produced a dosage of 2.0 mg/kg. Three additional cohorts of mice (n = 4) were not treated with dexamethasone, but were injected with fluorescent probe (either Tetra-Zn₂BDPA-SR, Bis-Zn₂BDPA-SR, or Tracer-653 in H₂O). All animals were euthanized by either CO₂ asphyxiation or cervical dislocation under anesthesia.
After sacrifice, selected rat tissues were excised, placed on a transparent imaging tray, and imaged using a Carestream Health In-Vivo Multispectral Imaging System FX equipped with a $630 \pm 10$ nm excitation filter and $700 \pm 20$ nm emission filter.

5.4.7. PAIII Subcutaneous Tumor Rat Model

Eight-week old Lobund Wistar rats (Freimann Life Science Center; 250 g, n = 4) were injected subcutaneously into the right flank with $1 \times 10^6$ Prostate Adenocarcinoma III (PAIII) cells, suspended in 300 µL of DMEM medium. Tumors grew for 14 days prior to injection of the probe. Lobund Wistar rats were anesthetized (1.5 % isoflurane inhalation) and injected intravenously via the tail vein with 2.0 mg/kg of either Tetra-Zn$_2$BDPA-SR, Bis-Zn$_2$BDPA-SR, or Tracer-653 in H$_2$O. Twenty-four hours post-injection, rats were anesthetized and sacrificed. The skin on the flank of each rat was removed and fluorescence images were acquired using a Carestream Health In-Vivo Multispectral Imaging System FX (Rochester, NY) equipped with a $630 \pm 10$ nm excitation filter and $700 \pm 20$ nm emission filter. Following imaging, selected tissues were excised, placed on a transparent imaging tray, and optically imaged as described above. The tumors, from each animal, were then separated into halves by slicing along the longest axis. The tumor halves were placed onto a transparent imaging tray so that the inner cores faced the CCD. Fluorescence images were acquired for 3 minutes at a 30 mm field of view.

5.4.8. Fluorescence Image Analysis

Images were analyzed using ImageJ. The 16-bit images were imported, opened in sequential order, and converted to an image stack. Background subtraction was applied to the images using the rolling ball algorithm. The stack was then converted to a montage and pseudocolored as “Thai” (under the “Lookup Tables” menu). Region of
interest (ROI) analysis was performed on each in vivo image by drawing an identical area around the tumor (Target, T) and the same anatomical location on the contralateral side of the animal (Non-Target, NT). The mean pixel intensities for the target and non-target regions were measured and recorded for each animal. The resulting ROI values were plotted using Graphpad Prism 4. For ex vivo biodistribution images, a ROI was manually drawn to outline each tissue and the mean pixel intensity was recorded.
APPENDIX:

EVALUATION OF FLUORESCENT PHOSPHATIDYLSERINE SUBSTRATES FOR THE AMINOPHOSPHOLIPID FLIPPASE IN MAMMALIAN CELLS

A.1. Background

The distribution of phospholipids across the plasma membrane of mammalian cells is asymmetric. The zwitterionic phospholipids, phosphatidylcholine (PC) and sphingomyelin (SM), are enriched in the outer leaflet of the bilayer membrane, while the aminophospholipids phosphatidylserine (PS) and phosphatidylethanolamine (PE) are located on the cytoplasmic leaflet. This asymmetric distribution is critical for various biological functions including clearance of apoptotic cells, signal transduction and blood clotting. Maintenance of phospholipid distribution requires the concerted action of several membrane transport proteins called phospholipid translocases. These translocases can be divided into three categories: bi-directional non-energy dependent “scramblases”, and energy dependent “translocases” that move phospholipids toward or away from the inner leaflet of the membrane. One of the most studied translocases is the aminophospholipid flippase, which selectively transports PS, and to a much lesser extent PE, to the inner leaflet in the plasma membrane of human erythrocytes and most nucleated cells. Until recently, it has proven difficult to reconstitute this putative transport
system in artificial membranes and most studies in the literature have employed intact cells.\textsuperscript{197}

Substrate selectivity studies of the aminophospholipid flippase indicate a strong preference for the phosphatidylserine head group structure, with insensitivity to the composition of the acyl chains.\textsuperscript{198} Synthetic PS derivatives with acyl chains of varying length are accepted, as are derivatives containing spin or fluorescent probes in this lipophilic region.\textsuperscript{199} The most studied fluorescent substrate for the aminophospholipid flippase is compound I, a PS derivative with a 2-acyl chain containing a small and uncharged 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD) fluorophore.\textsuperscript{200} While PS-NBD conjugates are valuable for spectroscopy experiments and “single-acquisition” fluorescence microscopy studies, they have several photophysical limitations. The emission band of the NBD fluorophore is quite broad, which discourages multicolor imaging. The quantum yield changes substantially with solvent polarity and the fluorophore self-quenches upon aggregation.\textsuperscript{201} In addition, the fluorophore readily photobleaches, which means that PS-NBD conjugates are not suitable for intravital microscopy or long-term image acquisition studies using high intensity confocal microscopes.\textsuperscript{202} It is clear that future studies of phospholipid translocase activity in living cells and tissue would be greatly facilitated by the development of new, high performance, fluorescently labeled PS substrates for the aminophospholipid flippase, that are compatible with confocal one-photon and two-photon microscopy methods.

The goal of this present study was to determine if the putative aminophospholipid flippase(s) in nucleated mammalian cells could translocate phosphatidylserine derivatives
Figure A.1. Structures of fluorescent PS and PC conjugates and synthetic intermediates. Springer and Smith, B. A. et al. (2011) Evaluation of Fluorescent Phosphatidylserine Substrates for the Aminophospholipid Flippase in Mammalian Cells. *J. Fluoresc.* 22, 722-731, scheme 1 is given to the publication in which the material was originally published, by adding; with kind permission from Springer Science and Business Media.

with attached fluorophores that were slightly larger and more polar than NBD. This information would help to define the promiscuity of the amino-phospholipid flippase and guide the development of more useful, fluorescent PS substrates. Recently, we reported new synthetic chemistry methods that efficiently attach a fluorophore to the end of the 2-acyl chain of a glycerophospholipid, and here we have employed this chemistry to
prepare PS and PC derivatives 2–7 with three different fluorophores; NBD, coumarin, and carboxyfluorescein (Figure A.1).\textsuperscript{203} Using cell microscopy, we find that the endogenous aminophospholipid flippase activity in three different mammalian cell lines readily translocates the PS derivatives 2 and 4 with neutral NBD and coumarin fluorophores, respectively, but not the PS derivative 6 with a charged and highly polar carboxyfluorescein fluorophore. The coumarin fluorophore is quite bright and can be observed selectively in the blue channel of fluorescent microscopes and flow cytometers. Thus, fluorescent PS-coumarin conjugate 4 is a new translocation substrate for the aminophospholipid flippase and should be useful in modern confocal microscopy studies.

A.2. Results and Discussion

The syntheses of NBD and coumarin conjugates 2–5 have been recently reported.\textsuperscript{203} The new carboxyfluorescein probes 6 and 7 were prepared from the protected PS and PC precursors 8 and 9 by first removing the Fmoc protecting group and reacting the liberated amine with fluorescein isothiocyanate, followed by removal of the remaining protecting groups in the case of 6.\textsuperscript{203} Coumarins are well-known as bright and relatively stable blue-emitting fluorescent dyes, and the 3-triazole-coumarin fluorophore in probes 4 and 5 has been utilized several times in recent years for various chemical biology studies and also for two-photon microscopy.\textsuperscript{204} To quantify these favorable photophysical properties we prepared the organic soluble, non self-aggregating control dye 10 and found that it is very weakly solvatochromatic, with a large Stokes shift, and that its quantum yield hardly changes with solvent polarity. For example, the relevant photophysical properties in chloroform are $\lambda_{\text{abs}}$ 415 nm, log $\varepsilon$ 4.56, $\lambda_{\text{em}}$ 461 nm, $\Phi$ 0.46, and in 1:1 water:ethanol are $\lambda_{\text{abs}}$ 420 nm, log $\varepsilon$ 4.55, $\lambda_{\text{em}}$ 490 nm, $\Phi$ 0.38. A
Figure A.2. Internalization of phospholipid NBD derivatives in eukaryotic cells. Cells were incubated with 10 μM of either 1 (A), 2 (B), or 3 (C) at 37°C for 1 h, then washed and imaged at 60X. Springer and Smith, B. A. et al. (2011) Evaluation of Fluorescent Phosphatidylserine Substrates for the Aminophospholipid Flippase in Mammalian Cells. J. Fluoresc. 22, 722-731, figure 1 is given to the publication in which the material was originally published, by adding; with kind permission from Springer Science and Business Media.
Figure A.3. Internalization of PS-coumarin probe 4 (A) and membrane localization of PC-coumarin probe 5 (B) in three mammalian cell lines. Probe 4 or 5 (10 μM) were incubated with mammalian cells at 37°C for 1 h, then washed and imaged at 60X. Springer and Smith, B. A. et al. (2011) Evaluation of Fluorescent Phosphatidylserine Substrates for the Aminophospholipid Flippase in Mammalian Cells. J. Fluoresc. 22, 722-731, figure 2 is given to the publication in which the material was originally published, by adding; with kind permission from Springer Science and Business Media.

Photostability study in ethanol solvent found that the absorption spectrum of coumarin dye 10 was unaffected by 30 min of intense irradiation from a 150 W Xenon lamp, whereas the same conditions lead to significant photobleaching of the NBD dye 3. The superior photostability of the coumarin fluorophore was also apparent from the greatly decreased rates of photobleaching observed during the following cell microscopy studies. Fluorescence microscopy was used to evaluate the ability of each phospholipid probe to penetrate three eukaryotic cell lines: Chinese hamster lung fibroblasts (V79), Chinese hamster ovary cells (CHO), and human lung carcinoma cells (A549). Control studies
using the commercially available PS- NBD probe 1 showed that each cell line exhibited aminophospholipid flippase activity and was able to transport 1 into the cytosol Figure A.2 A). Not surprisingly, the structurally similar PS-NBD probe 2 with a triazole linker was also internalized (Figure A.2 B), whereas the PC-NBD probe 3 remained in the cell plasma membrane (Figure A.2 C), which agrees with the known selectivity of the aminophospholipid flippase to not translocate PC derivatives. The same phospholipid head group selectivity was obtained with coumarin probes 4 and 5. As shown in Figure A.3, PS- coumarin 4 was strongly internalized after incubation for 1 h at 37°C; whereas, PC-coumarin 5 remained concentrated at the plasma membrane. With each cell line, the
rate and extent of cell internalization of PS-coumarin 4 was similar to that observed with PS-NBD probes 1 and 2. As expected for amphiphilic compounds, it was possible to remove the probes 1–5 from inside the cells and also the plasma membrane by a back extraction process that washed the stained cells with excess BSA solution. The intracellular distribution of probe 4 appeared to be cell line dependent. Separate colocalization experiments with ER Tracker Red and Mito Tracker Red indicated that

**Figure A.5.** Inhibition of PS-coumarin probe 4 translocation by ATP depletion. Cells were incubated with 50 mM 2′-deoxyglucose and 0.1% sodium azide in glucose free media for 30 min at 37°C then treated with 10 μM of 4. After incubation at 37°C for 30 min, cells were washed and imaged at 60X. Springer and Smith, B. A. et al. (2011) Evaluation of Fluorescent Phosphatidylserine Substrates for the Aminophospholipid Flippase in Mammalian Cells. *J. Fluoresc.* 22, 722-731, figure 4 is given to the publication in which the material was originally published, by adding; with kind permission from Springer Science and Business Media.
4 preferred the endoplasmic reticulum in A549 cells, the mitochondria in CHO cells, and a mixture of both organelle locations in V79 cells. A possible explanation for the difference in staining patterns is different rates of intracellular metabolism of the PS-coumarin 4; specifically, decarboxylation of the PS head group to form a PE head group.\(^{207}\) In the case of the A549 cells, treatment with PS-coumarin 4 also produced

**Figure A.6.** Inhibition of PS-coumarin probe 4 translocation by covalent sulphydryl modification. Cells were incubated with 10 mM of iodoacetamide in media at 37°C for 30 min, then treated with 10 μM of 4. After incubation at 37°C for 30 min, cells were washed and imaged at 60X. Springer and Smith, B. A. et al. (2011) Evaluation of Fluorescent Phosphatidylserine Substrates for the Aminophospholipid Flippase in Mammalian Cells. *J. Fluoresc.* 22, 722-731, figure 5 is given to the publication in which the material was originally published, by adding; with kind permission from Springer Science and Business Media.
punctate staining in the cytosol, suggesting partial localization in endosomes. In addition to the normal endogenous mechanisms for probe uptake by endocytosis, it is possible that flippase-mediated transport of the probe from the outer to the inner leaflet of the plasma membrane can generate invaginations in the membrane resulting in endocytic vesicles.\textsuperscript{208}

Identical fluorescence microscopy analysis of cells treated separately with carboxyfluorescein conjugates $6$ and $7$ indicated that they are not substrates for the aminophosphalipid flippase. When incubated at 37°C for 1 h, both $6$ and $7$ localized to the plasma membranes of all three cell types (Figure A.4). Back extraction of the probe by washing with a solution of BSA allowed thin layer chromatography analysis of probe biochemical stability and showed that the recovered probes had not undergone any substantial metabolic decomposition. The lack of flippase promoted translocation with PS-carboxyfluorescein probe $6$ is attributed to the charge and hydrophilicity of the attached carboxyfluorescein dye. The size of the dye is not likely a major factor since PS conjugates with attached lipophilic pyrene fluorophores have been reported to translocate across mammalian plasma membranes.\textsuperscript{209}

Cell entry of PS-NBD probes $1$, $2$ and PS-coumarin probe $4$ was greatly inhibited when the incubation was conducted at 4°C, evidence that the membrane translocation is due to an active transport mechanism. Two sets of additional microscopy experiments were conducted to demonstrate that the selective cell entry of PS-coumarin probe $4$ was due to putative aminophospholipid flippase activity, and not a chemical process such as probe hydrolysis. There is growing evidence that the aminophospholipid flippase is a P4 ATPase, and cell culture conditions that deplete ATP levels are known to inhibit
translocation.\textsuperscript{210} As shown in Figure A.5, incubating separate samples of the three cell lines with a mixture of 2'-deoxyglucose and sodium azide for 30 min at 37°C (standard conditions to achieve intracellular ATP depletion) prior to the addition of PS-coumarin 4 prevented translocation of 4 such that most of the fluorescent signal remained concentrated in the plasma membrane. A second set of experiments inhibited probe translocation activity by direct chemical treatment. It is known that the aminophospholipid flippase is inhibited by sulfhydryl reactive reagents, and as shown in Figure A.6, pre-treatment of the three cell lines with iodoacetamide (10 μM) prevented cell entry of PS-coumarin probe 4 after a subsequent incubation at 37°C for 1 h.\textsuperscript{211}

A.3. Conclusions

The new fluorescent PS-coumarin probe 4 readily enters the cytosol of all three mammalian cell lines that were tested, and there is strong evidence that the process is due to selective translocation by the endogenous aminophospholipid flippase in the cell plasma membrane. PS-coumarin 4 exhibits bright fluorescence and much better photostability than the commercially available PS-NBD 1. Thus, it should be useful as a blue-emitting fluorescent translocation substrate for extended-imaging studies of flippase action in living cells using laser confocal microscopy. Furthermore, PS-coumarin 4 may have value as an energy donor in FRET experiments with NBD acceptor probes.\textsuperscript{212} The PS-carboxyfluorescein probe 6 is not a translocation substrate, indicating that the aminophospholipid flippase does not translocate PS derivatives with charged and highly polar groups attached to the 2-acyl chain. Together, these results suggest that the flippase catalyzed translocation process does not involve phospholipid passage through a non-selective hydrophilic channel. This mechanistic picture is in agreement with the latest
literature suggesting that the flippase is a P4 ATPase, perhaps associated with other proteins.\textsuperscript{197,213}

A.4. Methods and Materials

A.4.1. Materials

Culture media and bovine serum albumin (BSA) were purchased from Sigma Aldrich (St. Louis, MO). ER Tracker Red and Mito Tracker Red were purchased from Invitrogen (Carlsbad, CA). PS-NBD probe 1 was purchased from Avanti Polar Lipids (Alabaster, AL). Compounds 2–5 were prepared by the methods described previously (Lampkins, JOC). V79 (ATCC: CCL-93), CHO (ATCC: CCL-61), and A549 (ATCC: CCL-185) cells were certified and obtained from the ATCC.

A.4.2. Synthesis

A.4.2.1. PS-Carboxyfluorescein (6)

To a solution of 8 (0.04 g, 0.04 mmol) in CHCl\textsubscript{3} (10 mL) was added DBU (100 \(\mu\)L) and the reaction was stirred for 30 min at ambient temperature. Fluorescein isothiocyanate (FITC) (0.02 g, 0.05 mmol) was then added and the resulting mixture was allowed to stir overnight. The solvent was then evaporated and the crude residue purified using flash chromatography (4:1 CHCl\textsubscript{3}:MeOH eluent system) to afford the protected PS-carboxyfluorescein (31 mg, 67\%) as an orange solid: \textsuperscript{1}H NMR (2:1 CDCl\textsubscript{3}:CD\textsubscript{3}OD) \(\delta\) 0.68 (t, \(J=6.9\) Hz, 3H), 1.07 (m, 38H), 1.22 (s, 9H), 1.26 (s, 9H), 1.41 (m, 6H), 2.11 (m, 4H), 3.43 (m, 2H), 3.75 (m, 2H), 3.91 (m, 4H), 4.06 (m, 1H), 4.17 (m, 2H), 5.01 (m, 1H), 6.41 (m, 2H), 6.50 (s, 2H), 6.86 (d, \(J=8.4\) Hz, 2H), 6.94 (d, \(J=8.4\) Hz, 1H), 7.85 (m, 2H). \textsuperscript{13}C NMR (2:1 CDCl\textsubscript{3}:CD\textsubscript{3}OD) \(\delta\) 14.0, 22.7, 24.7, 24.9, 26.8, 27.8, 28.2, 28.9, 29.2, 29.3, 29.4, 29.5, 29.7, 29.7, 31.9, 34.1, 44.7, 55.0, 62.5, 63.8, 65.7, 70.3, 77.6, 80.2, 82.6,
103.1, 114.3, 131.0, 140.5, 169.7, 173.6, 173.9, 180.7. LRMS (FAB-) calcd for C_{64}H_{93}N_{17}O_{17}PS- (M-): 1238.6 found 1238.7. A solution of protected PS-carboxyfluorescein (0.02 g, 0.02 mmol) CH\textsubscript{2}Cl\textsubscript{2} (4 mL) and TFA (2 mL) was allowed to stir at room temperature for 12 h. The reaction was then concentrated to a crude residue which was taken up in 2:1 CHCl\textsubscript{3}: MeOH and washed with saturated NaHCO\textsubscript{3} and water. The organic layer was then concentrated and purified using flash chromatography (65:25:4 CHCl\textsubscript{3}:MeOH:H\textsubscript{2}O) to afford 6 (15 mg, 85%): R\textsubscript{f} = 0.05, Dittmer-Lester and ninhydrin stain positive. The NMR spectrum extremely broadened due to extensive aggregation in deuterated solvents; however, the PS headgroup was clearly deprotected because the t-Bu singlets at 1.20–1.30 ppm were absent. HRMS (FAB-) calcd for C\textsubscript{55}H\textsubscript{77}N\textsubscript{3}O\textsubscript{15}PS- (M-) 1082.4818, found 1082.4785.

A.4.2.2. PC-Carboxyfluorescein (7)

To a solution of 9 (0.04 g, 0.04 mmol) in CHCl\textsubscript{3} (10 mL) was added DBU (100 \mu L) and the reaction was stirred for 30 min at ambient temperature. FITC (0.02 g, 0.06 mmol) was then added and the resulting mixture was allowed to stir overnight. The solvent was removed using reduced pressure and the crude residue purified using flash chromatography (65:30:5 CHCl\textsubscript{3}:MeOH:H\textsubscript{2}O eluent system) to afford PC-carboxyfluorescein (31 mg, 65%) as an orange solid: \textsuperscript{1}H NMR (2:1 CDCl\textsubscript{3}:CD\textsubscript{3}OD) \delta 0.63 (t, J=6.9 Hz, 3H), 1.05 (m, 34H), 1.48 (m, 6H), 2.08 (m, 4H), 2.98 (s, 9H), 3.09 (m, 2H), 3.39 (m, 2H), 3.77 (t, J = 6.3 Hz, 2H), 3.92 (dd, J = 12.3 Hz, 6.9 Hz, 1H), 4.03 (m, 2H), 4.17 (dd, J=12.3 Hz, 3.3 Hz, 1H), 4.99 (m, 1H), 6.35 (m, 2H), 6.45 (m, 2H), 6.56 (m, 2H), 6.87 (m, 1H), 7.70 (m, 1H), 8.02 (s, 1H). \textsuperscript{13}C NMR (2:1 CDCl\textsubscript{3}:CD\textsubscript{3}OD) \delta 13.7, 19.0, 22.4, 23.5, 24.6, 26.2, 26.7, 28.6, 28.7, 28.8, 28.9, 29.1, 29.2, 29.2, 29.3, 29.4, 31.6.
32.3, 33.8, 33.9, 37.8, 44.3, 53.8, 54.2, 58.9, 62.3, 63.4, 66.1, 70.0, 70.1, 102.6, 111.3, 114.6, 125.5, 129.5, 141.0, 153.9, 165.9, 170.2, 173.3, 173.7, 180.5. HRMS (FAB+) calcd for C_{57}H_{84}N_{13}O_{13}PS (M+H+) 1082.5541, found 1082.5544.

A.4.2.3. Coumarin Triazole Dye (10)

The known precursor 3-azidocoumarin dye (21 mg, 80 μmol) and 1-octyne (27 mg, 240 μmol) were dissolved in dichloromethane (4 mL) and mixed with an aqueous solution (4 mL) of CuSO_{4}\cdot5H_{2}O (11 mg) and sodium L-ascorbate (13 mg). The reaction was allowed to stir overnight at room temperature and monitored by TLC. After completion the reaction was extracted with chloroform and washed with EDTA to remove the copper salts. The organic phase was evaporated and subjected to flash chromatography (0–3% MeOH in CHCl_{3}) to yield 10 as a dark yellow oil (24.3 mg, 82%). \(^1\)H NMR (CDCl_{3}): δ 0.89 (t, J=7.2 Hz, 3 H), 1.24 (t, J=7.1 Hz, 6 H), 1.36 (m, 6 H), 1.73 (m, 2 H), 2.78 (t, J=7.8 Hz, 2 H), 3.45 (q, J=7.2 Hz, 4 H), 6.55 (d, J=2.4 Hz, 1 H), 6.67 (dd, J= 8.9, 2.4 Hz, 1 H), 7.40 (d, J=8.9 Hz, 1 H), 8.28 (s, 1 H), 8.37 (s, 1 H). \(^{13}\)C NMR (CDCl_{3}) δ 12.6, 14.2, 22.7, 25.9, 29.1, 29.5, 31.8, 45.2, 97.2, 107.4, 110.2, 117.5, 121.80, 130.1, 134.5, 148.5, 151.6, 155.1, 157.3.

HRMS (ESI) calcd for C_{21}H_{29}N_{4}O_{2} (M+H+) 369.2285, found 369.2290. UV-Vis: λ_{max} (abs, CHCl_{3})=415 nm, (em, CHCl_{3})=461 nm, log ε (415 nm, CHCl_{3})=4.56, Φ (ex 341 nm, CHCl_{3})=0.46±5%, in reference to Coumarin 6 (CHCl_{3}, Φ=0.93). λ_{max} (abs, ethanol)=410 nm, (em, EtOH)=480 nm, Φ (ex 357 nm, EtOH)=0.44±5%, in reference to Coumarin 6 (EtOH, Φ=0.87), λ_{max} (abs, 1:1 water:EtOH)=420 nm, (em, 1:1 water: EtOH)=490 nm, Φ (ex 357 nm, 1:1 water: EtOH)=0.38±5%, in reference to Coumarin 6 (EtOH, Φ=0.87).
A.4.3. Fluorescent Phospholipid Preparation

The fluorescent phospholipids were dissolved in 2:1 CHCl₃:MeOH mixture and dried by vacuum. The film was resuspended in TES Buffer (5 mM TES, 145 mM NaCl, pH 7.4) to give a stock probe concentration of 1 mM.

A.4.4. Cell Culture

Monolayer cultures of V79 cells were grown in DMEM media supplemented with 10% fetal bovine serum (FBS), penicillin, streptomycin, and 2.25 mM L-glutamine. Mono-layer cultures of CHO and A549 cells were grown in HF-12 K media supplemented with 10% fetal bovine serum (FBS), penicillin, streptomycin, and 2.25 mM L-glutamine. Cells were grown in plastic culture flasks at 37°C in 5% CO₂ atmosphere, passed in 1:5 dilutions and seeded at 1:4 dilutions on chambered coverslips for microscopy studies.

A.4.5. Cell Microscopy

Brightfield and fluorescence microscopy was performed on a Nikon TE-2000U epifluorescence microscope equipped with filters that allowed detection of each probe. Filter sets were obtained from Nikon and included UV-2E/C filter (ex: 340/80, em: 435/85), EN GFP HQ filter (ex: 450/90, em: 500/50), Cy3 filter (ex: 535/50 nm, em: 610/75), and Cy5 filter (ex: 620/60, em: 700/75). Fluorescence images were captured using Metamorph software (Universal) and analyzed using ImageJ 1.40.

For probe translocation studies, cells were seeded on chambered coverslips at a 1:4 dilution following passage. Cells were grown to 80% confluency. The old media was removed and replaced with serum free media. The fluorescent phospholipids were added to the cells at a final concentration of 10 μM. The mixture was incubated at 37°C for 1 h.
followed by three washes with serum free media and then analysis by fluorescence microscopy.

**A.4.6. Metabolic Stability of Fluorescent Probes**

Probes 6 and 7 were added to separate samples of cells to give a final concentration of 10 μM. In each case, cells were incubated at 37°C for 15 min then trypsinized. The cells were transferred to an eppendorf tube and centrifuged for 1 min at 5000 g. The supernatant was removed and the fluorescent pellet was resuspended in 50 μL of H₂O, then transferred to 500 μL of 3% BSA and allowed to incubate on ice for 5 min. Following incubation and BSA-mediated extraction of all the fluorescent probe from the cells, the mixture was centrifuged for 30 s at 12000 g and the pellet was discarded. The supernatant containing the BSA and all of the extracted probe was added to MeOH:CHCl₃ (2:1, v/v), vortexed, and allowed to incubate in the dark at room temperature for 30 min. After incubation, the solution was centrifuged for 10 min at 2000 g, which allowed separation of the solvent layers. The cellular phospholipid components in the organic layer were analyzed by thin layer chromatography using CHCl₃:MeOH:H₂O (65:25:4 by volume) as the eluent and fluorescence emission to visualize the plate. Comparison of the chromatogram with a control plate containing pure material showed that cell incubation produced negligible (< 10%) loss in probe purity.

**A.4.7. Aminophospholipid Flippase Inhibition Studies**

ATP depletion was achieved by incubating cells in 0.1% sodium azide and 50 mM 2-deoxyglucose in glucose free media for 30 min at 37°C. Cells were then treated with 10 μM of fluorescent phospholipid probe and incubated for 30 min at 37°C. After incubation, the cells were washed three times with serum free media and imaged by
fluorescence microscopy. Chemical inhibition of aminophospholipid flippase activity was achieved by treating cells with 10 mM of iodoacetamide in serum free media followed by incubation at 37°C for 30 min. The media was removed and 10 μM of phospholipid probe was introduced and the cells incubated at 37°C for 1 h. After incubation, cells were washed three times with serum free media and imaged by fluorescence microscopy.
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