NEAR-INFRARED ENZYME-ACTIVATED FLUORESCENT PHOSPHOLIPIDS FOR MOLECULAR IMAGING

Theresa M. Mawn

A DISSERTATION

in

Bioengineering

Presented to the Faculties of the University of Pennsylvania

in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

2009

Dr. E. James Delikatny
Supervisor of Dissertation

Dr. Susan Margulies
Graduate Group Chairperson
COPYRIGHT

Theresa M. Mawn

2009
DEDICATION

To my Dad – the best dad in the whole world!

Thank you for always being there.
ACKNOWLEDGEMENTS

As I reflect on the time that I have spent obtaining my doctorate, what stands out most in my mind are the faces of all those that I have met along my journey. Some of these people offered me guidance and direction; some provided me the tools that I needed to reach my goal; and many others carried me to the end with their encouragement, love and prayers. These people have all touched my life and have contributed to the completion of this work, and to them I wish to extend my sincerest gratitude.

The person to whom I owe the most appreciation is my primary thesis advisor, Dr. E. James Delikatny, who has been a wonderful mentor, teacher, role-model and friend, and has affectionately become my “doctor-father.” I am most honored to have been his first student at the University of Pennsylvania. I would also like to thank the other members of my thesis committee, Dr. Andrew Tsourkas (Committee Chair), Dr. Daniel Hammer and Dr. Mark Elliott for their discussions and assistance in the final stages of my dissertation.

I am also grateful to my secondary advisor, Dr. John S. Leigh (now deceased), for his support over the years, Dr. Mark Elliott for his guidance, Dr. Ravinder Reddy, Dr. Ari Borthakur, Susan Colleluori, Allen Bonner, and the entire MMRRCC laboratory. I must also thank Dr. Britton Chance, with whom I studied during my first year as a graduate student and who introduced me to the world of biomedical optics.

I must thank all of my fellow members of the Lipid Metabolism Group, especially, Nancy Beardsley, our laboratory technician and my dear friend, who not only provided the skills necessary to perform our animal studies, but who also made those long days enjoyable. I
must also acknowledge my fellow lab-mate, Daniel-Joseph Leung, for many thoughtful science- and non-science-related discussions and for his friendship. I would like to thank Dr. Matthew Milkevitch, who mentored me in the early stages of this project, and Dr. Seung Cheol Lee, who is always willing to offer his valuable expertise. It has been my pleasure to be able to mentor Melissa Love in her rotation project and I thank her for her preliminary data mentioned in this work. I must also acknowledge David Nelson, who has been a valuable resource over the years, and Antoinette Salvatore, who manages to keep us organized. Finally, I would like to thank Dr. Jerry Glickson, the Director of the Molecular Imaging Laboratory.

This project would not have existed if it were not for the contributions of the Chemistry Core (Division of Molecular Imaging, Department of Radiology, University of Pennsylvania). In particular, I want to thank Dr. Klara Stefflova for her contributions to this work, Dr. Gang Zheng, who jointly conceived of this project with Dr. E. James Delikatny, and especially Dr. Anatoliy V. Popov, who synthesized the compounds investigated in this dissertation.

I would also like to give my appreciation to Yvette Liu at the Optical/Bioluminescence Sub-Core of the Small Animal Imaging Facility (University of Pennsylvania) for her assistance in performing animal studies, and Dr. Steve Pickup and Dr. Weixia Liu for their assistance at the NMR Core (University of Pennsylvania).

I recognize that this research would not have been possible without the financial assistance of the NIH through the grants of my mentor and the support of the MMRRCC through training grant and stipend support.
Finally, I want to thank my boyfriend, Jason Jeremias, for always believing in me and for his patience throughout the duration of this endeavor. I want to thank both my long-time friends from home and all of those who I have met while in graduate school for helping me through the hard times with your optimism and levity. Above all, I want to thank my father for the constant love and support that he has given me throughout the years. My father always taught me that I can do anything that I set my mind to do, and it has been that self-confidence which has allowed me to accomplish this goal.
ABSTRACT

NEAR-INFRARED ENZYME-ACTIVATED FLUORESCENT PHOSPHOLIPIDS FOR MOLECULAR IMAGING

Theresa M. Mawn
Dr. E. James Delikatny

With the aim of being able to detect disregulated choline lipid metabolism in cancer and elucidate the pathways involved in the malignant transformation, our group is designing and synthesizing a series of phospholipase-specific NIR molecular beacons. The focus of my dissertation has been to characterizing these NIR enzyme-activated fluorescent phospholipids and to assess their potential as suitable in vivo phospholipase molecular beacons. Several key criteria were necessary to be determined a successful in vivo phospholipase probe: 1) sensitivity and specificity to phospholipase activity, 2) adequate signal amplification, 3) high enzyme-substrate affinity allowing for sufficient in vivo activation, 4) ability to overcome biological delivery barriers, and 5) in vivo pharmacokinetics that allows for sufficient tumor absorption and activation.

These criteria have been explored here for the first enzyme-activated fluorescent phospholipid to be synthesized by our group, Pyro-PL-BHQ. Pyro-PL-BHQ is highly specific to phosphatidylcholine-specific phospholipase C (PC-PLC), responsible for catabolizing phosphatidylcholine directly to phosphocholine (PC). Incubation of Pyro-PL-BHQ with PC-PLC in solution demonstrated a 150-fold amplification. Enzyme kinetics determined an apparent $K_m$ of $1.4 - 1.9 \mu M$ and $V_{max}$ of $160.7 \pm 29.2$ nmol/min/mg. The PC-PLC inhibitor, tricyclodecan-9-yl xanthogenate (D609), inhibited
probe activation with an IC$_{50}$ of 34 ±8 µM. Pyro-PL-BHQ was internalized by DU145 human prostate cells, and subsequently activated. Tumor-bearing mice injected with Pyro-PL-BHQ, followed by \textit{in vivo} NIR imaging, resulted in a 4-fold tumor-specific increase in radiance over background. Tumor probe activation was inhibited with administration of D609. As Pyro-PL-BHQ has satisfied the above prerequisites, we present Pyro-PL-BHQ as the first NIR phospholipase-activated molecular beacon.

Preliminary characterization has been performed on the second enzyme-activated phospholipid synthesized, Pyro-C12-PL-BHQ. This substrate displays high specificity for secreted phospholipase A$_2$ type IB (sPLA$_2$ IB). Enzyme kinetics determined an apparent $K_m$ of 2 µM. Tumor-bearing mice injected with Pyro-C12-PL-BHQ presented activation specifically in the gut, an expected result given that sPLA$_2$ IB is primarily a digestive enzyme.

These results demonstrate the feasibility of designing a phospholipase-specific molecular imaging probe capable of directly, sensitively and quantitatively measuring phospholipase activity \textit{in vivo}, an important contribution to the study of lipid metabolism as it pertains to cancer.
# TABLE OF CONTENTS

COPYRIGHT ........................................................................................................ II

DEDICATION ....................................................................................................... III

ACKNOWLEDGEMENTS .................................................................................... IV

ABSTRACT ......................................................................................................... VII

TABLE OF CONTENTS ...................................................................................... IX

LIST OF TABLES ............................................................................................... XIII

LIST OF FIGURES ........................................................................................... XIV

CHAPTER 1: INTRODUCTION ........................................................................... 1

1.1. Molecular Imaging of Cancer ................................................................. 1

1.2. Enzyme-activated Molecular Beacons ................................................... 3

1.3. Lipid Metabolism in Cancer .................................................................... 7

1.4. Phospholipases ......................................................................................... 10

1.4.1. Phospholipase A \(_2\) ........................................................................... 11

1.4.2. Phospholipase C .............................................................................. 12

1.5. Thesis Contributions .............................................................................. 12

1.5.1. Background ..................................................................................... 12

1.5.2. Thesis Outline .................................................................................. 16

1.5.2.1. Chapter 2 .................................................................................. 16

1.5.2.2. Chapter 3 .................................................................................. 17

1.5.2.3. Chapter 4 .................................................................................. 17

1.5.2.4. Chapter 5 .................................................................................. 17

1.5.2.5. Chapter 6 .................................................................................. 17

1.5.3. Summary ......................................................................................... 18

CHAPTER 2: CHARACTERIZATION ............................................................... 19
### 2.1. Pyro-PL-BHQ

- 2.1.1. Synthesis ................................................................. 20
- 2.1.2. Specificity and Sensitivity ........................................... 22
- 2.1.3. Aggregation Process .................................................. 29
- 2.1.4. Calibration ............................................................. 35
- 2.1.5. Enzyme assays ......................................................... 36

### 2.2. Pyro-C12-PL-BHQ ........................................................... 41

- 2.2.1. Synthesis ................................................................. 41
- 2.2.2. Specificity and Sensitivity ........................................... 42
- 2.2.3. Enzyme Assays ......................................................... 46

### 2.3. Comparisons with Commercial Probes: PED-6 and B7701

- 2.3.1. PED6 ................................................................. 51
- 2.3.2. B7701 ................................................................. 53

### 2.4. Discussion ............................................................... 54

### CHAPTER 3: KINETICS ........................................................... 58

#### 3.1. Pyro-PL-BHQ ............................................................... 59

- 3.1.1. Surface Dilution Kinetics ........................................... 59
  - 3.1.1.1. Bulk Kinetics ................................................... 60
  - 3.1.1.2. Interfacial Kinetics ........................................... 61
- 3.1.2. Enzyme Inhibition .................................................. 62

#### 3.2. Pyro-C12-PL-BHQ ........................................................... 66

- 3.2.1. Surface Dilution Kinetics ........................................... 66

#### 3.3. Discussion ............................................................... 68

### CHAPTER 4: CELL STUDIES ..................................................... 71

#### 4.1. Cellular uptake of lipid probes ...................................... 71

#### 4.2. Activation of lipid probes in cells ....................................... 73

- 4.2.1. Confocal Imaging of PC-PLC using Pyro-PL-BHQ .................. 73
- 4.2.2. Confocal Imaging of PLA₂ and PC-PLC using PED6 ............... 75

#### 4.3. Validation of PC-PLC activity in DU145 cell extracts .................. 77
LIST OF TABLES

Table 1 Association constants and maximum fluorescent intensities for Pyro-PL dispersed in different surfactants. .................................................................32
Table 2 Physiological PBPK Model Parameters ..............................108
Table 3 Fractional volumes of neutral lipids, phospholipids and water contents of tissues in mice. ........112
Table 4 Optimized parameters in Pyro-PL PBPK model ................119
Table 5 Optimized parameters in Pyro-PL-BHQ PBPK model ........123
LIST OF FIGURES

Figure 1 Absorption coefficients of oxy-hemoglobin (HbO2), deoxy-hemoglobin (Hb), fat and water ........6
Figure 2 Biosynthetic (solid lines) and catabolic (dashed lines) pathways of choline phospholipid metabolism .......................................................... 10
Figure 3 Cleavage specificities of phospholipases on the phospholipid .......................................................... 11
Figure 4 Pyro $\lambda_{ex}/\lambda_{em}$ wavelength pairs used in vitro and in vivo .......................................................... 14
Figure 5 BHQ-3 absorption overlap with Pyro emission .......................................................... 16
Figure 6 Synthesis of Pyro-PL and Pyro-PL-BHQ .......................................................... 21
Figure 7 Absorbance spectra of Pyro acid, Pyro-PL and Pyro-PL-BHQ .......................................................... 21
Figure 8 Pyro-PL-BHQ specificity determined by TLC .......................................................... 23
Figure 9 Validation of PC-PLC Activation of Pyro-PL-BHQ by UV HPLC and MALDI-TOF .......................................................... 25
Figure 10 Sensitivity of Pyro-PL-BHQ to PC-PLC, PC-PLD, and SMase .......................................................... 26
Figure 11 Signal amplification after activation of Pyro-PL-BHQ .......................................................... 27
Figure 12 Comparing the sensitivity of Pyro-PL-BHQ toward the actions of PC-PLC (B. cereus) and PC-PLC (L. monocytogenes) .......................................................... 28
Figure 13 TLC of Pyro-PL-BHQ cleavage products after activation by B. cereus PC-PLC and L. monocytogenes PC-PLC .......................................................... 28
Figure 14 Linearity of emission with concentration in methanol .......................................................... 29
Figure 15 Effect of mole fraction on fluorescence .......................................................... 30
Figure 16 Effect of micelle concentration on Pyro-PL fluorescent emission .......................................................... 31
Figure 17 Pyro-PL Titration curve of the addition of PtdCho to Pyro-PL .......................................................... 33
Figure 18 Determination of MF correction factor .......................................................... 34
Figure 19 Correcting Pyro-PL fluorescence measurements with MF correction factor .......................................................... 34
Figure 20 Calibration curve to convert fluorescence (a.u.) to amount of cleaved product (pmol) .......................................................... 36
Figure 21 Inhibition of PC-PLC activity toward Pyro-PL-BHQ by Triton X-100 .......................................................... 37
Figure 22 PC-PLC activity toward Pyro-PL-BHQ in mixed lipid vesicles of PtdSer, PtdGro and cholesterol .......................................................... 38
Figure 23 PC-PLC activity toward Pyro-PL-BHQ in mixed lipid vesicles of containing PtdCho, PtdGro and cholesterol .......................................................... 39
Figure 24 PC-PLC (B. cereus) activity toward Pyro-PL-BHQ in PtdCho vesicles .......................................................... 40
Figure 25 PC-PLC (L. monocytogenes) activity toward Pyro-PL-BHQ in PtdCho vesicles .......................................................... 41
Figure 26 Synthesis of Pyro-C12-PL-BHQ .......................................................... 42
Figure 27 Pyro-C12-PL-BHQ specificity determined by TLC .......................................................... 44
Figure 28 Sensitivity of Pyro-C12-PL-BHQ to sPLA2, PC-PLC, PC-PLD, SMase, and PI-PLC .......................................................... 45
Figure 29 Specificity of Pyro-C12-PL-BHQ for sPLA2 IB .......................................................... 46
Figure 30 Enzyme assay for sPLA2 IB activity on Pyro-C12-PL-BHQ .......................................................... 47
Figure 31 Activity of sPLA2 on Pyro-C12-PL-BHQ in PtdCho and Triton X-100 .......................................................... 48
Figure 32 Lag period in sPLA2 activation of Pyro-C12-PL-BHQ mixed in PtdCho .......................................................... 49
Figure 33 Simultaneous measurements of egg-PtdCho and Pyro-C12-PL-BHQ hydrolysis .......................................................... 50
Figure 34 Specificity of PED6 by TLC .......................................................... 52
Figure 35 Enzyme specificity of PED6 by fluorescence spectroscopy .......................................................... 53
Figure 36 Enzyme specificity of B7701 by fluorescence spectroscopy .......................................................... 54
Figure 37 Case I: Bulk Kinetics of Pyro-PL-BHQ with PC-PLC .......................................................... 62
Figure 38 Case II: Interfacial Kinetics of Pyro-PL-BHQ with PC-PLC .......................................................... 62
Figure 39 Inhibition of PC-PLC activity toward Pyro-PL-BHQ using D609 .......................................................... 63
Figure 40 Dose-response curve of Pyro-PL-BHQ activation versus D609 concentration .......................................................... 64
Figure 41 D609 Inhibition of bulk kinetics of Pyro-PL-BHQ with PC-PLC .......................................................... 65
Figure 42 Lineweaver-Burk plots of D609 inhibition of PC-PLC activation of Pyro-PL-BHQ .......................................................... 65
Figure 43 Determination of D609 dissociation constant for PC-PLC activation of Pyro-PL-BHQ .......................................................... 66
Figure 44 Case I: Bulk Kinetics of Pyro-C12-PL-BHQ with sPLA2 IB .......................................................... 67
Figure 45 Lineweaver-Burk Plot of Pyro-C12-PL-BHQ Bulk Kinetics with sPLA2 IB .......................................................... 67
Figure 46 Confocal images of Pyro-PL and Pyro acid delivered to DU145 cells .......................................................... 72
CHAPTER 1: INTRODUCTION

1.1. MOLECULAR IMAGING OF CANCER

According to the American Cancer Society [1], one in four deaths in the United States is caused by cancer, at an alarming rate of 1,500 deaths per day. However, over the past two decades (1990 – 2004), we have finally started to see a significant decrease in cancer mortality rates – by 18.4% among men and by 10.5% among women – which is largely a reflection of improvements in early detection and treatment [2].

The field of molecular imaging has led to the development of novel, noninvasive molecular imaging techniques which offer the ability to detect cancer at a curable stage, to monitor and adjust patient treatments, and to improve the efficiency of cancer drug development [3]. The National Cancer Institute has identified in vivo molecular imaging as an extraordinary opportunity for studying diseases noninvasively and, often, quantitatively. While conventional anatomical and structural imaging is often insensitive to the presence of early cancer, imaging strategies which directly monitor a molecular target or cancer biomarker allow for earlier detection, and offer the detailed elucidation of key metabolic pathways and specific cellular processes.

The imaging modalities that have spurred rapid growth in noninvasive in vivo molecular imaging over the past decade are magnetic resonance, nuclear positron emission tomography (PET) and single photon emission computed tomography (SPECT) and in vivo NIR optical imaging [4]. They have the potential to reveal manifestations of malignancy much earlier than the routine anatomical clinical imaging methods. All of these molecular imaging modalities depend on endogenous molecules or on a molecular
imaging probe to provide the imaging signal or image contrast. Molecular imaging modalities that receive signals from endogenous molecules offer useful information. For example, with magnetic resonance spectroscopy (MRS), the metabolic distributions in living cells and tissues can be probed by receiving signals from endogenous $^1$H, $^{31}$P, or $^{23}$Na, which can be used to detect prognostic and diagnostic markers in cancer, such as choline [5-8] and mobile lipids [9-12]. Near-infrared (NIR) spectroscopy is used to measure the oxygenation of hemoglobin, which can also be mapped using diffuse optical tomography (DOT). Recent work has been done using optical imaging to detect breast tumors [13]. However, these modalities are limited by signal strength and many researchers have been developing methods to enhance signal using exogenous signals, such as hyperpolarized nuclei for MRS [14] and fluorescent agents for NIR optical tomography [15]. The molecular probes used in magnetic resonance imaging (MRI) are commonly a paramagnetic atom (e.g., gadolinium) or superparamagnetic iron oxide particle. In optical imaging the signal is provided by a fluorochrome or a bioluminescent molecule, and in nuclear imaging, i.e., PET and SPECT, a radiolabeled molecule emits a signal as the radioisotope decays.

There are generally three classes of molecular imaging probes that are used to image cancer: nonspecific agents, targeted conjugates, and smart probes [16]. Nonspecific probes depend largely on the leaky vasculature of tumors and the differential rates of perfusion to normal versus tumor tissue. The greatest difficulty with this class of probes is the poor tumor:background ratio. In order to overcome this limitation, targeted conjugates, such as monoclonal antibodies, have been developed which increase probe localization in cancer [17]. However, the signal is limited by the availability and density
of receptors, as well as clearance kinetics. In these cases, imaging must be carefully
timed, so that unbound ligands are given time to clear; otherwise, differentiation between
specifically bound and unbound probe is difficult.

The third class of molecular probes, smart probes, is the type of probe on which
this thesis is based. These smart probes, also known as molecular beacons [18], are only
detectable after a specific molecular interaction has caused a change in their physical
properties. Therefore, background signal due to nonspecific uptake is diminished, while
amplification is no longer limited by the density of the target but continues to occur with
each molecular interaction. In vivo optical smart probes are often based on a quenching-
dequenching effect, where, in the native state, the probe is optically silent (quenched), but
becomes highly fluorescent when altered by a specific molecular interaction, often due to
the action of an enzyme.

1.2. ENZYME-ACTIVATED MOLECULAR BEACONS

The original molecular beacon consisted of a single DNA strand to which a
fluorophore and a quencher are covalently attached to either end [19]. When the DNA
strand is unhybridized, it forms a hairpin shape with a short matched base pair stem and
loop which brings the fluorophore and quencher at opposite ends close together, causing
the fluorescence to be quenched. The loop contains a sequence that is complementary to
the target sequence. Thus, when the strand unfolds to hybridize to the target DNA or
RNA, the fluorophore and quencher are separated, which allows for the release of
fluorescence. In this way, these probes act as a switch, which is only turned on after this
specific interaction has occurred. However, the inherent limitation in this scenario is that
the fluorescence generated is based on the interaction between one target and one probe in a 1:1 stoichiometric ratio, i.e., one target can only activate one probe.

In an attempt to create signal amplification, many molecular probes are being structured to be sensitive to enzyme activities so that cleavage of the fluorescent moiety from the quencher moiety causes the release of fluorescence. For example, quenched NIR molecular probes that are activated by matrix metalloproteinases (MMPs) have been developed by covalently coupling a fluorochrome to a poly-L-lysine backbone onto which specific synthetic peptide substrates are coupled [20]. This signal-amplifying mechanism increases the sensitivity of molecular beacons because one enzyme can catalyze many cycles of conversions, which leads to an accumulation of optical signals [21]. Weissleder et al. [22-24] have demonstrated the use of NIR fluorescent, optically quenched probes to detect tumor-associated lysosomal protease activity. They have demonstrated a 12-fold signal amplification using protease-activated NIR fluorescent probes in vivo [25, 26]. Tsourkas et al. have reported signal-to-background ratios of up to 50, using dual fluorescence resonance energy transfer (FRET) molecular beacons [27]. In this case, the fluorescence released is dependent on the distance between the fluorophore and the quencher, so compared to a fluorophore and quencher that are separated by the length of a DNA strand, the separation between fluorophore and quencher is far greater when a molecular probe is cleaved by an enzyme. An increase in detection sensitivity would lead to improved disease diagnosis and therapy, and also reduces the extent of invasive monitoring of therapeutic response.
In probing surface structures, the level of sensitivity offered by optical imaging methods is unmatched by any other imaging technique. The limit of tumor detection in animal models has been reduced to fewer than 1,000 cells [28]. Optical contrast agents can be detected at subpicomolar concentrations [29], while MRI and MRS require micromolar to millimolar concentrations of contrast agent in order to be detected. However, signal amplification is particularly important for in vivo imaging of deeper organs because of the absorption and scattering effects of biological tissue, which inhibit depth penetration and resolution. Therefore, the use of fluorescent molecules with excitation and emission frequencies in the NIR spectral region (650 – 900 nm) must be employed. Biological tissue is more transparent to NIR light than to any other wavelength because water, hemoglobin, and fat are least absorbent in the NIR window (650 – 900 nm, Figure 1), and tissue autofluorescence is minimized. Whereas fluorescent light in the visible range can only penetrate 1 – 2 mm [30], under certain conditions fluorescence imaging in the NIR range can penetrate several centimeters through biological tissue [31]. Optical methods also offer the potential for multiplexing [32] by assigning fluorophores of various wavelengths to probes specific to different activities, thereby offering the ability to monitor multiple events simultaneously in vivo. Optical imaging offers a further advantage in that the technology is relatively inexpensive and smaller than other imaging methods, which allows for the potential of portability and bedside imaging.
In this work, we have tested and validated NIR fluorescent molecular probes that were specifically synthesized to be sensitive to the activity of phospholipases. These probes are phospholipid analogs, which can be designed to have specificity for a particular phospholipase of interest by adjusting the placement of a fluorophore and quencher on the phospholipid. The close proximity of the fluorophore and the quencher on the phospholipid results in both contact and FRET quenching in the native state [36]. After hydrolysis by the target phospholipase, the fluorescent moiety is separated from the quencher moiety and the fluorescence is recovered. Phospholipase probes of this sort in the visible range have been previously reported. Thuren et al. used a pyrene-labeled phospholipid analog (λ$_{ex}$ = 343 nm, λ$_{em}$ = 400 nm), sensitive to phospholipase A$_2$ (PLA$_2$) [37] and phospholipase C (PLC) [38]. Babitskaya et al. [39] used a modified pyrene-
labeled phospholipid analog that was also sensitive to PLA₂, and Hendrickson et al. [40] who used a BODIPY-labeled PLA₂-sensitive phospholipid analog (λ_{Ex} = 500 nm, λ_{Em} = 512 nm). These substrates have been successful in several applications, such as measuring PLA₂ activity in the sera from patients with suspected pancreatitis or sepsis [41] and measuring PLA₂ activity in living zebrafish embryos [40]. Yet, the unavailability of NIR phospholipase-sensitive conjugates has prevented the application of these probes toward in vivo molecular imaging in small animals or humans.

Therefore, the major contribution of this work is the development and characterization of intramolecularly-quenched phospholipase-activated molecular probes, which incorporate a NIR fluorophore. These probes are suitable for detecting and monitoring phospholipase activity in vivo. With the growing evidence of deregulated lipid metabolism in the progression and development of cancer, phospholipases are intricately involved and may prove to be an important molecular imaging target and a marker for this disease.

1.3. LIPID METABOLISM IN CANCER

Lipids play an important role in the regulation of cellular responses and cellular communication [42], so it is not surprising that in a variety of cancers the lipid metabolic profile has been found to be altered. The development of probes for the detection or imaging of lipid metabolism has been relatively unexplored, but holds great promise for resolving and monitoring specific molecular targets for anticancer therapy.

Phospholipids are the principal component of all biological membranes. The hydrolysis of membrane phospholipids is catalyzed by phospholipases, which, in turn,
generates a variety of important lipid second messengers. These lipid-derived products are necessary for both physiological and pathological processes. It has been reported that the lipid metabolites of the major membrane phospholipid, phosphatidylcholine (PtdCho), in particular, are essential for the mitogenic signal transduction in cells [5, 43-47], in processes such as differentiation, cell survival/apoptosis, and initiating and maintaining a growth signal [44, 48]. Many hormones, growth factors and related agonists that play a role in malignant transformation have been implicated in inducing the synthesis and breakdown of PtdCho [45, 49, 50].

Proton MRS ($^1$H-MRS) and phosphorus MRS ($^{31}$P-MRS) have been extensively used to study the biochemistry of cancer cells and solid tumors by observing changes in the lipid metabolites of PtdCho. The ultimate goal of these studies is to gain the ability to detect cancer at an early stage and to assess the response to therapy [51-60]. Over the past two decades, MRS studies have consistently revealed the presence of increased phosphocholine (PC) and total choline (tCho) levels in breast [61-64], prostate [6, 64-68], and brain [7, 64-66, 69] cancer cells and solid tumors. Elevated levels of PC have been correlated with the degree of malignancy in cancer [50, 70], and it has been shown that PC levels decrease in response to successful chemotherapeutic treatment [71]. Additionally, there has been in vitro evidence of altered phospholipid metabolism associated with growth arrest or apoptosis, such as accumulation of PC and glycerophosphocholine (GPC) [6, 12, 68, 72]. Prominent levels of mobile lipids have been observed in a majority of cancers, as well [12, 72, 73].
In spite of these studies, the biochemical origins leading to alterations in these resonances have not been fully elucidated, since these lipid metabolites can arise through several signaling or metabolic pathways. For example, it has been hypothesized that increases in PC are due to enhanced choline transport and choline kinase (ChoK) activity in cancer cells [62], presumably to fuel cell membrane synthesis and enhanced cell growth, since PC is the substrate for the rate-limiting step in the Kennedy pathway synthesis of PtdCho. However, increased levels of PC have also been attributed to the catabolism of PtdCho, mediated by elevations in phosphatidylcholine-specific phospholipase C (PC-PLC) [74, 75], which hydrolyzes PtdCho to yield PC and diacylglycerol (DAG). PC can also be produced indirectly through phospholipase D (PLD), or by the sequential actions of PLA$_2$ and/or phospholipase A$_1$ (PLA$_1$), followed by glycerophosphocholine phosphodiesterase (GPC:PDE, Figure 1). In these indirect pathways, PC is replenished via the phosphorylation of choline (Cho) by ChoK [56].

It is of growing importance to resolve the sources of choline lipid metabolites, as potential markers for the malignant phenotype [61, 76], or as markers for successful response to therapy. Therefore, there is a strong rationale to develop molecular imaging probes that target phospholipases and other enzymes involved in choline phospholipid metabolism.
1.4. PHOSPHOLIPASES

Phospholipases are categorized as A₁, A₂, C and D based on their site of action (Figure 3). PLA₁ and PLA₂ removes fatty acid chains from the sn-1 and sn-2 positions of the glycerol backbone of a variety of phospholipids [78, 79]. PLC specifically hydrolyzes the P-O bond adjacent to the glycerol sn-3 position to produce DAG and the corresponding phosphorylated head group. PLD hydrolyzes the O-P bond adjacent to the head group, releasing the head group and a molecule of PA. Phospholipases are involved in signal transduction, for the maintenance and turnover of membranes, as mediators of inflammation and immunity, and as digestive enzymes both at the cellular lysosomal level and for absorption of nutrients through the gut [78, 79].
Figure 3 Cleavage specificities of phospholipases on the phospholipid.

In this work, we have characterized in detail a NIR probe, specific to the actions of the phosphatidylcholine-specific PLC (PC-PLC). We have also performed preliminary experiments to characterize a second NIR probe, specific to the actions of a secreted PLA2 (sPLA2). Both of these enzymes are involved in many cellular functions and play large role in generating important second messengers involved in cellular signaling. These enzymes are discussed in more detail below.

1.4.1. Phospholipase A2

PLA2 is a critical modulator of inflammation, regulator of immune function, a controlling factor in signal transduction and is important in membrane re-modeling. PLA2 levels are increased during inflammation, hyperproliferation [79-81] and apoptosis [82, 83]. PLA2 is responsible for arachidonic acid release and formation of lysophospholipids, which are signaling molecules involved in differentiation, intercellular communication, cell invasion, and more recently, in the regulation of apoptosis [84]. Importantly,
arachidonic acid is a precursor to prostaglandins and leukotrienes, critical modulators in inflammation and modulators of growth and differentiation [85].

There are numerous isoforms of PLA₂ that are divided into three categories on the basis of molecular weight and requirement for calcium. The low molecular weight (13-16 kDa) secretory PLA₂ is Ca²⁺ dependent (Types II, V, X and XII). The high molecular weight Ca²⁺-dependent (type IV, IX, MW 55-100 kDa), are generally cytoplasmic and require calcium for translocation, but not for activity. The calcium-independent (type VI-VII) PLA₂ vary in molecular weight and can be secretory or cytoplasmic.

1.4.2. Phospholipase C

The two major types of PLC are the PtdIns-specific (PI-PLC) and PtdCho specific (PC-PLC) [86-88]. PI-PLC is the more widely known PLC, involved in G-protein and tyrosine kinase mediated signal transduction pathways. PI-PLC specifically hydrolyzes a phosphorylated form of PtdIns (PIP2) into inositol triphosphate (IP3) and DAG. Both DAG and IP3 are important second messengers; DAG activates protein kinase C and IP3 modulates ras, Akt and mTOR signaling pathways. PC-PLC hydrolyzes a broad range of phospholipids at the head group, but has a preference for hydrolyzing PtdCho, releasing choline and DAG. PC-PLC is particularly interesting as it has been implicated in the production of PC in tumors [74, 89].

1.5. THESIS CONTRIBUTIONS

1.5.1. Background

Our group is in the process of designing and synthesizing a series of optically quenched, lipid-based NIR fluorescent molecular beacons for the detection of specific
phospholipase activities. These probes are synthesized from a 1-palmitoyl phosphatidylethanolamine, on which the NIR fluorophore, pyropheophorbide a (Pyro) is attached to the sn-2 position and the NIR Black Hole Quencher 3 (BHQ-3) is conjugated to the phospholipid head group. The close proximity of the quencher to the fluorophore in this native state results in fluorescence quenching. Upon enzymatic cleavage of the lipid by a phospholipase, the fluorescent moiety is separated from the quencher moiety and NIR fluorescence is restored. Thus, due to this enzyme-activated mechanism, these probes have the ability to augment their signal each time a phospholipase cleaves a fluorescent substrate, and so we are only limited by the amount of probe that can be delivered.

This concept stems from the recent advances by Farber et al. [40, 74], who have developed a BODIPY-labeled, self-quenching phospholipid probe to detect PLA\textsubscript{2} activity in zebrafish during embryogenesis. The critical modification that our group has made is to incorporate a NIR fluorophore, Pyro; this is essential for future translation to the clinical setting. Pyro is a neutral NIR photosensitizer derived from chlorophyll a. Pyro can be excited at either 420 nm or 660 nm, where it absorbs strongly and emits over the 670-730 nm wavelength region, with the major emission peak at 675 nm and a smaller shoulder peak at 720 nm (Figure 4).
Figure 4 Pyro $\lambda_{Ex}/\lambda_{Em}$ wavelength pairs used in vitro and in vivo.

Pyro absorption spectrum is shown in blue and emission spectrum is shown in red. For in vitro studies, the 420/675 nm wavelength pair (blue) can be used with less spectral overlap. The 660/720 nm wavelength pair (red) is optimal for in vivo studies with maximum tissue penetration. Inset is of structure of pyropheophorbide a.

It is the 670 nm excitation band that allows for NIR in vivo imaging, where deeper tissue penetration is necessary. Although the major emission peak at 675 nm overlaps with this excitation band, a 660 nm excitation/720 nm emission filter combination is commonly used to detect Pyro fluorescence in vivo. The 420 nm excitation/675 nm emission filter combination is often used in vitro or in solution studies due to better separation of excitation and emission light.

In addition to its NIR qualities, Pyro was also chosen because, as a porphyrin, it is preferentially taken up by tumors. Although the reasons for preferential uptake in tumors is unknown, it has been suspected that characteristic properties of tumor tissue, such as reduced lymphatic drainage, large interstitial space, high amounts of collagen, and leaky vasculature, contribute much to the phenomenon [90-92]. The fact that Pyro is an uncharged molecule is a further advantage and requirement with the goal of synthesizing
a fluorogenic lipid probe; a neutral fluorophore is necessary for incorporation into the hydrophobic portion of the lipid molecule.

Fluorescence quenching is achieved via the mechanism of Förster resonance energy transfer (FRET). Typically, self-quenching molecular probes utilize a pair of fluorophores, one reporter and one quencher. The reporter fluorophores absorbs excitation light, which lifts it to its excited state. As the reporter dye returns to the ground state, it emits a photon, which is nonradiatively transferred to the quencher fluorophore. The quencher is subsequently excited and as it returns to the ground state, it emits a photon at a longer wavelength. Using optical filters, the fluorescence emitted from the quencher fluorophore can be cut off. However, overlap between the emissions of the two fluorophores can result in significant background signal.

We have implemented the use of a dark quencher for our probes, BHQ-3 (Figure 5A). The Black Hole Quenchers are dark quenchers, i.e., they have no native fluorescence. As these quenchers return to the ground state, they release energy in the form of heat rather than fluorescence, thereby significantly reducing background signal. We have found that BHQ-3 is a very efficient quencher of Pyro. The absorbance of BHQ-3 overlaps well with the emission of Pyro (Figure 5B). There is evidence that this FRET quenching may also be enhanced by the effect of accompanied static quenching [93], also known as contact quenching, however this has not been investigated in this study.
Figure 5 BHQ-3 absorption overlap with Pyro emission.
(A) Structure of BHQ-3, (B) BHQ-3 (red, $\lambda_{\text{Abs}}$: 620 – 730 nm) overlaps well with Pyro emission (green, $\lambda_{\text{Em}}$ = 670 – 730 nm) resulting in efficient fluorescence quenching.

1.5.2. Thesis Outline

In this work, we present the results of extensive characterization and in vitro and in vivo testing of the first probe in a series of NIR phospholipase-activated probes to be synthesized by our group, Pyro-PL-BHQ [l-palmitoyl-2-pyropheophorbide-sn-glycero-3-BHQ-3], which is sensitive to the phosphatidylcholine-specific PLC (PC-PLC).

Some preliminary studies have also been done to characterize the second probe to be synthesized by our group, Pyro-C12-PL-BHQ, which is sensitive to the secreted isoform of PLA$_2$ (sPLA$_2$), including preliminary in vivo testing. Some of these studies were performed by a rotation student, Melissa Love, while under my mentorship, and will be noted as such in the text.

1.5.2.1. Chapter 2

We characterize the phospholipase specificity and sensitivity of the NIR probes synthesized by our group, resolve the effects of aggregation and fluorescence
reabsorption in fluorescence measurements in order to obtain quantitative measurements of activity, and we determine the optimal conditions for hydrolysis. Comparisons are made to other commercially available probes.

1.5.2.2. Chapter 3

An assay for determining the activity of phospholipases toward molecular imaging probes is developed based on the surface dilution method and Lineweaver-Burk plots. The effect of inhibition on enzyme activity is also determined.

1.5.2.3. Chapter 4

In cultured DU145 human prostate cells, we demonstrate cellular uptake and subsequent activation of Pyro-PL-BHQ using confocal imaging. Comparisons are made to activity observed using a commercially available probe.

1.5.2.4. Chapter 5

We demonstrate the ability to image PC-PLC activity in vivo in a DU145 human prostate tumor xenograft model. Moreover, we demonstrate that this fluorescence can be attenuated using tricyclodecan-9-yl xanthogenate (D609), a specific inhibitor to PC-PLC, both in solution and in vivo.

1.5.2.5. Chapter 6

Finally, using pharmacokinetic models, we have characterized the substrate disposition, tissue absorption, metabolism, and excretion of Pyro-PL-BHQ in order to deconvolve fluorescence due to activation and recirculated accumulation of activated probe.
1.5.3. **Summary**

The major contribution of this work is to design and develop the framework for employing NIR phospholipase-activated molecular beacons for detecting and quantifying phospholipase activity in solution and *in vivo*, with the hope that these probes may aid in the discovery and validation of biomarkers of tumor development and/or successful drug treatment. These phospholipase-activated fluorescent probes will be able to identify the mechanisms of specific phospholipases in cancer and monitor the effects of cancer therapeutics on phospholipase activity. This is essential for establishing the importance of selected lipid biomarkers in cancer. The ability to visualize the molecular effects of drug treatment will aid in the optimization of drug doses and in exploring drug pharmacokinetics. These enzyme-activated fluorescent probes will offer a noninvasive method of acquiring precise, quantitative information about lipid metabolism and help elucidate the marked relationship of lipids to cancer. The development of new and better cancer therapies depends greatly on the identification of true cancer biomarkers [71, 94].
CHAPTER 2: CHARACTERIZATION

The first aim of this project was three-fold: 1) to characterize phospholipase specificity and sensitivity of the NIR probes synthesized by our group, 2) to resolve the effects of aggregation and fluorescence reabsorption in fluorescence measurements, and 3) to determine the optimal conditions for hydrolysis.

In order to determine specificity and sensitivity, the probes were subjected to several *in vitro* assays in order to compare enzyme affinities, testing each probe with an array of commercially available phospholipases. Thin-layer chromatography (TLC), high performance liquid chromatography (HPLC) and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) studies were done to validate probe specificity.

These lipid substrates must be solubilized in lipid vesicles in order to enable kinetic reactions to occur. Pyro is a planar molecule, which tends to stack and self-quench at high concentrations. The effect of this aggregation is that fluorescence measurements will not be proportional to the amount of fluorescent product. Thus, in order to eliminate intermolecular quenching artifacts in kinetic measurements, the substrate must be completely incorporated. The aggregation process of the probes in various lipid formulations was investigated in order to correct for these intermolecular quenching artifacts. A calibration curve was also constructed so that fluorescence could be quantitatively converted to moles of fluorescent product.
Finally, a range of enzyme assays were performed with various lipid carrier vesicles, enzyme concentrations and reaction buffers in order to optimize the conditions necessary for doing a full kinetic analysis.

2.1. PYRO-PL-BHQ

2.1.1. Synthesis

The prototype self-quenching NIR molecular beacon, Pyro-PL-BHQ, was designed and synthesized by our collaborator, A.V. Popov, Ph.D. The synthesis of Pyro-PL and Pyro-PL-BHQ is presented in Figure 6. Briefly, Pyro-PL and Pyro-PL-BHQ, were synthesized by substituting Pyro onto the $sn$-2 position of the glycerol backbone in place of the fatty acid normally present. This was performed by acylating N-Boc 1-palmitoyl-$sn$-glycero-3-lyso-PtdEtn (Lyso-PE-NBoc) with Pyro acid after Boc-deprotection to give the permanently fluorescent analog (Pyro-PL). A second N-acylation of Pyro-PL with BHQ gave rise to the desired probe, Pyro-PL-BHQ.

This prototype, Pyro-PL-BHQ, serves as the basis for synthesizing future probes in the series. The intermediate permanently fluorescent phospholipid analog that was produced, Pyro-PL, is crucial for approximating various photophysical characteristics of the optically quenched Pyro-labeled phospholipid probes.
Figure 6 Synthesis of Pyro-PL and Pyro-PL-BHQ.

(A) Pyro, EDC, DMAP, CH$_2$Cl$_2$, 25°C 72 h, argon (Ar); (B) TFA, CH$_2$Cl$_2$, 0°C, 4h, Ar; (C) BHQ-3-SU·PF$_6^-$, Et$_3$N, CH$_2$Cl$_2$, 25°C 12 h, Ar.

Figure 7 Absorbance spectra of Pyro acid, Pyro-PL and Pyro-PL-BHQ.
Figure 7 shows the absorbance spectra for Pyro acid, the permanently fluorescent Pyro-PL, and the quenched construct, Pyro-PL-BHQ. The absorbance of BHQ-3 can be clearly seen in the spectrum of the Pyro-PL-BHQ construct.

2.1.2. Specificity and Sensitivity

The specificity of Pyro-PL-BHQ was determined by TLC for a range of enzymes: PC-PLC (Bacillus cereus), phosphatidylinositol-specific phospholipase C (PI-PLC, B. cereus), sphingomyelinase (SMase, B. cereus), phosphatidylcholine-specific phospholipase D (PC-PLD, Streptomyces chromofuscus), type IA sPLA₂ (naja mossambica mossambica), type IB sPLA₂ (porcine pancreas), and type IB sPLA₂ (bovine pancreas). TLC analysis after enzyme incubation for 24 h showed that Pyro-PL-BHQ could be activated by PC-PLC, and to a lesser extent, SMase and PC-PLD (Figure 8).

The cleaved fluorescent Pyro moieties could be clearly seen under ultra-violet (UV) light (385 nm) as red spots. The fluorescent product released by PC-PLC and SMase, was found at a retention factor (Rf) of 0.9 (Columns 2 and 4). The sample that was exposed to PC-PLD (Column 5) resulted in two fluorescent products, found at Rf 0.9 and 0.05, while the uncleaved Pyro-PL-BHQ and the cleaved non-fluorescent product, phospho-BHQ-3, were visible to the eye as dark spots at Rf 0.5 and 0.0, respectively. No activity was observed with PI-PLC (Column 3) or type IA sPLA₂ (data not shown). Type IB (porcine) sPLA₂, however, released one non-fluorescent product, found at Rf 0.3 (Column 6).
Figure 8 Pyro-PL-BHQ specificity determined by TLC.

Pyro-PL-BHQ/egg-PtdCho lipid dispersions were incubated for 24 h with various enzymes and the products were separated on a TLC plate using chloroform:methanol (5:1): 1, Control (no enzyme); 2, PC-PLC; 3, PI-PLC; 4, SMase; 5, PC-PLD; 6, sPLA₂ (IB, porcine). BHQ moieties were visible to the eye as blue spots, while fluorescent Pyro moieties were observed as red spots under UV excitation (385 nm). No enzyme activity was observed by PI-PLC or sPLA₂ (type IA or bovine type IB, data not shown). The observed cleaved products confirmed by HPLC followed by MALDI-TOF (Figure 9) are as follows: Rf=0.9, 1-acyl-2-pyropheophorbol glycerol (Lanes 2, 4 and 5); Rf=0.5, Pyro-PL-BHQ; Rf=0.3, Lyso-Pyro-PL-BHQ (Lane 6); Rf=0.05 (red), 1-acyl-2-pyropheophorbol 3-phosphate (Lane 5); Rf=0.0 (blue), phospho-BHQ-3 (Lanes 2, 4 and 5).

Analysis of the TLC samples by HPLC with UV-visible detection and MALDI-TOF mass spectrometry was performed by our collaborator, Klara Stefflova, Ph.D., to validate these results (Figure 9). In Figure 9A, the HPLC trace of Pyro-PL-BHQ shows two closely separated peaks at retention times (RT) 56.4 and 57.9 min, possibly corresponding to free and vesicle-encapsulated probes. Upon treatment with PC-PLC, two cleavage products were observed (Figure 9B). The corresponding UV-visible spectra (Figure 9C) shows that the first product (RT=20.972) exhibited the absorbance spectrum of Pyro and the second exhibits the absorbance spectrum of BHQ-3 (RT = 53.38 min).
MALDI-TOF mass spectrometry further validated that Pyro-PL-BHQ (Figure 9D; calculated molecular weight, 1498.22 Da; found, 1497.80 \textit{m/z}) was cleaved similarly by PC-PLC and SMase into two products that had molecular weights consistent with 1-palmitoyl-2-pyropheophorbol glycerol (calculated, 846.53 Da; found, 846.19 \textit{m/z}) and phospho-BHQ-3 (calculated, 669.28 Da; found, 669.67 \textit{m/z}), shown in Figure 9E and F, respectively. This analysis also confirmed the existence of two fluorescent products in the sample treated with PC-PLD, the expected 1-acyl-2-pyropheophorbol 3-phosphate (calculated, 926.11 Da; found, 926.36 \textit{m/z}), and 1-acyl-2-pyropheophorbol glycerol, the product released by PC-PLC (data not shown), suggesting a cross reactivity or a PC-PLC impurity. The non-fluorescent product released by sPLA2 (type IB, porcine) was found to be the result of PLA1 activity (data not shown) having a molecular weight consistent with 2-pyropheophorbol 3-BHQ-3 (calculated, 1260.42 Da; found, 1258.45 \textit{m/z}), presumably also due to lack of enzyme specificity or enzyme impurity.
Figure 9 Validation of PC-PLC Activation of Pyro-PL-BHQ by UV HPLC and MALDI-TOF.

HPLC traces of (A) Pyro-PL-BHQ and (B) cleavage products of Pyro-PL-BHQ from the actions of PC-PLC; (C) corresponding UV spectra of cleaved products; MALDI-TOF mass spectra and chemical structures of (D) Pyro-PL-BHQ and PC-PLC derived products: (E) 1-palmitoyl-2-pyropheophorbol glycerol and (F) phospho-BHQ-3.
Although PC-PLC, SMase and PC-PLD all demonstrated the ability to hydrolyze Pyro-PL-BHQ, measurement of the time course of these reactions by fluorescence spectroscopy revealed that Pyro-PL-BHQ exhibits a remarkable sensitivity for PC-PLC (Figure 10). The enzyme (10 U) was added to sonicated lipid dispersions of 1 μM Pyro-PL-BHQ in egg-PtdCho vesicles at a mole fraction (MF = [Substrate]/([Lipid]+[Substrate])) of 0.02 and the time-dependent release of fluorescence was measured using a SpectraMax M5 fluorescent plate reader. A 30-fold increase in fluorescence was observed that began immediately after addition of PC-PLC. In contrast, the rate of fluorescence release by PC-PLD and SMase was negligible, even out to 1 h. As shown in Figure 11, the hydrolysis of Pyro-PL-BHQ by PC-PLC fully restored the fluorescence that had been quenched by BHQ. The cleavage of Pyro-PL-BHQ resulted in a ~150-fold increase in fluorescence over baseline, the equivalent fold increase of Pyro-PL over Pyro-PL-BHQ at equal concentrations.

![Figure 10 Sensitivity of Pyro-PL-BHQ to PC-PLC, PC-PLD, and SMase.](image)

Time-dependent increase in fluorescence of 1 μM Pyro-PL-BHQ incubated with 10 U each of PC-PLC, PC-PLD or SMase (λ<sub>ex</sub> 418 nm, λ<sub>em</sub> 675 nm).
Figure 11 Signal amplification after activation of Pyro-PL-BHQ.

Fold increase in fluorescence ($\lambda_{ex} = 418$ nm, $\lambda_{em} = 665$ nm) of 1 μM Pyro-PL-BHQ in PtdCho vesicles (MF 0.02) before and after complete activation by PC-PLC, as compared to that of Pyro-PL at equal concentrations (error bars represent s.d., n=3).

We compared the activity of *B. cereus* PC-PLC with the isoform of PC-PLC isolated from *Listeria monocytogenes*, which was provided to us as a gift from Dr. Howard Goldfine (Department of Microbiology, University of Pennsylvania). Activation of Pyro-PL-BHQ was measured by fluorescence spectroscopy with equal amounts (mg) of enzyme (Figure 12). The probe was incubated at 37 °C with equivalent amounts of both types of PC-PLC for 24 hours and the cleaved products were separated on a TLC plate (chloroform/methanol, 5:1). As shown in Figure 13, the *B. cereus* PC-PLC (Column 2) completely cleaved Pyro-PL-BHQ to release 1-palmitoyl-2-pyropheophorbol glycerol (Rf=0.9) and phospho-BHQ-3 (Rf=0.0), whereas the *L. monocytogenes* PC-PLC (Column 3) only partially cleaved the probe. The remaining uncleaved Pyro-PL-BHQ can be seen at Rf=0.3, as compared to the control in Column 1.
Figure 12 Comparing the sensitivity of Pyro-PL-BHQ toward the actions of PC-PLC (B. cereus) and PC-PLC (L. monocytogenes).

Activation was measured by fluorescence spectroscopy ($\lambda_{ex}$ 418 nm, $\lambda_{em}$ 675 nm) after the addition of 0.001 mg of enzyme.

Figure 13 TLC of Pyro-PL-BHQ cleavage products after activation by B. cereus PC-PLC and L. monocytogenes PC-PLC.

Pyro-PL-BHQ was incubated for 24 h with no enzyme (Column 1), with PC-PLC (Column 2, B. cereus), and PC-PLC (Column 3, L. monocytogenes). After 24 h, the cleaved products were separated by TLC.
2.1.3. Aggregation Process

Pyro, and other derivatives of chlorophyll, are known to form aggregates at high concentrations or in aqueous solutions, which results in fluorescence reabsorption and intermolecular quenching effects [95]. However, in a solvent, such as methanol or chloroform, the substrate is soluble and does not form aggregates. Figure 14 shows the emission spectra (A) and the peak emission (B) of the addition of Pyro-PL from 0 to 80 pmol in methanol. The peak emission at 665 nm is directly linear with concentration in this case.

![Figure 14](image.png)

*Figure 14 Linearity of emission with concentration in methanol.*

Increasing amounts of Pyro-PL was added to methanol: (A) fluorescence emission spectra, (B) fluorescent emission at 665 nm.

In an aqueous environment, lipid vesicles are used to disperse and separate the Pyro-labeled substrates from one another. However, if the Pyro:lipid ratio is not small enough, the Pyro substrates will not be uniformly dispersed and will aggregate within the lipid vesicles. For example, Figure 15 demonstrates that although the concentration of Pyro-PL is increasing (measured by absorbance), the fluorescence measured does not
correlate with concentration. As the MF of Pyro-PL suspended in sonicated mixed vesicles (20 μM of lipid consisting of PtdCho, phosphatidylethanolamine (PtdEtn) and cholesterol (2:1:1)) is incremented from 0 – 0.1, the absorbance is linear (Figure 15A), while the fluorescence is not (B). This data demonstrates the need to correct for these quenching artifacts, especially when performing enzyme kinetic measurements.

Figure 15 Effect of mole fraction on fluorescence.

[Pyro-PL] is varied in 20 μM of lipid (PtdCho, PtdEtn and cholesterol (2:1:1)); (A) Absorbance is linear with increases in Pyro-PL MF, while (B) fluorescence is not correlated with Pyro-PL concentration at high MF (error bars are s.e.).
These aggregates must be forced to disperse uniformly in the lipid vesicles far enough from each other that they do not intermolecularly quench one another. This is done by increasing the lipid concentration, or decreasing the Pyro:lipid MF. The surface area of the lipid vesicles increases with bulk lipid concentration, until the intermolecular quenching is negligible [96]. Figure 16 shows the increase in fluorescence as Pyro-PL (1 μM) molecules are dispersed with increased surface area. With the addition of PtdCho, Tween-80 or Triton X-100, the Pyro-PL molecules separate from one another and the fluorescent signal increases until they no longer cause intermolecular quenching and the fluorescent signal reaches a plateau. The point of complete incorporation is found at the start of the fluorescent plateau with the addition of 200 μM PtdCho (MF 0.005) and 1 mM Tween-80 (MF 0.001). The point of complete incorporation of Pyro-PL does not occur up to 2 mM Triton X-100.

Figure 16 Effect of micelle concentration on Pyro-PL fluorescent emission.
As lipid or surfactant is added to 1 μM Pyro-PL, aggregates are incorporated and separated causing fluorescence to increase until there is no longer intermolecular quenching (λ<sub>Ex</sub> = 410 nm, λ<sub>Em</sub> = 675 nm).
Figure 16 demonstrates that the probe interacts with each surfactant differently, but since Pyro-PL-BHQ is a neutral probe, incorporation is primarily driven by the hydrophobic effect. The association constant, $K_a$, describing the affinity for which the probe binds to a surfactant can be obtained by fitting titration curves to Equation 1 [97],

$$\frac{I}{I_0} = 1 + \frac{K_a[S]}{1 + K_a[S]}(I_{\text{max}} - 1),$$

where $I$ is the intensity of fluorescence at various concentrations of surfactant, $I_0$ is the minimum fluorescence intensity at MF = 1 (determined by extrapolation), $I_{\text{max}}$ is the maximum fluorescence intensity in the absence of self-quenching, and $[S]$ is the concentration of surfactant. The data in Figure 16 was fit to this equation using KaleidaGraph and the parameters for $K_a$ and $I_{\text{max}}$ were determined for the binding of Pyro-PL to PtdCho, Tween-80, and Triton X-100. The results are shown in Table 1.

### Table 1 Association constants and maximum fluorescent intensities for Pyro-PL dispersed in different surfactants.

<table>
<thead>
<tr>
<th>Surfactant</th>
<th>PtdCho</th>
<th>Tween-80</th>
<th>Triton X-100</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_a$ (mM)$^{-1}$</td>
<td>3.10 ± 1.52</td>
<td>0.48 ± 0.30</td>
<td>0.65 ± 0.04</td>
</tr>
<tr>
<td>$I_{\text{max}}$</td>
<td>89.7 ± 21.0</td>
<td>125.7 ± 38.5</td>
<td>63.5 ± 2.6</td>
</tr>
</tbody>
</table>

Although, ideally, all enzyme assays should be performed at a MF below which intermolecular quenching effects are an issue, it became apparent that some experiments would need to be performed at higher MFs above the point of complete probe incorporation in order to calculate the kinetic parameters, $V_{\text{max}}$ and $K_m$. However, if the amount of fluorescence to be reabsorbed is known, these quenching artifacts can be
corrected with an appropriate MF correction factor, \( cf \). The \( cf \) was calculated based on the incorporation of Pyro-PL in PtdCho vesicles using the data from the titration curve in Figure 17.

![Figure 17 Pyro-PL Titration curve of the addition of PtdCho to Pyro-PL.](image)

PtdCho was added to 1 \( \mu \text{M} \) Pyro-PL. The fluorescence is plotted as a function of PtdCho (blue diamonds) and Pyro-PL MF (red squares).

For those experiments that were done at high MFs, we fit the fluorescence versus MF data to an inverse power curve and normalized the maximum fluorescence to 1.0 (Figure 18). Fluorescence measurements that were later collected at high MFs (> 0.005) were divided by the corresponding \( cf \) according to the equation, \( y = 0.0002x^{-1.606} \), where \( x \) is the MF and \( y \) is the \( cf \). This method is similar to that described by Liu et al. [98] to correct for inner filter effects of FRET substrates.
The $cf$ was used to correct for self-quenching artifacts during kinetic experiments at high MFs.

To test the effectiveness of using a MF $cf$ method, Pyro-PL was again mixed in sonicated PtdCho vesicles at a range of MFs, such that the concentration of Pyro-PL was held constant while the concentration of PtdCho was varied. In Figure 19, a MF-specific $cf$ was applied and the fluorescence signal at MFs $> 0.005$ was corrected.

Pyro-PL (1 μM) dispersed in PtdCho vesicles at various MFs. For MF $> 0.005$, the $cf$ was used to correct the observed Pyro-PL fluorescence.
2.1.4. Calibration

The accuracy of kinetic calculations also depends on well-defined calibration curves, which are used to convert the arbitrary units of fluorescence intensity to moles of hydrolyzed product. To achieve an accurate calibration, the conditions of a true kinetic experiment were mimicked, using Pyro-PL as an analog to the released fluorescent product (Figure 20). The fluorescence was measured as Pyro-PL was incremented from 0 to 25 pmol and mixed with Pyro-PL-BHQ in PtdCho vesicles (MF 0.003), such that the concentration of substrate ([Pyro-PL] + [Pyro-PL-BHQ]) was held constant at 100 pmol. This was compared to the measured fluorescence, as Pyro-PL was incremented from 0 to 25 pmol in the absence of Pyro-PL-BHQ. The fluorescence measured in the presence of Pyro-PL-BHQ was much lower than that measured without Pyro-PL-BHQ. This indicates that there is a considerable amount of intermolecular quenching between the bound quencher moieties on the uncleaved substrate and the cleaved fluorescent products.

However, from this modeled experiment, the conversion factor of 16.816 a.u./pmol was determined. This factor was applied to all kinetic assays to convert the measured initial velocity in units of a.u./min to pmol/min.
Figure 20 Calibration curve to convert fluorescence (a.u.) to amount of cleaved product (pmol).
The fluorescence was measured ($\lambda_{\text{ex}} = 418$ nm, $\lambda_{\text{em}} = 675$ nm) as Pyro-PL was incremented from 0 to 25 pmol and mixed with Pyro-PL-BHQ in PtdCho vesicles (MF 0.003), such that the concentration of total substrate ([Pyro-PL] + [Pyro-PL-BHQ]) was held constant at 100 pmol.

2.1.5. Enzyme assays

Enzyme kinetics must be performed using an enzyme concentration in the linear range of activity, i.e., where activity is proportional to concentration. Enzyme assays were done to determine the optimal enzyme concentration for reactions and carrier vesicle. The dependence of activity on enzyme concentration was determined by adding increasing concentrations of PC-PLC (*B. cereus*) to Pyro-PL-BHQ mixed in surfactant or various sonicated lipid dispersions. The activity of PC-PLC with Pyro-PL-BHQ was measured as the rate of hydrolyzed product per minute (pmol/min), as determined by fluorescence spectroscopy ($\lambda_{\text{ex}} = 418$ nm, $\lambda_{\text{em}} = 675$ nm) using the conversion factor determined from Figure 20. All assays were performed at a pH of 7.4 and at 37°C, which is the optimal temperature for PC-PLC activity.
In an ideal analysis of enzyme kinetics, there is one substrate in the reaction and the activity of the enzyme is characterized by the rate that product is formed from the substrate. As we have shown, Pyro-PL-BHQ must be incorporated into a carrier vesicle at a suitable MF in order to prevent aggregation and fluorescent measurement artifacts. Thus, the ideal carrier vesicle would be a surfactant that is not a substrate for PC-PLC. Unfortunately, we found that PC-PLC activity on Pyro-PL-BHQ was greatly inhibited by surfactant vesicles.

In Figure 21, Pyro-PL-BHQ was incorporated into sonicated Triton X-100 vesicles at MF 0.001 and serial dilutions of the bulk lipid ([Pyro-PL-BHQ]+[Triton X-100]) were aliquotted into 100 μL volumes in a 96-well plate, such that the concentration of Triton X-100 was varied from 1 mM to 0.2 mM. PC-PLC (1 U) was added to each of these solutions. The only reaction that occurred was at 0.2 mM Triton X-100, which is the critical micelle concentration (CMC) of Triton X-100 and not a suitable situation for performing enzyme kinetic assays.

![Figure 21 Inhibition of PC-PLC activity toward Pyro-PL-BHQ by Triton X-100.](image)

PC-PLC reactions with Pyro-PL-BHQ incorporated in Triton X-100 (MF 0.001) over a range of dilutions where Triton X-100 is varied from 1 – 0.2 mM.
The activity of PC-PLC (*B. cereus*) on Pyro-PL-BHQ was investigated using a variety of different lipid dispersions. Figure 22A shows a sample enzyme assay of increasing PC-PLC on Pyro-PL-BHQ mixed into sonicated lipid vesicles containing phosphatidylserine (PtdSer), phosphatidylglycerol (PtdGro), and cholesterol (10:2:3). The initial rates were plotted versus enzyme concentration (mg), as shown Figure 22B. The enzyme assay for PC-PLC acting on Pyro-PL-BHQ mixed into lipid dispersions of PtdCho, PtdGro, and cholesterol (10:1:2) is shown in Figure 23. Both of these conditions showed good linearity of activity with enzyme amounts up to 0.05 mg of PC-PLC.

![Figure 22](image.png)

Figure 22 PC-PLC activity toward Pyro-PL-BHQ in mixed lipid vesicles of PtdSer, PtdGro and cholesterol.

Pyro-PL-BHQ (50 nM) mixed in sonicated vesicles of PtdSer:PtdGro:cholesterol (10:2:3) at MF 0.003. (A) Time-dependent curves with increasing amounts of enzyme; (B) activity versus enzyme concentration.
Figure 23 PC-PLC activity toward Pyro-PL-BHQ in mixed lipid vesicles of containing PtdCho, PtdGro and cholesterol.

Pyro-PL-BHQ (665 nM) mixed in sonicated vesicles of PtdCho:PtdGro:cholesterol (10:1:2) at MF 0.0015. (A) Time-dependent curves with increasing amounts of enzyme; (B) activity versus enzyme concentration.

The activity of PC-PLC (B. cereus) on Pyro-PL-BHQ mixed in sonicated vesicles of egg-PtdCho proved to be more efficient than the other lipid dispersions tested. Figure 24 shows the time-dependent curves of activation and the initial rates versus enzyme concentration. Activity was linear up to 0.002 mg of PC-PLC.

It is not surprising that the greatest activity was observed when using egg-PtdCho vesicles. It is well-known that PC-PLC (B. cereus) has a high affinity for PtdCho, and as
such is generally known as "phosphatidylcholine-specific phospholipase C." However, it can also hydrolyze other lipid substrates, and has also been called "broad-range phospholipase C." It hydrolyzes PtdCho, PtdEtn, and PtdSer with specificity constants in the ratio of 10:7:1, respectively [99, 100].

![Figure 24](image_url)  
Figure 24 PC-PLC (B. cereus) activity toward Pyro-PL-BHQ in PtdCho vesicles. Pyro-PL-BHQ (1 μM) mixed in sonicated egg-PtdCho vesicles (MF 0.003). (A) Time-dependent curves with increasing amounts enzyme; (B) activity versus enzyme concentration.

Although Pyro-PL-BHQ was capable of being hydrolyzed by L. monocytogenes PC-PLC (Figure 13), the activity on Pyro-PL-BHQ/egg-PtdCho vesicles was found to be much lower than that of PC-PLC (B. cereus) (Figure 25).
Figure 25 PC-PLC (L. monocytogenes) activity toward Pyro-PL-BHQ in PtdCho vesicles. Pyro-PL-BHQ (1 μM) was mixed in egg-PtdCho vesicles (MF 0.003). Activity versus enzyme concentration.

These enzyme assays demonstrated that Pyro-PL-BHQ is hydrolyzed most efficiently when presented to PC-PLC (B. cereus) in egg-PtdCho vesicles. In Chapter 3, we have determined the kinetic parameters for Pyro-PL-BHQ under these conditions using a PC-PLC (B. cereus) concentration of 0.002 mg (0.5 U).

2.2. PYRO-C12-PL-BHQ

2.2.1. Synthesis

Pyro-C12-PL-BHQ is an analog of the Pyro-PL-BHQ, but contains a C12 spacer, omega-aminolauric acid (NH$_2$(CH$_2$)$_{11}$CO$_2$H), between the fluorescent Pyro moiety and the sn-2 position of the glycerol backbone. The C12 spacer was inserted in order to investigate whether the enzyme specificity of the probe could be modulated. The preparation of Pyro-C12-PL-BHQ, which was done by our collaborator, A.V. Popov, Ph.D., can be concisely described as follows. Pyro-C12-acid was coupled to the sn-2 position of the glycerol backbone of N-Boc protected 1-palmitoyl-sn-glycero-3-
phosphoethanolamine. Next, the continuously fluorescent phosphoethanolamine
derivative Pyro-C12-PL was prepared by N-Boc deprotection. The final acylation of
Pyro-C12-PL with BHQ-NHS in the presence of TEA resulted in the target 1-palmitoyl-
2-(pyropheophorbide-ω-amidolauryl)-sn-glyceryl-3-phosphoethanolamide of BHQ-3
acid (Pyro-C12-PL-BHQ).

![Chemical structures](image)

Figure 26 Synthesis of Pyro-C12-PL-BHQ.

(A) NHS, EDC, DMAP, CH₂Cl₂, Ar, 25°C, 4 h; (B) H₂N(CH₂)₃CO₂H, CH₂Cl₂, Ar, 25°C, 72 h; (C) EDC,
DMAP, CH₂Cl₂, Ar, 25°C, 72 h; (D) TFA, CH₂Cl₂, Ar, 0°C, 4 h; (E) BHQ-3-SU⁺PF₆⁻, Et₃N, CH₂Cl₂, 25°C
12 h, Ar.

2.2.2. Specificity and Sensitivity
A TLC analysis of Pyro-C12-PL-BHQ was performed by Melissa Love, a rotation student in Pharmacology, in order to determine enzyme specificity. Ms. Love was trained by me and these studies were performed under my supervision. Pyro-C12-PL-BHQ was incubated for 24 h with a range of enzymes: PC-PLC, PI-PLC, SMase, PC-PLD, and sPLA2 IB (porcine) and sPLA2 IB (bovine). TLC analysis showed that Pyro-C12-PL-BHQ could be activated by sPLA2 IB from both porcine and bovine sources (Figure 27).

The fluorescent product released by sPLA2, was found at a retention factor (Rf) of 0.8 (Columns 2 and 5). A fluorescent moiety was observed in all samples at Rf 0.2, including the Control (Column 1), which indicates the presence of an impurity, likely Pyro-C12-PL without the quencher. The uncleaved Pyro-C12-PL-BHQ was visible to the eye as dark spots at Rf 0.6. No activation of Pyro-C12-PL-BHQ was observed with PC-PLC (Column 3), PI-PLC (Column 4), PC-PLD (Column 6), or SMase (Column 7). However, note that the impurity was hydrolyzed by both sPLA2 (both sources), as well as by PC-PLC.
Figure 27 Pyro-C12-PL-BHQ specificity determined by TLC.

Pyro-C12-PL-BHQ/egg-PtdCho lipid dispersions were incubated for 24 h with various enzymes and the products were separated on a TLC plate using chloroform:methanol (5:1): SMase. Uncleaved Pyro-C12-PL-BHQ was visible to the eye as blue spots (Rf 0.6), while fluorescent Pyro moieties were observed as red spots (Rf 0.8) under UV excitation (385 nm). A fluorescent impurity was observed at Rf 0.2. No activation of Pyro-C12-PL-BHQ was observed by PC-PLC, PI-PLC, PC-PLD, or SMase.

The specificity and sensitivity of Pyro-C12-PL-BHQ was analyzed by fluorescence spectroscopy (Figure 28). Enzyme (1 U) was added to sonicated lipid dispersions of 1 μM Pyro-C12-PL-BHQ in egg-PtdCho vesicles at a MF of 0.006 and the time-dependent release of fluorescence was measured. Pyro-C12-PL-BHQ showed a marked specificity for sPLA2 IB (porcine pancreas) compared to the other phospholipases. PC-PLC (B. cereus) partially hydrolyzed Pyro-C12-PL-BHQ, but not to completion. The other phospholipases, PC-PLD, SMase, and PI-PLC, demonstrated no affinity for Pyro-C12-PL-BHQ.
Although Pyro-C12-PL-BHQ demonstrated a high sensitivity to sPLA₂ type IB, we wanted to test probe specificity across sPLA₂ isoforms. We added 1 U of sPLA₂ IB as a positive control, sPLA₂ (III, bee venom) and sPLA₂ (human recombinant, type V) to 1 μM Pyro-C12-PL-BHQ in egg-PtdCho vesicles (MF 0.006). As shown in Figure 29, Pyro-C12-PL-BHQ exhibited a high specificity for sPLA₂ IB, and no sensitivity toward sPLA₂ III or sPLA₂ V.
Figure 29 Specificity of Pyro-C12-PL-BHQ for sPLA2 IB.
Time-dependent increase of 1 μM Pyro-C12-PL-BHQ in PtdCho (MF 0.006) with the addition of 1 U of sPLA2 IB as a positive control, sPLA2 III and sPLA2 V.

2.2.3. Enzyme Assays

An enzyme assay was done to determine the optimal enzyme concentration. The dependence of activity on enzyme concentration was determined by adding increasing concentrations of sPLA2 IB to Pyro-C12-PL-BHQ mixed in sonicated lipid dispersions. The activity of sPLA2 with Pyro-C12-PL-BHQ was measured as the rate of hydrolyzed product per minute (pmol/min). All assays were performed at a pH of 7.4 and at 37°C.
Figure 30 Enzyme assay for sPLA$_2$ IB activity on Pyro-C12-PL-BHQ.

(A) Time-dependent increase in fluorescence of Pyro-C12-PLBHQ (1 μM) in PtdCho (MF 0.003) with the addition of increasing amounts of sPLA$_2$ IB (porcine pancreas); (B) activity versus enzyme concentration.

The activity of sPLA$_2$ (IB) on Pyro-C12-PL-BHQ was also investigated with various reaction buffers. Figure 31 shows the effect of increasing sPLA$_2$ IB concentration on the time-dependent enzymatic fluorescent release of Pyro-C12-PL-BHQ mixed into sonicated PtdCho (MF 0.003). In these experiments, the effects of (A) the addition of 10 mM CaCl$_2$ and 100 mM KCl, (B) the addition of 10 mM CaCl$_2$, 100 mM KCl and 300 μM Triton X-100, and (C) the measured activity (pmol/min) as a function of sPLA$_2$ were investigated. We also tested a reaction buffer in which 100 mM KCl was substituted with 150 mM NaCl, which yielded near equivalent results (Figure 31C).
Figure 31 Activity of sPLA₂ on Pyro-C12-PL-BHQ in PtdCho and Triton X-100.

(A) Pyro-C12-PL-BHQ (1 μM) mixed in sonicated egg-PtdCho vesicles (MF 0.003) in buffer containing 150 mM NaCl (A) without Triton X-100 or (B) with the addition of 300 μM Triton X-100, and (C) activity versus enzyme in various reaction buffers.

The enzyme assay containing 300 μM Triton X-100 allowed for the completion of all reactions, whereas without Triton X-100, the PtdCho vesicles would collapse due to enzyme hydrolysis before the reactions could be completed. This is particularly evident...
by looking at the activity versus enzyme plots where the activity is linear with enzyme concentration, even out to 2 U, in the reaction containing Triton X-100 (Figure 31C). This was a surprising result, since Triton X-100 inhibited PC-PLC reactions with Pyro-PL-BHQ.

At very low concentrations of enzyme, a lag period was noticed, followed by a sudden burst in activity (Figure 32). We hypothesized that a percentage of the PtdCho vesicles must be hydrolyzed in order to loosen the vesicles and allow for the hydrolysis of Pyro-C12-PL-BHQ.

![Figure 32 Lag period in sPLA2 activation of Pyro-C12-PL-BHQ mixed in PtdCho.](image)

Hydrolysis of 1 μM Pyro-C12-PL-BHQ in egg-PtdCho (MF 0.006), showing a lag period in activity with the addition of 0.1 U sPLA2.

In order to better understand these results, we added small amounts of enzyme (0.04 – 0.08 U of sPLA2 IB) to Pyro-C12-PL-BHQ/PtdCho vesicles (MF 0.006) and measured the hydrolysis of both Pyro-C12-PL-BHQ and PtdCho simultaneously using the fluorescence polarization mode on the SpectraMax M5 microplate reader. We
measured the fluorescence intensity parallel to the plane of excitation light using two pairs of $\lambda_{\text{Ex}}/\lambda_{\text{Em}}$ wavelengths: 410/675 nm for Pyro-C12-PL-BHQ hydrolysis and 500/520 nm for PtdCho vesicle hydrolysis. Figure 33 shows the time-dependent curves of PtdCho hydrolysis (black) and Pyro-C12-PL-BHQ hydrolysis (red). Circles mark the points on both curves at which the activation of Pyro-C12-PL-BHQ began. Surprisingly, we found that ~75% of the PtdCho vesicles were hydrolyzed before the enzyme began to hydrolyze Pyro-C12-PL-BHQ.

Figure 33 Simultaneous measurements of egg-PtdCho and Pyro-C12-PL-BHQ hydrolysis. Pyro-C12-PL-BHQ (1 µM) was dispersed in egg-PtdCho vesicles (MF 0.006). Parallel-polarized fluorescence was used to measure simultaneously the hydrolysis both egg-PtdCho ($\lambda_{\text{Ex}}/\lambda_{\text{Em}}$: 500/520 nm) and Pyro-C12-PL-BHQ ($\lambda_{\text{Ex}}/\lambda_{\text{Em}}$: 410/675 nm).
2.3. COMPARISONS WITH COMMERCIAL PROBES: PED-6 AND B7701

Invitrogen supplies two enzyme-activated fluorescent phospholipids designed to detect PLA₁/PLA₂ activity. These probes are PED6 [N-((6-(2,4-dinitrophenyl)amino)hexanoyl)-2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-1-hexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt] and B7701 [bis-BODIPY FL C11-PC]. The fluorescence of both probes is detected using $\lambda_{Ex} = 488$ nm and $\lambda_{Em} = 530$ nm.

PED6 is an activatable fluorescent substrate sensitive to the detection of PLA₂. It consists of a BODIPY FL C5 dye conjugated to the sn-2 acyl chain and a dinitrophenyl quencher at the head group [101]. Hydrolysis by PLA₂ results in the separation of the fluorophore from the quencher and the fluorescence is recovered. PED6 has been shown to be sensitive to both sPLA₂ and cPLA₂, as well as platelet-activating factor acetylhydrolase [101].

The other probe, B7701, is designed to be sensitive to both PLA₁ and PLA₂, as it contains two BODIPY FL C11 fatty acids that are conjugated to both the sn-1 and sn-2 positions of glycerophosphocholine. Self-quenching is obtained by the close proximity of the two fluorophores. Cleavage of the probe by either PLA₁ or PLA₂ results in increased fluorescence with the release of two fluorescent products [102].

2.3.1. PED6

We tested the specificity of PED6 with a range of enzymes: PC-PLC, PI-PLC, SMase, PC-PLD, and type IA sPLA₂. TLC analysis after enzyme incubation for 24 h
showed that PED6 could be activated by PC-PLC, sPLA$_2$, and to a lesser extent, SMase (Figure 34).

Figure 34 Specificity of PED6 by TLC.
The hydrolyzed products of PED6 were separated by TLC after 24 h incubation with 1 U en enzyme. (Column 1) Control, (Column 2) PC-PLC, (Column 3) PI-PLC, (Column 4) SMase, (Column 5) PC-PLD, and (Column 6) sPLA$_2$ IB.

The sensitivity of PED6 was analyzed by fluorescence spectroscopy (Figure 35). Enzyme (0.5 U) was added to sonicated lipid dispersions of 1 µM PED6 in egg-PtdCho vesicles at a MF of 0.006 and the time-dependent release of fluorescence was measured. PED6 showed a high sensitivity for sPLA$_2$ IB and PC-PLC, although the reaction with sPLA$_2$ IB was faster. The other phospholipases, PC-PLD, SMase, and PI-PLC, demonstrated no affinity for PED6.
Figure 35 Enzyme specificity of PED6 by fluorescence spectroscopy.

Time-dependent increase in fluorescence of 1 μM PED6 mixed in PtdCho vesicles (MF 0.006) with the addition of 0.5 U of sPLA$_2$ IB, PC-PLC, PC-PLD, SMase, and PI-PLC, as compared to no enzyme.

2.3.2. B7701

An identical fluorescence spectroscopy experiment was performed to test the effectiveness of B7701 as a phospholipase probe (Figure 36). We found that compared to Pyro-PL-BHQ and PED6, this probe had a very low fold increase in fluorescence over background, likely due to the lack of quencher moiety and the poor intramolecular FRET quenching arising from two identical fluorophores. It is possible that at a higher MF, a higher fold increase would be observed due to further intermolecular quenching due to the closer proximity of probes in the PtdCho vesicles.
Figure 36 Enzyme specificity of B7701 by fluorescence spectroscopy.

Time-dependent increase in fluorescence of B7701 (1 μM) mixed in PtdCho vesicles (MF 0.006) with the addition of 0.5 U of sPLA2, PC-PLC, PC-PLD, SMase, and PI-PLC as compared to no enzyme.

2.4. DISCUSSION

These results demonstrate the ability to synthesize NIR intramolecularly-quenched phospholipase probes that have high specificity for a particular isoform of phospholipase. We have shown by TLC and UV HPLC and MALDI-TOF analysis that the first construct to be synthesized, Pyro-PL-BHQ, is highly specific to PC-PLC, yielding a ~150-fold increase in signal:background at MF 0.02 (Figure 11) and a ~40-fold increase in fluorescence at MF 0.003 with 1 U of enzyme (0.002 mg), as shown in Figure 24.

The optimal conditions for performing enzyme kinetic experiments was thoroughly investigated, including the probe/lipid concentrations necessary to reduce quenching effects due to aggregation and the most effective lipid carrier composition for performing enzyme reactions. Additionally, a method was developed to correct for
quenching artifacts due to aggregation at high MFs. Calibration studies were performed to convert fluorescence units to moles, in order to quantify enzyme activity.

In testing probe specificities, we have tried to use mammalian sources whenever possible. However, due to the commercial unavailability of mammalian isoforms, some bacterial isoforms of phospholipases were used. In the particular case of PC-PLC, a mammalian isoform has not yet been cloned. However, there is substantial evidence that a mammalian PC-PLC exists with functional similarity to the bacterial isoform. It has been shown that exposure of eukaryotic cells to *B. cereus* PC-PLC mimics the mitogenic response by activation of protein kinase C (PKC) [103]. Moreover, NIH 3T3 cells transfected with the gene encoding PC-PLC from *B. cereus* induced DNA synthesis and progressed through the cell cycle in the absence of mitogenic stimulus [104]. PC-PLC activity, as measured by PC release from PtdCho, has been demonstrated in cytoplasmic extracts of NIH-3T3 cells [75]. High titer polyclonal antibodies against *B. cereus* PC-PLC were used further to detect the translocation of a cross-reactive mammalian PC-PLC component from the cytosol in control cells to the plasma membrane in oncogene-transformed cells [75]. An increased plasma membrane PC-PLC expression was later shown to accompany tumor progression in ovarian cancer cell lines [105].

Therefore, the *B. cereus* isoform of PC-PLC was used in these enzyme assays and in the kinetic studies in the following chapter to estimate the activity of the mammalian PC-PLC toward Pyro-PL-BHQ. Given that the activity of *B. cereus* PC-PLC toward Pyro-PL-BHQ was much greater than that of *L. monocytogenes* PC-PLC, Pyro-PL-BHQ may have a high specificity for the mammalian isoform, as well.
The second construct to be synthesized, Pyro-C12-PL-BHQ, contains a C12 spacer between Pyro and the sn-2 position of the glycerol backbone, which altered its enzyme specificity. Analysis by TLC and HPLC MALDI-TOF demonstrated that this substrate is highly specific to sPLA2, particularly type IB. Various reaction mixtures were tested in order to optimize conditions for performing enzyme kinetic studies. A ~7-fold increase in signal:background was found with activation at MF 0.003 and 1 U of enzyme (Figure 31). The difference in signal:background for Pyro-C12-PL-BHQ versus Pyro-PL-BHQ is possibly due to more efficient quenching by the Pyro-PL-BHQ construct since, on this molecule, Pyro is in closer proximity to BHQ-3. In the Pyro-C12-PL-BHQ construct, the C12 spacer separates the Pyro fluorophore from BHQ-3, which increases the background fluorescence.

Finally, these in-house phospholipase probes were compared to commercially available phospholipase probes: PED6 and B7701. The substrates synthesized by us proved to be superior in enzyme specificity, as well as signal:background amplification. PED6 was shown to have high specificity for both PLA2 and PC-PLC, which would lead to difficulties in vivo when trying to distinguish these enzyme activities. The signal:background offered by PED6 at MF 0.006 was ~4-fold. Although B7701 may be specific to PLA2, the signal:background amplification was negligible with activation at MF 0.006. When used at higher MFs, intermolecular quenching may lead to higher signal:background amplification, however, at high MFs, it is more difficult to quantify activation because of the intermolecular quenching effects of aggregation.
Overall, these studies provide a method by which future substrates can be characterized and optimized for use *in vitro* and *in vivo*, as well as compared against one another in order to determine their efficiency as phospholipase probes.
CHAPTER 3: KINETICS

Although phospholipases are known to be critically involved in lipid signaling pathways in that they generate lipid second messengers, much is unknown of the mechanism of activity, substrate specificity, and the regulation of activity of these enzymes. Phospholipases are interfacial enzymes, i.e., they act on hydrophobic or amphipathic lipid substrates which aggregate in water. The kinetic reactions of phospholipases, thus, include both a three-dimensional bulk step in which the enzyme first binds to the vesicle surface, and a two-dimensional interfacial step in which the enzyme binds to and hydrolyzes a substrate in the interface. The “surface dilution model” was developed by Dennis and co-workers [106, 107] to incorporate both of these interactions and is summarized in Equation 2.

\[
\begin{align*}
\text{Bulk Step} & \\
E + A & \xrightarrow{k_1} EA & & \text{Interfacial Step} \\
& \xrightarrow{k_{-1}} & \xrightarrow{k_2} EAB & \xrightarrow{k_3} E + A + P
\end{align*}
\]

The surface dilution model can be adapted to all types of interfaces, including micelles, vesicles, liposomes, or membranes. For example, if the enzyme binds non-specifically to the interface, the model is known as the “surface-binding model” and \( A \) represents the sum molar concentration of all components in the surface. Likewise, if the enzyme only binds to a specific substrate, the model is known as the “phospholipid binding model” and \( A \) represents the bulk molar concentration of the substrate [108].

The second surface interaction is comprised of two steps: the surface-associated enzyme first binds to an individual phospholipid molecule \( B \) in the catalytic site to form
the $EAB$ complex and, subsequently, the catalytic turnover is performed regenerating $EA$ and releasing the product $P$ [109, 110]. This two-dimensional surface interaction is highly dependent on the initial surface-binding step and on the local concentrations of substrate at the interface, in that the amount of time spent at the interface and the close proximity of substrate to the enzyme determines the number of catalytic turnover reactions [111-113].

By holding the surface concentration (or mole fraction) constant, the bulk three-dimensional kinetics can be assayed, as the surface dilution model reduces to Michaelis-Menten kinetics. Here, we have used surface dilution kinetics to analyze the enzyme kinetics of PC-PLC on Pyro-PL-BHQ and Pyro-C12-PL-BHQ in a carrier vesicle of PtdCho in order to obtain the maximal reaction velocity ($V_{\text{max}}$), the dissociation constant for binding the enzyme to the vesicle surface ($K_s$), and the interfacial Michaelis constant ($K_m$). Enzyme inhibition was also performed using traditional Lineweaver-Burk plot methods in order to obtain the half maximal inhibitory concentration ($IC_{50}$) and the dissociation constant of the inhibitor ($K_i$).

3.1. PYRO-PL-BHQ

3.1.1. Surface Dilution Kinetics

The surface dilution model, described in more detail in the Methods, was implemented in order to determine the kinetic properties of PC-PLC when hydrolyzing Pyro-PL-BHQ. The kinetic equation for the velocity at steady state is shown in Equation 3,
\[
    v = \frac{V_{\text{max}} X_s S_0}{K_m K_s + K_m S_0 + X_s S_0},
\]

where \( v \) (mole/volume/time) is the measured initial velocity, \( X_s \) (mole fraction, unitless) represents the surface concentration of substrate being hydrolyzed ([Pyro-PL-BHQ]/([Pyro-PL-BHQ]+[PtdCho])), \( S_0 \) (mole/volume) represents the bulk concentration of substrate ([Pyro-PL-BHQ]), \( V_{\text{max}} \) (mole/volume/time) is the true \( V_{\text{max}} \) at infinite \( X_s \) and infinite \( S_0 \), \( K_s \) (mole/volume) is the dissociation constant for binding the enzyme to the vesicle surface, and \( K_m \) (mole fraction, unitless) is the interfacial Michaelis constant.

Thus, in order to carry out a surface dilution kinetic analysis, \( S_0 \) must be varied while at a fixed \( X_s \) (Case I) in order to obtain \( v \) as a function of \( S_0 \), and \( X_s \) must be varied while holding \( S_0 \) constant (Case II) in order to obtain \( v \) as a function of \( X_s \). We have used egg-PtdCho as a carrier lipid in our surface dilution model, which has been thoroughly characterized in Chapter 2 with regards to overcoming probe aggregation and has been shown to be an efficient carrier for enzyme reactions.

3.1.1.1. Bulk Kinetics

In Case I, in order to vary \( S_0 \) while holding \( X_s \) constant, the molar concentrations of egg-PtdCho and Pyro-PL-BHQ were varied proportionally (Figure 37). The bulk lipid concentration was varied for two values of \( X_s \), 0.05 and 0.003. For the data at \( X_s = 0.05 \), a MF cf was applied to correct for aggregate quenching effects according to the method described previously in Chapter 2. The plot of \( v \) as a function of \( S_0 \) was sigmoidal and the data was fit to the Hill equation \( [114, 115] \),

\[
    v = V_{\text{max(app)}} S_0 / \left( K_{m(app)}^h + S_0^h \right),
\]

where \( V_{\text{max(app)}} = V_{\text{max}} X_s / (K_m + X_s) \) and \( K_{m(app)} = K_m K_s / (K_m + X_s) \).
3.1.1.2. Interfacial Kinetics

In Case II, $X_s$ was varied independently of $S_0$ by increasing the concentration of egg-PtdCho from 50 μM to 1 mM, while holding the concentration of Pyro-PL-BHQ constant at 1 μM, thus varying $X_s$ from 0.02 to 0.001 (Figure 38). At high MFs, $X_s > 0.005$, an inner filter correction was applied by dividing the initial rate by the respective MF cf. The initial rate, $v$, was plotted as a function of $X_s$ and fitted to the Michaelis-Menten equation, $v = V_{\text{max}} X_s / (K_m^{(app)} + X_s)$, where $V_{\text{max}}$ is the true $V_{\text{max}}$ at infinite $S_0$ and infinite $X_s$, and $K_m^{(app)} = K_m (K_s / S_0 + 1)$.

The program, KaleidaGraph, was used to obtain the empirical parameters from the fitted data. The parameter $V_{\text{max}}$ was found from Case II to be 160.7 (± 29.2) nmol min$^{-1}$ mg$^{-1}$, which was substituted into the equation for $V_{\text{max}^{(app)}}$ in Case I to yield a value for $K_m$ for both $X_s = 0.05$ and $X_s = 0.003$, where $V_{\text{max}^{(app)}} = 151$ and 79 nmol/min/mg, respectively. A value for $K_s$ was found for both $X_s = 0.05$ and $X_s = 0.003$ by substituting the corresponding value for $K_m$ into the equation for $K_m^{(app)}$ in Case I, where $K_m^{(app)} = 1.90$ and 1.45 μM, respectively. The values for $K_m$ and $K_s$ were similar for both MFs and are reported as the average of the two values: $K_m = 0.003 ± 0.00007$ (mole fraction, unitless), and $K_s = 0.35 (± 0.03)$ μM. The turnover number, $k_{cat}$, was calculated from the equation, $V_{\text{max}} = k_{cat} E_s$, to be 0.045 min$^{-1}$. 

61
Figure 37 Case I: Bulk Kinetics of Pyro-PL-BHQ with PC-PLC.
Total Lipid ([PtdCho] + [Pyro-PL-BHQ]) is varied while holding $X_s$ constant at 0.003 and 0.05.

Figure 38 Case II: Interfacial Kinetics of Pyro-PL-BHQ with PC-PLC.
$X_s$ is varied by increasing [PtdCho] and holding $S_0$ ([Pyro-PL-BHQ]) constant.

3.1.2. Enzyme Inhibition

The activity of PC-PLC on Pyro-PL-BHQ was tested with the competitive enzyme inhibitor, D609, to obtain the IC$_{50}$ and $K_i$. In order to find these parameters, the
substrate concentration and inhibitor concentration were varied to obtain a matrix of kinetic data points. Lineweaver-Burk plots revealed values for $K_{m(app)}$ and $V_{\text{max(app)}}$ at the intersection of the abscissa axis and the ordinate axis, respectively. A replot of $K_{m(app)}/V_{\text{max(app)}}$ versus inhibitor concentration result in a line intersecting the abscissa axis at $-K_i$ [116].

Inhibition of PC-PLC using 100 µM D609, reduced the rate of hydrolysis of Pyro-PL-BHQ, as shown in Figure 39. Varying concentrations of D609 were added to 1 µM Pyro-PL-BHQ in egg-PtdCho dispersions (MF = 0.003) with the addition of 1 U PC-PLC. Activity was measured by fluorescence spectroscopy and the percentage of full activity was plotted as a function of inhibitor concentration. Complete inhibition was achieved with 500 µM D609 and an IC$_{50}$ of 34 ± 7.9 µM was determined (Figure 40).

![Graph showing inhibition of PC-PLC activity towards Pyro-PL-BHQ using D609.](image-url)

**Figure 39** Inhibition of PC-PLC activity toward Pyro-PL-BHQ using D609.

The time-dependent reaction is shown of 1 U PC-PLC added to 1 µM Pyro-PL-BHQ in egg-PtdCho vesicles (MF=0.003) with or without the addition of 100 µM D609.

63
Figure 40 Dose-response curve of Pyro-PL-BHQ activation versus D609 concentration.

Plot of the initial rate of PC-PLC activity toward 1 μM Pyro-PL-BHQ/egg-PtdCho (MF 0.003) versus log[D609] (IC₅₀ = 34 +/- 7.86 μM).

The total lipid concentration ([Pyro-PL-BHQ] + [PtdCho], MF = 0.003) was varied, such that [Pyro-PL-BHQ] was equal to 1, 1.36, 2.15, or 5 μM, and mixed with a constant concentration of D609 (0.008, 0.05 or 0.1 mM), shown in Figure 41.

Lineweaver-Burk plots of 1/ν versus 1/S₀ revealed kinetics consistent with a mixed substrate solution (Figure 42), i.e., $V_{\text{max(app)}}$ varies, as well as the apparent $K_{m(app)}$. This is to be expected, since the reaction contains two substrates of PC-PLC, PtdCho and Pyro-PL-BHQ. A plot of $K_{m(app)}/V_{\text{max(app)}}$ versus [D609] was used to determine a $K_i$ of 59 μM (Figure 43).
Figure 41 D609 Inhibition of bulk kinetics of Pyro-PL-BHQ with PC-PLC.
Plot of $v$ versus $S_0$ at $[D609] = 0.1$ mM (diamonds), $[D609] = 0.05$ mM (squares), and $[D609] = 0.008$ mM (triangles).

Figure 42 Lineweaver-Burk plots of D609 inhibition of PC-PLC activation of Pyro-PL-BHQ.
Plots of $1/v$ versus $1/S_0$ to determine $V_{\text{max(app)}}$ and $K_{m(app)}$ at $[D609] = 100$ μM (diamonds), $[D609] = 50$ μM (squares), and $[D609] = 8.3$ μM (triangles).
3.2. **PYRO-C12-PL-BHQ**

3.2.1. **Surface Dilution Kinetics**

A Case I bulk kinetic analysis of Pyro-C12-PL-BHQ activation by sPLA2 IB was performed by Melissa Love (Figure 44). Pyro-C12-PL-BHQ was suspended in egg-PtdCho vesicles at $X_s = 0.003$ and the total lipid ([Pyro-C12-PL-BHQ]+[PtdCho]) was varied, such that $S_0$ ([Pyro-C12-PL-BHQ]) ranged from 0 – 5 μM. A Lineweaver-Burk plot (Figure 45) of this data was used to determine a $V_{\text{max(app)}} = 100.5$ pmol/min/unit and $K_{m(app)} = 12.3$ μM.
Figure 44 Case I: Bulk Kinetics of Pyro-C12-PL-BHQ with sPLA$_2$ IB.
Total Lipid ([PtdCho] + [Pyro-PL-BHQ]) is varied while holding $X_s$ constant at 0.003.

Figure 45 Lineweaver-Burk Plot of Pyro-C12-PL-BHQ Bulk Kinetics with sPLA$_2$ IB.
Plot of $1/v$ versus $1/S_0$ (with inset) for Pyro-C12-PL-BHQ in egg-PtdCho vesicles at $X_s = 0.003$ yields a $V_{\text{max(app)}}$ of 100.5 pmol/min/unit and $K_{m(app)} = 12.3$ µM.

$y = 122.7x + 9.9456$
3.3. DISCUSSION

In order to compare enzyme activities across various phospholipase probes, it is helpful to calculate the Michaelis-Menten parameters, \( V_{\text{max}} \) and \( K_m \), for each enzyme-substrate pair. The Michaelis-Menten analysis relates the initial reaction rate \( v \) to the substrate concentration \([S]\), where \( V_{\text{max}} \) is the maximum reaction rate at saturating \([S]\) and \( K_m \) is the concentration of substrate when \( V_{\text{max}} / 2 \) is achieved.

The enzyme kinetics of phospholipases adds an element of complexity because they interact on the surface of lipid bilayers or micelles. Therefore, a normal Michaelis-Menten kinetic analysis is not suitable. To overcome this problem, the surface dilution method was developed, which allows for the characterization of both the bulk kinetic reaction, where the enzyme first binds to the surface of the bulk lipid, and the interfacial kinetic reaction, where the enzyme interacts with a substrate along the surface. Here, we have utilized this method to calculate the surface dilution kinetic parameters for Pyro-PL-BHQ/PC-PLC: \( V_{\text{max}} = 160.7 \pm 29.2 \text{ nmol min}^{-1} \text{ mg}^{-1} \), \( K_m = 0.003 \pm 0.00007 \) (mole fraction, unitless), \( K_s = 0.35 \pm 0.03 \) \( \mu \text{M} \), and \( k_{\text{cat}} = 0.045 \text{ min}^{-1} \). Preliminary analysis of Pyro-C12-PL-BHQ/sPLA2 (type IB) bulk surface dilution kinetics (Case I) yielded \( V_{\text{max(app)}} = 100.5 \text{ pmol/min/unit} \) and \( K_{m(app)} = 12.3 \) \( \mu \text{M} \). These results are very different from the Case I results found for Pyro-PL-BHQ/PC-PLC (\( V_{\text{max(app)}} = 151 \) and 79 nmol/min/mg and \( K_{m(app)} = 1.90 \) and 1.45 \( \mu \text{M} \)).

An inhibition study using D609 to inhibit PC-PLC activity toward Pyro-PL-BHQ was performed at a mole fraction of \( K_m \), yielding IC\(_{50} \) of 34 ± 7.9 \( \mu \text{M} \). The \( V_{\text{max(app)}} \) and
\(K_{m(app)}\) was found under bulk kinetics for a range of D609 concentrations, which were used to determine a \(K_i\) of 59 \(\mu\)M.

Traditionally, mixed micelles containing an uncharged detergent, such as Triton X-100, have been usefully employed. Neutral diluters are often used because they generally do not interact with the enzyme, and they do not dramatically affect the physical properties of the surface. By simply increasing the concentration of surfactant, \(X_s\) can be systematically varied independently of \(S_0\) and solutions for the kinetic parameters, \(K_m\), \(K_s\), and \(V_{max}\), can be determined.

However, in the case of enzyme reactions involving the hydrolysis of Pyro-PL-BHQ by PC-PLC, our preliminary studies have shown that Pyro-PL-BHQ/detergent-mixed micelles affected the catalytic activity. We found that even small quantities of Triton X-100 (Figure 21) or Tween-80 greatly inhibited the reaction. The reason for this is unknown, but it is presumed that detergent has an effect on the conformation of Pyro-PL-BHQ, which prevents it from being hydrolyzed. Nevertheless, this has prevented us from performing surface dilution kinetics under traditional conditions.

The use of phospholipids as a carrier vesicle has proven to be a suitable substitution for Triton X-100. We found that reactions were greatly improved when using carrier vesicles composed of a mixture of lipids, or contained 20 - 30 % cholesterol (Figure 22 and Figure 23). This result is supported by several studies which have found that PC-PLC activity is highly sensitive to the physical properties of the bilayer [117-119]. It has previously been found that PC-PLC activity on egg-PtdCho bilayers is
enhanced dose-dependently upon the addition of up to ~12 % cholesterol or PtdEtn, and that a "burst" in activity occurs after the production of ~10 % DAG, presumably due to structural defects created in the liposome [119]. We have found superior activity of PC-PLC toward Pyro-PL-BHQ when using egg-PtdCho as a carrier vesicle (Figure 24).

Thus, we have settled on using egg-PtdCho as a carrier lipid in our surface dilution model for PC-PLC acting on Pyro-PL-BHQ, due to the excellent activity observed in our preliminary studies. Despite the fact that it is not an ideal model, PtdCho is the most abundant lipid found in biological membranes, and egg-PtdCho has been widely studied as a membrane model. Egg-PtdCho vesicles contain a mixture of saturated and unsaturated phosphatidylcholine molecules varying in chain length, which are known to form an asymmetric transmembrane lipid packing geometry [120]. Perhaps this asymmetry allows the Pyro-PL-BHQ to be presented to the enzyme in a more favorable position for hydrolysis.

These calculated parameters are helpful in determining optimal reaction conditions and the effectiveness of enzyme inhibitors. Furthermore, the ability to calculate kinetic parameters for phospholipase activities toward self-quenched lipid substrates allows for a more effective determination of whether newly synthesized probes meet sensitivity requirements as successful in vivo molecular beacons.
CHAPTER 4: CELL STUDIES

The use of cultured cells facilitates the optimization of delivery vehicles and the assessment of toxicity, as well as the determination of cell types that can internalize and activate these fluorescent substrates. In this chapter, the uptake of fluorescent substrates was investigated using the DU145 prostate cancer cell line. More specifically, the intracellular location of probe accumulation and phospholipase activity were determined by analyzing the uptake of Pyro-PL and Pyro acid by confocal microscopy.

PC-PLC activity in cells was monitored using the PC-PLC-sensitive probe, Pyro-PL-BHQ. These results were compared to the fluorescence observed using the PLA_2/PC-PLC-sensitive probe, PED6. Enzyme activity was modulated by treating cells with phenylbutyrate (PB), which has been previously studied in our group and known to up-regulate lipid metabolic changes in DU145 cells [68, 121]. Finally, in order to deconvolve PC-PLC activity from PLA_2 activity in cells treated with PED6, the activities of both cPLA_2 and iPLA_2 were potently inhibited by arachidonyl trifluoromethyl ketone (AACOCF3).

4.1. CELLULAR UPTAKE OF LIPID PROBES

The first objective was to determine the ability DU145 cells to take up Pyro-labeled substrates. Two unquenched compounds were tested, Pyro acid and Pyro-PL. We wished to determine whether uptake of the phospholipid construct, Pyro-PL, would be different from that of the free fluorophore. After being incubated for 1 h with Pyro acid or Pyro-PL (5 μM), DU145 cells exhibited a very strong fluorescence, localized in the cytoplasm and perinuclear region (Figure 46A). The fluorescent images were taken over
a span of several wavelengths from 660 – 714 nm (Figure 46B). The strongest signal was seen between 671 and 681 nm, demonstrating that the Pyro signal spectrum does not shift upon entering cells (how do we know it is in the nuclear membrane, In retrospect, it would have been interesting to use lower concentrations).

Figure 46 Confocal images of Pyro-PL and Pyro acid delivered to DU145 cells. (A) DU145 cells after being incubated for 1 h with Pyro acid or Pyro-PL (5 μM); (B) fluorescence spectrum of DU145 cells incubated with Pyro-PL.
In cells incubated with Pyro acid for 24 h (Figure 47B) versus 3 h (A), fluorescence was perinuclear, highly concentrated around the nuclear membrane. Also, after 24 h, fluorescence appeared punctate in some regions and was coincident with vesicles that could be seen in the respective bright-field images.

Figure 47 DU145 cells stained with Pyro acid.
DU145 cells incubated with Pyro acid for (A) 3 h or (B) 24 h. Fluorescence images are shown on the left with the respective bright field image on the right.

4.2. ACTIVATION OF LIPID PROBES IN CELLS

4.2.1. Confocal Imaging of PC-PLC using Pyro-PL-BHQ

Our goals in investigating the activation of Pyro-PL-BHQ in DU145 cells were to 1) detect any intrinsic PC-PLC activity within these cells, and 2) to modulate PC-PLC activity in these cells and the subsequent activation of Pyro-PL-BHQ. Therefore, in order to investigate the activation of Pyro-PL-BHQ in cells under different conditions of phospholipase activity we treated DU145 cells with the differentiating agent, phenylbutyrate (PB). In perfused DU145 cells observed by $^1$H- and $^{31}$P-NMR, PB (10
mM) was found to induce increases in PtdCho metabolites and mobile lipids, indicating an upregulation of phospholipase activity. These effects were accompanied by a significant increase in cytoplasmic lipid droplets as observed by morphometric analysis of Oil Red O-stained cells. Figure 48 shows the paired fluorescence and bright-field confocal microscopy images of DU145 cells incubated with Pyro-PL-BHQ for (A) 3 h or (B) 24 h, PB-treated cells incubated with Pyro-PL-BHQ for (C) 3 h or (D) 24 h, or (E) control cells not incubated with Pyro-PL-BHQ.

Figure 48 DU145 control and PB-treated cells incubated with Pyro-PL-BHQ.

Images are shown as pairs of fluorescence and bright field images. Cells were pre-treated for 16 h with (A, B, E) saline or (C, D) with PB (10 mM), followed by incubation with Pyro-PL-BHQ for (A, C) 3 h or (B, D) 24 h., or (E) no fluorescent substrate as a control.

The fluorescence pattern observed in cells incubated for 3 h (A) was concentrated mostly in the perinuclear region. After 24 h, fluorescence can be seen diffusing outward.
into other areas of the cytoplasm. The fluorescence was also punctate in areas coincident with vesicles observed in the bright-field image. In PB-treated cells, the cytoplasmic punctate staining pattern is more pronounced and is coincident with the increase in lipid droplets visible in the respective bright-field image. In PB-treated cells incubated with Pyro-PL-BHQ for 24 h, the perinuclear fluorescence has increased, while in regions of many lipid droplets, the fluorescence is brighter and more diffuse. Cells not incubated with Pyro-PL-BHQ did not display any fluorescence.

4.2.2. Confocal Imaging of PLA₂ and PC-PLC using PED6

In order to better understand these results and validate that the fluorescence observed using Pyro-PL-BHQ was due to PC-PLC activity, we also incubated control and PB-treated DU145 cells with the BODIPY-labeled phospholipase probe, PED6. Although PED6 has been reported to be a specific cPLA₂ probe [40], our in vitro studies have shown that PED6 is activatable by both PLA₂ and PC-PLC. Therefore, we expected that when incubated with PED6, fluorescence would be released as a result of both PLA₂ and PC-PLC activities. We then investigated the effect of PLA₂ inhibition using the cPLA₂ and iPLA₂ inhibitor, AACOCF3. Figure 49 shows the paired fluorescence and bright-field confocal microscopy images of DU145 control cells incubated for 3 h with PED6 (A) with no inhibitor, (B) with the addition of 10 μM AACOCF3, or (C) 100 μM AACOCF3; and PB-treated cells incubated for 3 h with PED6 (D) with no inhibitor, (E) with the addition of 10 μM AACOCF3, or (F) 100 μM AACOCF3.

Unlike cells incubated with Pyro-PL-BHQ, the fluorescence pattern observed in control cells incubated with PED6 without inhibitor (A) was diffuse and extended...
throughout the cytoplasm. With the addition of 10 μM AACOCF3 (B), the fluorescence signal was weaker and more perinuclear. With the addition of 100 μM AACOCF3 (C), fluorescence was only observed in the perinuclear region and cytoplasmic fluorescence was diminished. In PB-treated cells incubated with PED6 without inhibitor (D), cytoplasmic fluorescence is stronger and pronounced in areas where many lipid droplets are present. With the addition of 10 μM AACOCF3 (E), although the number of lipid droplets is decreased, the fluorescence is highly localized in the lipid droplets present and also in the perinuclear region. With the addition of 100 μM AACOCF3 (F), although some lipid droplets are still visible in the bright-field image, they are not fluorescent. Fluorescence is only observed in the perinuclear region.

Figure 49 DU145 control and PB-treated cells incubated with PED6 ± PLA2 inhibitor
Images are of paired fluorescence and bright-field confocal microscopy images of DU145 control cells incubated with PED6 (A) with no inhibitor, (B) with the addition of 10 μM AACOCF3, or (C) 100 μM AACOCF3; and PB-treated cells incubated with PED6 (D) with no inhibitor, (E) with the addition of 10 μM AACOCF3, or (F) 100 μM AACOCF3.
4.3. Validation of PC-PLC Activity in DU145 Cell Extracts

4.3.1. Amplex Red Assay for PC-PLC Activity

In order to validate the presence of PC-PLC activity in DU145 cells, the Amplex Red PC-PLC Assay Kit (Invitrogen, Eugene, OR) was used to measure PC-PLC activity in whole cell extracts. PC-PLC activity (RFU/min) was shown to increase linearly as a function of total supernatant protein (Figure 50) as detected by increases in resorufin fluorescence.

![Graph showing the relationship between PC-PLC activity and protein concentration. The equation y = 380.32x + 9.6317 is given.]

Figure 50 Amplex Red assay of PC-PLC activity in DU145 whole cell extracts.
Whole cell extracts of DU145 cells were prepared and tested for PC-PLC activity using the Amplex Red PC-PLC assay kit.

4.3.2. $^{31}$P-MRS Assay for PC-PLC Activity

The presence of PC-PLC activity in DU145 cell extracts was also measured using $^{31}$P-MRS by observing the conversion of PtdCho to PC with the addition of DU145 whole cell extracts. Figure 51 shows the $^{31}$P-MR spectra of sonicated egg-PtdCho.
vesicles (A) after incubation at 37°C with PC-PLC, (B) DU145 extracts, (C) DU145 extracts ± D609, or (D) vesicles alone. $^{31}$P-MRS of PtdCho vesicles shows a strong resonance at 3.8 ppm. Incubation of PtdCho with PC-PLC resulted in a decrease in PtdCho and an increase in PC at 6.4 ppm. Incubation with cell extracts yielded the same decrease in PtdCho and increase in PC, and D609 inhibited this conversion, validating the presence of PC-PLC activity in DU145 cells.

![Figure 51 $^{31}$P-MRS Spectra.](image)

Sonicated PtdCho vesicles after incubation at 37°C for 2 h with 2 U PC-PLC (A), with whole cell DU145 extracts (B), with whole cell DU145 extracts and D609, a specific inhibitor of PC-PLC (C), compared to baseline (D).

**4.4. DISCUSSION**

The results of these studies demonstrate: 1) the ability for DU145 cells to internalize Pyro-labeled self-quenched phospholipase probes, 2) that the phospholipase probes are activated by specific phospholipases in DU145 cells, and 3) that modulation of
phospholipase activity with a differentiating drug or phospholipase inhibitor can be
detected using these probes.

The fluorescence observed in cells incubated with Pyro-PL-BHQ exhibited an 8-fold weaker signal than those incubated with Pyro-PL or Pyro acid, likely due to the efficiency of BHQ as a fluorescence quencher. The intracellular patterns of fluorescence observed in these experiments indicate the action of phospholipases on these fluorescent substrates. Previous studies have shown that incubation of Chinese hamster V79 fibroblasts at 2°C with fluorescent analogs of PtdCho, PtdEtn and SM results in fluorescent staining that is restricted to the plasma membrane [122]. This group also showed that when these cells were incubated with a fluorescent analog of PtdCho, followed by treatment with PC-PLC, fluorescence labeling of intracellular membranes was observed [123]. Identical results were obtained in cells incubated with a fluorescent analog of DAG. Therefore, it is highly unlikely that the observed Pyro fluorescence is solely background fluorescence from uncleaved substrate, as it is not localized in the plasma membrane. The localization of Pyro-PL indicates that this substrate is also being metabolized to 1-acyl-2-pyropheophorbol glycerol.

In control cells incubated with PED6, increased inhibition of PLA₂ by AACOCF₃ caused an expected change in the observed fluorescence: more concentrated in the perinuclear region and resembling that observed in cells incubated with Pyro-PL-BHQ. This is particularly evident by comparing Figure 49C and Figure 48A, which although faint, indicate the localization of native PC-PLC activity in this region. These fluorescence images are consistent with those observed by Ramoni et al. [124] who have
used high titer polyclonal antibodies, raised in a rabbit against *B. cereus* PC-PLC, to detect PC-PLC in NIH-3T3 fibroblasts. They have reported that fixed, quiescent fibroblasts incubated with the anti-PC-PLC antibodies and imaged by indirect immunofluorescence epimicroscopy express a fluorescent pattern that is confined to the perinuclear region.

The cytoplasmic fluorescence signal seen in DU145 control cells incubated with PED6, and more intensely in PB-treated cells (Figure 49A, D) is reduced with PLA2 inhibition and is relatively undetectable in cells incubated with Pyro-PL-BHQ (Figure 48A). This indicates that the cytoplasmic fluorescence is due to BODIPY fatty acid, which is released from PED6 by the action of PLA2. This result is consistent with a study in which human embryonic kidney 293 (HEK293) cells were labeled with PED6 and treated with a purified recombinant human group V PLA2 (hPLA2 V) [125]. In this study, confocal microscopy was used to show initial BODIPY fluorescence of intact PED6 at the plasma membrane reaching a peak signal at ~2 min, followed by the appearance of signal at the nuclear envelope reaching a plateau at ~4 min, and, finally, a rise in cytoplasmic signal continuing up to 6 min. The resulting diffuse cytoplasmic fluorescence was believed to be the result of the diffusion of the short-chain BODIPY fatty acid from the nuclear membranes.

It is interesting that in PB-treated cells incubated with Pyro-PL-BHQ, fluorescence was concentrated in cytoplasmic lipid droplets, as well as in PB-treated cells incubated with PED6 with partial inhibition of PLA2 (10 μM). From this, we can hypothesize that either the action of PC-PC occurs at the surface of the lipid droplet, or
that 1-acyl-2-pyropheophorbol glycerol, the fluorescent product released by PC-PLC, preferentially incorporates into lipid droplets. This metabolite is a fluorescent analog to diacylglycerol (DAG), which is known to be a key participant in the formation of lipid droplets as the direct metabolic precursor to triacylglyceride (TAG), so there is evidence that supports this hypothesis. It is known that lipid droplets are stores for neutral lipids consisting largely of TAG and cholesteryl esters surrounded by a phospholipid monolayer. The synthesis of TAG is catalyzed by the action of diacylglycerol acyltransferases, which use acyl-CoA as acyl donor and DAG as acceptor. Thus, it is possible that the fluorescent DAG analogs are being converted to TAG in this manner, causing fluorescence to accumulate on lipid droplets. However, a recent study following TAG biosynthesis and targeting using fluorescence imaging in living 3T3-L1 adipocytes and COS7 fibroblasts found that DAG also accumulates on the surface of lipid droplets [126].

There are two main observations in PB-treated cells incubated with PED6 and with 100 μM AACOCF3: 1) there are fewer lipid droplets, and 2) although lipid droplets are still visible in the bright-field image, they are not fluorescent (Figure 49F). Rather, the fluorescence is more concentrated surrounding the nuclear envelope, as it is in cells not treated with PB. One possible reason for this is that with the inhibition of PLA2, there is a reduction in available free fatty acids, which are necessary to convert DAG to TAG. Yet, a recent study suggests that cPLA2, specifically group IVA cPLA2α, plays a larger role in the biogenesis of lipid droplets than simply generating fatty acids for TAG and cholesteryl ester synthesis [127]. It was shown that, although exogenous fatty acids
(arachidonic acid, oleate or palmitate) induced the appearance of lipid droplets, this action could be prevented with PLA$_2$ inhibition. It seems from our results that PLA$_2$ inhibition prevents the incorporation of fluorescent DAG analogs into lipid droplets.

PC-PLC activity was detected in DU145 whole cell extracts using the Amplex Red assay for PC-PLC and, also, by observing with $^{31}$P-MRS the conversion of egg-PtdCho to PC with the addition of whole cell extracts. Taken together, the results here offer evidence that PC-PLC is present and active in DU145 cells, and that Pyro-PL-BHQ is hydrolyzed by PC-PLC to 1-acyl-2-pyropheophorbol glycerol, which is then translocated to intracellular membranes. A study to further validate these results would be to incubate DU145 cells with Pyro-PL-BHQ in the presence of D609 in order to observe the effect of PC-PLC inhibition. We would expect that with PC-PLC inhibition, a background fluorescent signal would be observed at the plasma membrane due to intact substrate with little intracellular signal.
CHAPTER 5:  **IN VIVO IMAGING**

The application of fluorescent molecular beacons to *in vivo* imaging is very exciting in that it allows diagnostic information to be obtained noninvasively. The main objectives in performing *in vivo* experiments were to 1) demonstrate efficient delivery of the probe to the target tissue (tumor) in order to make it available for activation; 2) determine probe biodistribution; 3) demonstrate the ability for probes to be activated *in vivo*; and 4) demonstrate that activation is due to specific enzymes, determined by using specific enzyme inhibitors.

The accomplishment of these objectives is a key step in developing the phospholipase probes described in this work as suitable candidates for *in vivo* molecular imaging. We have employed for these purposes the tumor xenograft animal model derived from the DU145 cell model studied in Chapter 4. DU145 cells were grown as xenografts in the left hind flanks of nude mice. The permanently fluorescent analog, Pyro-PL, was first used to determine efficient probe tumor delivery and biodistribution. The PC-PLC-specific probe, Pyro-PL-BHQ, was then utilized to image PC-PLC activity *in vivo*, particularly in the tumor. The activation of Pyro-PL-BHQ by PC-PLC was validated using the PC-PLC inhibitor, D609. Preliminary work has also been done to investigate the *in vivo* activation of the sPLA$_2$ IB-specific probe, Pyro-C12-PL-BHQ.

### 5.1. PYRO-PL-BHQ

#### 5.1.1. Delivery and Biodistribution

We have chosen to use a DU145 human prostate tumor xenograft model in nude mice to investigate the effectiveness of Pyro-PL-BHQ as a tumor-targeting phospholipase
imaging agent. The design of Pyro-PL-BHQ, itself, allows us to take advantage of several tumor characteristics, i.e. it is lipophilic and it contains a porphyrin fluorophore, which contributes to the preferential probe uptake by tumors. Porphyrins, such as Pyro, have an inherent ability to passively target tumors. It is well established that porphyrin derivatives have a high affinity for tumorigenic cells and solid tumors [128-130]. The reason for tumor preference is not fully understood. However, a combination of several tumor characteristics is implicated in tumor selectivity, including a large interstitial space, leaky vasculature, and compromised lymphatic drainage, which allows for increased tumor permeation and greater probe retention. Furthermore, tumors have high levels of newly formed collagen, which binds to porphyrins [131-133] and a large quantity of lipid. Lipid binds lipophilic photosensitizers, so the affinity of the probe for tumors increases with the degree of probe hydrophobicity [134]. The acidic environment of tumor tissue also increases lipophilicity and, thus, tumor specificity. Thus, lipid constructs, such as Pyro-PL-BHQ, benefit from these tumor characteristics and offer a special advantage in tumor imaging.

As a permanently fluorescent analog to Pyro-PL-BHQ, Pyro-PL was utilized to determine the efficacy of probe delivery to the tumor and the differences in probe biodistribution associated with delivery vehicle. For these experiments, 80 nmol of Pyro-PL was dissolved in either 0.1% Tween-80 or PtdCho (MF = 0.001) and injected via the tail vein into nude mice bearing subcutaneous DU145 tumors on the left hind flank. Images were taken frequently from 0 to 6 h after injection. The average radiance was measured from both the tumor and normal muscle tissue over time. Regardless of delivery vehicle, Pyro-PL was observed in the tumors within 20 min. Although the use of
PtdCho vesicles resulted in slower overall accumulation and clearance than Tween-80 delivery, both methods reached maximum tumor accumulation between 1 – 2 h post-injection and were nearly, if not completely, cleared from the tumor by 6 h (Figure 52A – C). The tumor/muscle ratio shows that accumulation occurred similarly for both delivery vehicles (Figure 52D).

![Figure 52 Comparing Tween-80 and PtdCho as delivery vehicles.](image)

(A) Images of mice at prescan (before injection) at ~1 h and ~6 h after i.v. injection of Pyro-PL delivered in 0.1 % Tween-80 or (B) PtdCho (MF 0.001), (C) measurement time courses for both delivery methods, and (D) tumor/muscle ratios over time for both delivery methods (error bars represent standard deviation).

Although both delivery vehicles led to efficient tumor probe accumulation, the use of Tween-80 micelles was chosen for future studies, since micelles are generally favored for their small size. Micelles are known to be superior to liposomes in their ability to accumulate in the tumor interstitial space and penetrate the leaky tumor vascular endothelium [135, 136].

85
Using the same DU145 tumor xenograft model, Pyro-PL was delivered i.v. in Tween-80 and fluorescence images were acquired over the first 6 hours and followed up the next day between 24h and 30 h. As shown in Figure 53, Pyro-PL exhibited fast circulation throughout the body, accumulating primarily in the tumor, liver, stomach and intestines. By 6 h, the substrate was being cleared via the digestive tract, indicated by the bright intensity accumulating in the intestines.

Figure 53 In vivo imaging of Pyro-PL in DU145 tumor-bearing nude mouse. Location of tumor is circled in red. Mice were injected i.v. with 80 nmol of Pyro-PL. Fluorescence images were acquired using the Cy5.5 filter set. Time points are noted in the top left corner of each image.

The fluorescence-time curve of average radiance originating from the tumor and contralateral muscle tissues after i.v. injection of Pyro-PL is shown in Figure 54. As seen in the insert, the tumor and muscle tissues both reached a maximum tumor radiance of
~10 fold over baseline between 30 min and 2 h. The probe accumulated preferentially in the tumor tissue with a tumor/muscle ratio of ~1.5 at peak accumulation time. The probe exhibited a fast washout period between 2.5 h and 3 h as the fluorescent signal fell steeply during this time. By 24 h, the substrate was cleared from the mouse and fluorescence had returned to prescan levels.

Figure 54 Time-dependent tumor and muscle Pyro-PL measurements. Average measurements after i.v. injection of 80 nmol Pyro-PL. Error bars represent standard error (n=5).

In order to analyze the biodistribution of Pyro-PL at peak tumor accumulation time, the organs were harvested at 2 h post-injection of Pyro-PL and imaged in a 24-well plate. The total flux (photons/sec) in each organ was compared to the background flux due to autofluorescence in organs taken from a control mouse that received no fluorescent substrate. The largest increases in Pyro-PL fluorescence over control were found in the liver (6-fold). In the Pyro-PL treated mouse, a tumor/muscle ratio of 2 was calculated from the tissue harvested at 2 h post-injection.
Figure 55 Biodistribution of Pyro-PL.

(A) Fluorescence in ex vivo organs at 2 h post-injection of Pyro-PL (top) as compared to background autofluorescence (bottom). In each plate, (A,3) adrenal, (A,5) kidneys; (B,2) liver, (B,4) spleen, (B,6) heart; (C,7) intestines, (C,3) lung, (C,5) muscle; (D,2) stomach, (D,4) tumor, (D,6) pancreas. (B) Quantified fluorescence in organs from (A) at 2 h post-injection of Pyro-PL (red) compared to background autofluorescence (blue).
5.1.2. Enzyme Activation and Inhibition

Assuming that Pyro-PL-BHQ has the same biodistribution as its permanently fluorescent analog, Pyro-PL, we predicted that Pyro-PL-BHQ would be well circulated throughout the body, and be taken up by the tumor. We injected DU145 tumor-bearing nude mice with Pyro-PL-BHQ. Since Pyro-PL-BHQ itself is dark, any increase in fluorescence should be due to the increase of Pyro-containing hydrolyzed product. An increase in radiance was observed, specifically in the tumor, over the first 6 h post-injection (Figure 56 A).

In order to validate that the increase in tumor radiance after Pyro-PL-BHQ injection was induced by PC-PLC activation, we inhibited probe activation using D609. Due to its short half-life in vivo [137, 138], we administered D609 (50 μg/g body weight, intraperitoneally) to mice 30 min prior to injection of Pyro-PL-BHQ, as well as 30, 60, and 120 min post-injection. As expected, D609 treatment caused an overall attenuation of tumor radiance as soon as 2 h after injection (Figure 56 B) and continued to suppress probe activation until clearance.
Figure 56 *in vivo* imaging of PC-PLC activation and inhibition with Pyro-PL-BHQ.

Representative *in vivo* imaging of DU145 tumor-bearing nude mice injected with (A) Pyro-PL-BHQ and (B) Pyro-PL-BHQ + D609 treatment

From the mice in Figure 56, the fluorescence-time curve of average radiance was measured from the tumor and contralateral muscle tissues and this data is shown in Figure 57. Pyro-PL-BHQ tumor radiance gradually increased ~4-fold over baseline during the first 4 h and was maintained continually up to 24 h before beginning to clear from the tumor (Figure 57 A). From 2 – 24 h, the tumor radiance increases to about 2.5-fold greater than in the contralateral muscle. Pre- and post-treatment with D609 caused an overall attenuation of tumor radiance as soon as 2 h after injection, and continued to suppress probe activation until clearance at 28 h (Figure 57 B). Note that the maximum Pyro-PL radiance detected *in vivo* is about 4-fold higher than that of the cleaved Pyro-PL-
BHQ radiance, which suggests that complete hydrolysis of Pyro-PL-BHQ is not being achieved or that residual quenching effects from cleaved BHQ-3 remain.

Figure 57 Time-dependent Pyro-PL-BHQ fluorescence ± D609 treatment.
Average radiance was quantified from the tumor and contralateral muscle tissue after i.v. injection of 80 nmol Pyro-PL-BHQ (A) without D609 treatment or (B) with D609 treatment.
After *in vivo* imaging, the tumor and contralateral muscle were excised and imaged. Figure 58 shows a side-by-side comparison of tissue samples taken mice injected with Pyro-PL, Pyro-PL-BHQ and Pyro-PL-BHQ with D609 treatment. Samples imaged from mice injected with Pyro-PL exhibited low tumor radiance relative to contralateral muscle, confirming effective washout by 28 h post-injection Figure 58 A. In contrast, Figure 58 B shows a 5-fold increase in tumor radiance relative to the contralateral muscle in a sample excised at 30 h post-injection of Pyro-PL-BHQ. As shown in Figure 58 C, we found that D609 significantly reduced tumor radiance to less than half of that observed without D609 treatment.

**Figure 58** Comparison of excised tumor and contralateral muscle tissue.

Fluorescence images were acquired from *ex vivo* tumor and muscle tissues (A) at 28 h post-injection of Pyro-PL, (B) at 30 h post-injection of Pyro-PL-BHQ and (C) at 31 h post-injection of Pyro-PL-BHQ + D609 treatment.
Tumor:muscle average radiance ratios were calculated \textit{in vivo} over the time course of these experiments and these results are presented in Figure 59. At 24 h, the tumor:muscle ratio of mice injected with Pyro-PL-BHQ was significantly higher than that of mice injected with Pyro-PL. Furthermore, D609 treatment had significantly inhibited the activation of Pyro-PL-BHQ (p<0.05) by 24 h, decreasing the tumor:muscle ratio back to baseline.

![Figure 59 Tumor:Muscle average radiance.](image)

The average tumor radiance was normalized to muscle at 10 min, 1 h, 5 h and 24 h post-injection of Pyro-PL (n=5), Pyro-PL-BHQ (n=5) and Pyro-PL-BHQ + D609 treatment (n=3). Error bars represent s.d. * Significantly (p=0.011) different than Pyro-PL. † Significantly (p=0.024) different than Pyro-PL-BHQ without D609 treatment.

5.1.3. Validation of PC-PLC activity in extracts of DU145 tumor xenografts

To validate the presence of PC-PLC activity in DU145 tumor xenografts, the Amplex Red PC-PLC Assay Kit (Invitrogen, Eugene, OR) was used to measure PC-PLC activity in extracts of excised tumors. PC-PLC activity was shown to increase linearly as
a function of total supernatant protein (Figure 60A) as detected by increases in resorufin fluorescence. This activity was shown to decrease as a function of D609 concentration (Figure 60B).

![Graph](image)

**Figure 60 PC-PLC activity measured by Amplex Red.**
(A) Activity (RFU/min) measured as a function of protein (mg) from extracts of DU145 tumor xenografts. (B) Inhibition of PC-PLC activity in supernatants by D609.

5.2. **PYRO-C12-PL-BHQ**

5.2.1. **Preliminary In Vivo Imaging**

The activation of Pyro-C12-PL-BHQ was also tested *in vivo* in DU145 tumor-bearing nude mice and compared to the results obtained with Pyro-PL-BHQ. Our *in vitro* experiments have shown that Pyro-C12-PL-BHQ is highly specific to sPLA₂ IB (Figure 27), which is purified primarily from the pancreas. It is synthesized in pancreatic acinar cells and secreted into the pancreatic juice where its primary function is for the digestion of dietary phospholipids [139]. Therefore, it would be expected that this probe might not show fluorescence enhancement in the tumor, but rather in the digestive organs. We injected nude mice bearing DU145 tumors *i.v.* with 80 nmol of Pyro-C12-PL-BHQ and fluorescence images were acquired over a 24 h period (Figure 61). We found a small global increase in fluorescence, likely due to the background fluorescence of the
substrate, over the whole body within 5 min. By 1 h post-injection, fluorescence in the skin and muscle tissue had dissipated, leaving concentrations of fluorescence in the region of the adipose tissue, tumor tissue, and liver and small intestine. By 5 h post-injection, fluorescence in the tumor and adipose tissues had decreased while, in regions of the liver and small intestine, fluorescence had increased. At 24 h post-injection, fluorescence was 6 fold higher in the digestive tract organs than in the peripheral skin and muscle tissues as the substrate was cleared from the body and hydrolyzed.

Figure 61 In vivo imaging after injection of Pyro-C12-PL-BHQ.

The substrate is not cleaved by PC-PLC and does not show activation in the tumor. By 24 h, it is nearly all cleared from the tumor tissue and is being eliminated via the digestive tract.

5.3. DISCUSSION

These results demonstrate that Pyro-labeled phospholipase probes can be efficiently delivered to tumors, that these probes can be activated by specific enzymes in vivo, and that probe activation can be modulated with enzyme inhibition.

The permanently-fluorescent probe analog, Pyro-PL, was helpful in estimating the biodistribution of these probes, and validating that these probes are taken effectively delivered to the tumor tissue. Although fluorescence was observed throughout the body of the mouse, the highest intensities were initially (Figure 53) observed in the tumor and
in regions of fat along the spine. As time progressed, the pooling of the probe in the gut (liver and intestines) was observed. No signal was observed in the kidneys or bladder. A biodistribution study showed that by 2 h the probe had largely accumulated in the liver. However, a 2-fold increase in fluorescence was observed in the tumor relative to contralateral muscle. Tumor uptake is likely helped by the properties of tumor tissue, which allow for the preferential uptake of porphyrins, such as Pyro, as well as the properties of the chosen delivery vehicle, Tween-80, which forms small micelles capable of penetrating the tumor interstitial space.

Injection of tumor-bearing mice with Pyro-PL-BHQ led to a ~4-fold increase in signal over background, which persisted up to 24 h post-injection, yielding a tumor:muscle ratio of ~2.5 from 2 – 24 h post-injection. PC-PLC inhibition with D609 significantly decreased probe activation, yielding a tumor:muscle ratio of 1~1.5 at 24 h.

To validate the presence of PC-PLC in DU145 tumor xenografts, PC-PLC activity was measured in extracts of tumor xenografts using an Amplex Red assay. Activity was found to increase with protein concentration and decrease with D609 concentration.

Injection of the sPLA2 (IB)-specific probe, Pyro-C12-PL-BHQ, led to signal increases primarily in the gut, showing bright intensities in the liver and intestines, which is expected since sPLA2 IB is a digestive enzyme. These results are exciting in that, while demonstrating a specificity, in vivo, for digestive PLA2, Pyro-C12-PL-BHQ has also further validated the results observed with Pyro-PL-BHQ. These probes are very similar in structure, except for a 12-carbon chain connecting Pyro at the sn-2 position of the glycerol backbone. Assuming that these probes have similar biodistribution, the fact that
Pyro-C12-PL-BHQ is not well-hydrolyzed by PC-PLC further validates that the tumor fluorescence enhancement observed in the Pyro-PL-BHQ imaging study is not due solely to probe accumulation in the tumor, but is caused by enzymatic hydrolysis of the probe.

Most importantly, however, the *in vivo* results described in this work indicate that PC-PLC may be an important molecular marker for cancer, in that Pyro-PL-BHQ was activated specifically in the tumor. The additional co-registration of images obtained using phospholipase-activated NIR fluorescent probes with choline metabolite levels obtained from *in vivo* MRS could critically impact the field of cancer detection by offering a sensitive and specific method of examining the lipid catabolic pathways linked to the malignant phenotype.
CHAPTER 6: PHYSIOLOGICALLY BASED PHARMACOKINETIC MODELS

The main advantage of using self-quenched enzyme-activated fluorescent probes in vivo is that fluorescence will only be observed in the presence of enzyme activity, so the location of this activity can be pin-pointed easily. However, as the activated fluorescent moiety is taken back up into the blood and re-circulated throughout the body, often the background fluorescence increases and it can be difficult to deconvolve newly activated probe from accumulation of re-circulating activated probe. Moreover, since the inactivated probe is quenched and dark, it is difficult to determine how much of the injected probe has been activated and how much has been eliminated from the body. Thus, the use of these probes in determining the amount of enzyme activity in vivo is largely qualitative. In order to overcome some of these limitations and extract more quantitative results from our in vivo imaging data, we developed a physiologically-based pharmacokinetic (PBPK) model to describe how the substrates are absorbed, distributed, metabolized and excreted. The development of this PBPK model is described in three phases: model representation, model parameterization, and model simulation.

In developing a PBPK model, the first step is to identify a conceptual model consisting of the organs or tissues that contribute significantly to the uptake and disposition of the drug in the body. A PBPK model considers these different organs and tissues in the model as a set of interconnected compartments. From this conceptual representation, a mathematical representation can be derived in the form of mass balance differential equations. Within each tissue compartment, the change in amount of drug
present is dependent on a series of terms describing the rates of drug uptake into the tissue and the rates of drug elimination from the tissue.

The second step in developing a PBPK model is to incorporate physiological, physiochemical, and biochemical parameters, many of which can be found in the published literature, into the mathematical representation of the model. The rates of uptake and disposition are functions of several parameters, such as cardiac output, tissue perfusion, partition coefficient, tissue volume, and tissue metabolic capacity.

Finally, the set of equations in the system can be solved simultaneously to determine the rate processes involved and the amount of drug in each compartment over time. Generally, a PBPK model is fit to real in vivo data in order to obtain certain pharmacokinetic parameter values. A computer program can be used to apply a curve fitting process, such as the method of least squares, to fit the set of equations in the model to the measured raw data. For example, the model parameters may be iteratively varied until the compartment representing the central compartment (blood) is best fit to actual drug concentrations measured from plasma samples taken over time.

6.1. MODEL REPRESENTATION

6.1.1. PBPK Model for Pyro-PL

Since the distribution of the permanently fluorescent Pyro-PL compound is visible by in vivo fluorescence imaging, we first developed the PBPK model for Pyro-PL. Using the pharmacokinetic parameters determined from this model, the Pyro-PL model can be
built upon to develop a model for the disposition of Pyro-PL-BHQ and the fluorescent metabolite of Pyro-PL-BHQ.

The PBPK model is useful when analyzing real *in vivo* data because the model is conceptualized in terms of drug concentration rather than drug amount. The amount of drug in the central compartment cannot be measured by taking a blood sample, for instance, but the concentration can be measured. In our case, we have the ability to measure average radiance over an ROI, which is the maximum photons per second per centimeter squared per steridian (photons·s⁻¹·cm⁻²·sr⁻¹). This measurement of average radiance is proportional to the concentration of substrate in the region measured. Further, in a PBPK model, the rate processes are dependent on physiological parameters, such as tissue perfusion rates, tissue partition coefficients and tissue volumes, which can often be calculated or found in the literature.

The PBPK model that we have developed to model Pyro-PL pharmacokinetics is shown in Figure 62. This model consists of a tumor compartment, a periphery (slowly perfused) compartment, a liver/gastrointestinal (GI) compartment, and a central (rapidly perfused) compartment. The periphery compartment includes muscle, fat and skin tissue. The rapidly perfused tissues, such as the lungs, viscera, kidneys, and brain, are lumped into the central compartment. Elimination is represented as occurring via the liver/GI as a saturable process that follows Michaelis-Menten kinetics. Since Pyro-PL was never observed in the bladder or kidneys, this excretion pathway has not been included.

The central compartment in this PBPK model is divided into arterial and venous blood. In this model, the substrate kinetics are assumed to occur after a rapid *i.v.* injection
of the dose, shown as the input for the central compartment (venous blood), which is mixed instantaneously in the blood. This is a common assumption since the actual mixing time is within a few minutes and insignificant by the time imaging begins. The venous blood leaving each tissue is assumed to mix simultaneously where it becomes the substrate concentration in the arterial blood \((C_a)\) flowing at a rate equal to the cardiac output \((Q_c)\) with blood volume, \(V_b\).

![Figure 62 PBPK Model for Pyro-PL](image)

Each tissue compartment is characterized by a tissue concentration \((C_t)\), a tissue-blood partition coefficient \((P_t)\) and a tissue volume \((V_t)\). The concentration of substrate in the arterial blood \((C_a)\) flows into the compartment at the tissue perfusion rate \((Q_t)\) and the concentration that flows out in the venous blood \((C_{vt})\) leaves at the same rate. The diffusion-limited tissue compartments, substrate diffuses into the CM at the rate of the tissue permeation area cross product, \(P_{A_t}\). A Michaelis-Menten rate \((V_{maxB}, K_{mB})\) describes excretion via biliary transport from the liver compartment.
The mass balance equations for each tissue compartment are simply described by the rates of substrate flowing into the tissues from the arterial blood \((Q_t C_a)\) and out of the tissues into the venous blood \((Q_t C_v)\), and also include loss or gain of substrate due to cellular matrix (CM) diffusion or substrate metabolism \((V^,\), \((V_max, K_m)\). The equations can be represented in terms of tissue concentration \((C_t)\), as shown, using the relationship, \(A_t = V_t C_t\).

In the slowly-perfused periphery tissue compartment and the tumor compartment, the diffusion-limited case is assumed, \(i.e.,\) the rate of diffusion of the substrate from the tissue blood to the cellular matrix is assumed to be slow with respect to tissue perfusion. Thus, the compartments are divided into two subcompartments, the tissue (venous) blood compartment \((vt)\) and the tissue cellular matrix compartment \((cmt)\). Thus, the rate of change of amount of substrate in these tissues must be represented by two equations:

\[
V_{vt} \frac{dC_{vt}}{dt} = Q_t(C_a - C_{vt}) - PA_t \left( C_{vt} - \frac{C_{cmt}}{P_t} \right)
\]

\[\text{(4)}\]

\[
V_{cmt} \frac{dC_{cmt}}{dt} = PA_t \left( C_{vt} - \frac{C_{cmt}}{P_t} \right)
\]

\[\text{(5)}\]

where \(C_{cmt}\) is the concentration of substrate in the cellular matrix and \(PA_t\) (mL/min) is the tissue permeation area cross product and, essentially, the volume flow rate of substrate into the cellular matrix from the venous blood. The concentration of substrate flowing out of the cellular matrix is given by \(C_{cmt} / P_t\), where \(P_t\) is the tissue-blood partition coefficient. The partition coefficient describes the distribution of substrate
throughout the tissue as the ratio of substrate concentration found in the tissue relative to
the concentration found in the blood. The rate of change of concentration of substrate in
the tissue blood ($C_{vt}$) is described by the rate of substrate flowing into the tissue from the
arterial blood and out of the tissue into the venous blood, as well as the net flux from the
cellular matrix. The amount of substrate in the tissue compartment was taken as the sum
of the amounts in both compartments, such that

$$V_t C_t = V_{vt} C_{vt} + V_{cmt} C_{cmt}$$

(6)

Since the liver/GI compartment is considered to be perfusion-limited, i.e., a
homogenous, well-mixed compartment. Thus, the concentration of substrate flowing out
of the tissue in the venous blood is considered to be in equilibrium with the tissue
concentration, such that $C_{vt} = C_t / P_t$. The liver/GI compartment functions in eliminating
substrate, yielding an additional term representing clearance of substrate by fecal
excretion, such that

$$V_{ig} \frac{dC_{ig}}{dt} = Q_{ig} (C_a - C_{vig}) - \frac{V_{maxb} C_{ig}}{K_{mb} + C_{ig}}$$

(7)

The rate of excretion was considered to be dependent on the maximum rate of biliary
transport, $V_{maxb}$ (nmol/min), and the Michaelis-Menten constant, $K_{mb}$ (nmol/mL).

6.1.2. PBPK Model for Pyro-PL-BHQ

The PBPK model for Pyro-PL-BHQ is equivalent to that of Pyro-PL, except for
the metabolism pathway (Figure 63). Since fluorescence was observed primarily in the
tumor, we made the assumption that the Pyro metabolite was formed exclusively in the
tumor cellular matrix following Michaelis-Menten kinetics, such that the equations for
the concentration of Pyro-PL-BHQ parent compound in the tumor blood \( C_{\text{vtumP}} \) and
tumor cellular matrix \( C_{\text{cntumP}} \) compartments are:

\[
V_{\text{vtum}} \frac{dC_{\text{vtumP}}}{dt} = Q_{\text{tum}} \left( a_P - C_{\text{vtumP}} \right) - PA_{\text{tumP}} \left( C_{\text{vtumP}} - \frac{C_{\text{cntumP}}}{P_{\text{tumP}}} \right) \tag{8}
\]

\[
V_{\text{cntum}} \frac{dC_{\text{cntumP}}}{dt} = PA_{\text{tumP}} \left( C_{\text{vtumP}} - \frac{C_{\text{cntumP}}}{P_{\text{tumP}}} \right) - \frac{V_{\text{maxM}} C_{\text{cntumP}}}{K_{\text{mM}} + C_{\text{cntumP}}} \tag{9}
\]

where \( V_{\text{maxM}} \) and \( K_{\text{mM}} \) are the maximum metabolic rate and the metabolic Michaelis-
Menten constant, respectively. The rate of Pyro-PL-BHQ metabolism is the dose input
for the Pyro metabolite submodel, where the equations for the concentration of
metabolite in the tumor blood \( C_{\text{vtumM}} \) and tumor cellular matrix \( C_{\text{cntumM}} \) are:

\[
V_{\text{vtum}} \frac{dC_{\text{vtumM}}}{dt} = Q_{\text{tum}} \left( a_M - C_{\text{vtumM}} \right) - PA_{\text{tumM}} \left( C_{\text{vtumM}} - \frac{C_{\text{cntumM}}}{P_{\text{tumM}}} \right) \tag{10}
\]

\[
V_{\text{cntum}} \frac{dC_{\text{cntumM}}}{dt} = \frac{V_{\text{maxM}} C_{\text{vtumP}}}{K_{\text{mM}} + C_{\text{vtumP}}} + PA_{\text{tumM}} \left( C_{\text{vtumM}} - \frac{C_{\text{cntumM}}}{P_{\text{tumM}}} \right) \tag{11}
\]

The equations for the concentration of the parent compound and the metabolite in
the liver/GI tissue compartments \( C_{\text{lgP}} \) and \( C_{\text{lgM}} \), respectively) are analogous to those in
the Pyro-PL PBPK model and of the form:

\[
V_{\text{lg}} \frac{dC_{\text{lg(P,M)}}}{dt} = Q_{\text{lg}} \left( a_{\text{lg}(P,M)} - C_{\text{lg}(P,M)} \right) - \frac{V_{\text{maxB(P,M)}} C_{\text{lg}(P,M)}}{K_{\text{mB(P,M)}} + C_{\text{lg}(P,M)}} \tag{12}
\]
where $C_{a(P,M)}$ and $C_{v(P,M)}$ are the concentrations of compound in the arterial blood and the venous blood, respectively.

![Pyro-PL-BHQ and Pyro metabolite PBPK model](image)

**Figure 63** Pyro-PL-BHQ and Pyro metabolite PBPK model

The Pyro-PL-BHQ parent/metabolite PBPK model are identical to the Pyro-PL model, with the addition of a metabolism component, through which the two models are connected.

### 6.2. MODEL PARAMETERIZATION

The advantage of utilizing a PBPK model to analyze *in vivo* data is that many of the parameters have well-defined physiological or physical meaning and can be found in the literature or determined experimentally, which minimizes the number of unknowns and adds constraints to the parameters in the mass balance differential equations. We have tried to use as many known parameters as possible in performing these model simulations.
6.2.1. Physiological Parameters

The physiological parameters used in these PBPK models include cardiac flow rate, tissue perfusion rates, tissue volumes, rate of metabolism, and rate of excretion. The tissue perfusion rates, given as fraction of cardiac output \((FQ_i)\) and tissue volumes, given as fraction of body weight \((FV_t)\) were obtained from the data tabulated by El-Masri and Portier [140], except for the tumor perfusion rate \((Q_{\text{tum}})\), which was obtained from Davies and Morris [141], the tumor volume \((V_{\text{tum}})\), which was averaged from those measured experimentally, and the blood volume, which was obtained from Brown et al. [142]. The BW was averaged as 22 g and tissue volume was calculated using an estimated tissue density of 1 g/mL.

Our model assumes that the periphery compartment is a lumped compartment, consisting of muscle, skin and fat tissue, so the tissue perfusion and tissue volume is calculated as the lumped sum of all three compartments. The liver/GI compartment is also considered to be a lumped compartment, consisting of both liver and GI properties, and the tissue perfusion and volume is calculated similarly.

There seems to be some discrepancy in the literature with respect to \(Q_c\) in mice; it seems that open-chest measurements yield values of 3 – 6 mL/min, whereas closed-chest measurements made by conductivity dilution and radioactive microspheres are on the order of ~16 mL/min [143]. Variations in \(Q_c\) are also expected with the degree of anesthetization. Therefore, we have decided to allow this parameter to be optimized for each subject.
For the diffusion-limited case, the permeation area cross product ($PA_t$) was allowed to be optimized by the model. The equations in this case also require the use of the tissue blood volume ($V_{tr}$) and the tissue cellular matrix volume ($V_{cm} = 1 - V_{tr}$). The tumor and periphery tissue blood volumes were optimized by the model, with an initial starting value of 0.04, found in the literature for muscle blood volume [144]. A list of the parameters used in the simulations, the notation for each parameter and their values are presented in Table 2.
<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>NOTATION</th>
<th>VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compartment Volume (mL) $^b$:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$V_t = FV_t \times BW$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>BW</td>
<td>22.0</td>
</tr>
<tr>
<td>Blood</td>
<td>$FV_b$</td>
<td>0.049 $^c$</td>
</tr>
<tr>
<td>Tumor</td>
<td>$V_{\text{tum}}$</td>
<td>0.002 $^d$</td>
</tr>
<tr>
<td>Periphery</td>
<td>$V_\text{p}$</td>
<td>$(FV_m + FV_s + FV_f) \times BW$</td>
</tr>
<tr>
<td>Muscle</td>
<td>$FV_m$</td>
<td>0.384</td>
</tr>
<tr>
<td>Skin</td>
<td>$FV_s$</td>
<td>0.165</td>
</tr>
<tr>
<td>Fat</td>
<td>$FV_f$</td>
<td>0.070</td>
</tr>
<tr>
<td>Liver/GI</td>
<td>$V_{\text{lg}}$</td>
<td>$(FV_l + FV_g) \times BW$</td>
</tr>
<tr>
<td>Liver</td>
<td>$FV_l$</td>
<td>0.046</td>
</tr>
<tr>
<td>Gut</td>
<td>$FV_g$</td>
<td>0.042</td>
</tr>
<tr>
<td>Tissue Blood/CM Volumes (mL):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$V_v = FV_v \times V_t$, $V_{\text{cm}} = (1 - FV_v) \times V_t$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor</td>
<td>$FV_{\text{tum}}$</td>
<td>Optimized</td>
</tr>
<tr>
<td>Periphery</td>
<td>$FV_{\text{pp}}$</td>
<td>Optimized</td>
</tr>
<tr>
<td>Tissue Perfusion Rate (mL/min):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$Q_t = FQ_t \times Q_c$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cardiac Output (mL/min)</td>
<td>$Q_c$</td>
<td>Optimized</td>
</tr>
<tr>
<td>Tumor</td>
<td>$Q_{\text{tum}}$</td>
<td>0.1 $^e$</td>
</tr>
<tr>
<td>Periphery</td>
<td>$Q_p$</td>
<td>$(FQ_m + FQ_s + FQ_f) \times Q_c$</td>
</tr>
<tr>
<td>Muscle</td>
<td>$FQ_m$</td>
<td>0.521</td>
</tr>
<tr>
<td>Skin</td>
<td>$FQ_s$</td>
<td>0.058</td>
</tr>
<tr>
<td>Fat</td>
<td>$FQ_f$</td>
<td>0.070</td>
</tr>
<tr>
<td>Liver/GI</td>
<td>$Q_{\text{lg}}$</td>
<td>$(FQ_l + FQ_g) \times Q_c$</td>
</tr>
<tr>
<td>Liver</td>
<td>$FQ_l$</td>
<td>0.161</td>
</tr>
<tr>
<td>Gut</td>
<td>$FQ_g$</td>
<td>0.141</td>
</tr>
<tr>
<td>Tissue Permeation Area Cross Product (mL/min)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$PA_t = FPA_t \times Q_t$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor</td>
<td>$FPA_{\text{tum}}$</td>
<td>Optimized</td>
</tr>
<tr>
<td>Periphery</td>
<td>$FPA_p$</td>
<td>Optimized</td>
</tr>
</tbody>
</table>

$^a$ Obtained from El-Masri and Portier [140].
$^b$ Converted to volume by assuming a density of 1 g/mL.
$^c$ Obtained from Brown et al. [142].
$^d$ Estimated from measured tumors.
$^e$ Obtained from Davies and Morris [141].
6.2.2. Partition Coefficients

The tissue-blood partition coefficients \( P_t \) are important parameters in the PBPK model because they represent the amount of substrate that is absorbed by the tissue versus the amount that remains in the blood. Here we have tried to estimate the tissue-blood partition coefficients based on the calculated membrane-water partition coefficient of the substrate, \( K_{mw} \). From this value, an estimation of the 1-octanol-water partition coefficient \( (K_{ow}) \) was determined, which, when combined with the tissue-specific lipid and water fractions, could be used to calculate an estimated value for the tissue \( P_t \).

A technique described by Huang et al. [145] to determine \( K_{mw} \) for fluorescent probes between the aqueous phase and the phospholipid membranes was used to determine the \( K_{mw} \) for Pyro-PL. The membrane partition coefficient can be defined as

\[
K_{mw} = \frac{S_b/L}{S_f/W},
\]

where \( S_b \) and \( S_f \) are the molar concentrations of substrate bound in the membrane and free in the water phase, respectively. \( L \) is the concentration of lipid and \( W \) is the concentration of water. Since Pyro forms aggregates and self-quenches in the aqueous phase, the increase in fluorescence \( (I) \) observed with an increase in lipid (Figure 17) is proportional to \( S_b \). Thus, the following relationship is true:

\[
I = I_0 L/(W/K_{mw} + L),
\]
where \( I_0 \) is the maximum intensity observed when all substrate is incorporated into the phospholipid membrane. Thus, this equation can be rearranged such that

\[
\frac{1}{I} = \left[ \frac{W}{K_{mw}I_0} \right] \frac{1}{L} + \frac{1}{I_0}
\]  

(15)

Thus, by making a double reciprocal plot of \( 1/I \) versus \( 1/L \), \( K_{mw} \) can be calculated as \( W \times (x - \text{intercept}) \). The concentration of water is known as 55.6 M and can be substituted for \( W \) in this equation. The double reciprocal plot of the data in Figure 17, where Pyro-PL fluorescence increases with an increase in PtdCho concentration, is shown below in Figure 64.

![Graph](image)

**Figure 64 Determination of \( K_{mw} \) for Pyro-PL in PtdCho.**

Inverse reciprocal plot of Pyro-PL fluorescence vs PtdCho (\( K_{mw} = 1.9 \times 10^6 \)).

The PtdCho concentrations below the CMC (100 \( \mu \)M) have been ignored since below the CMC there is still a significant amount of aggregation and self-quenching. This data yields a \( K_{mw} \) of \( 1.9 \times 10^6 \).
The 1-octanol-water coefficient (\(K_{ow}\)) is most commonly used to measure the tissue-blood partition coefficient of a compound. This is found by measuring the concentration of a compound in each phase of a mixed solution containing octanol and water. However, since hydrophobic compounds associate mostly with biological membranes, the use phospholipid vesicles is a more accurate model than the 1-octanol water system. Dulfer \textit{et al.} [148] has shown that a quadratic relationship can be drawn between the log\(K_{mw}\) and the log\(K_{ow}\) of a compound, such that for a substrate binding to diarachidonoyl phosphatidylcholine (DAPC),

\[
\log K_{mw(DAPC)} = -0.383(\pm0.063)\log K_{ow}^2 + 5.67(\pm0.063)\log K_{ow} - 15.53(\pm2.04) \tag{16}
\]

Although our experiments utilized egg-PtdCho phospholipid vesicles, we assumed that this relationship would be similar. Using this equation, we have found an estimated log\(K_{ow}\) of 12.7.

An algorithm developed by Poulin and Krishnan [147] can be used to predict a tissue-blood partition coefficient by taking into account the \(K_{ow}\) of a compound and the fractional volumes of neutral lipids (\(F_n\)) phospholipids (\(F_p\)) and water (\(F_w\)) in the tissue (sub-t) and blood (sub-b), such that

\[
P_t = \frac{K_{ow}(F_{nt} + 0.3F_{pt}) + (F_{wt} + 0.7F_{pt})}{K_{ow}(F_{nb} + 0.3F_{pb}) + (F_{wb} + 0.7F_{pb})} \tag{17}
\]

A list of the lipid and water volume fractions used and the tissue:blood partition coefficients determined from this equation are shown in Table 3. The lipid and water
volume fractions for the periphery tissue and the liver/GI tissue are taken as the scaled lipid and water content from the included tissues as follows (Verner, et al. [148]):

\[
F = \frac{\sum_{i=1}^{n} V_i \times F_i}{\sum_{i=1}^{n} V_i},
\]

where \( F \) is the fractional volume of lipid or water in the combined compartment, and \( F_i \) and \( V_i \) are the fractional volumes of lipid or water and the tissue volumes of the individual tissues.

The periphery and liver/GI tissue-blood partition coefficients, calculated from the lipid and water tissue volume fractions, are found in Table 3. These were the partition coefficient values used as initial values for \( P_p \) and \( P_{lg} \). The lipid and water volume fractions for the tumor compartment were unknown, however, so \( P_{tum} \) was initialized with the same value calculated for the periphery. These partition coefficients were, then, optimized by the model from these starting points.

Table 3 Fractional volumes of neutral lipids, phospholipids and water contents of tissues in mice.*

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Water Content (fraction of tissue vol)</th>
<th>Total Lipid Content (fraction of tissue vol)</th>
<th>Neutral Lipids (fraction of total lipids)</th>
<th>Phospholipids (fraction of total lipids)</th>
<th>Partition Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Periphery</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muscle</td>
<td>0.67</td>
<td>0.044</td>
<td>0.38</td>
<td>0.62</td>
<td>37.6</td>
</tr>
<tr>
<td>Skin</td>
<td>0.70</td>
<td>0.12</td>
<td>0.57</td>
<td>0.43</td>
<td></td>
</tr>
<tr>
<td>Fat</td>
<td>0.12</td>
<td>0.855</td>
<td>0.853*</td>
<td>0.002*</td>
<td></td>
</tr>
<tr>
<td>Liver/GI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>0.75</td>
<td>0.16</td>
<td>0.711</td>
<td>0.23</td>
<td>35.9</td>
</tr>
<tr>
<td>GI</td>
<td>0.72</td>
<td>0.17</td>
<td>0.68</td>
<td>0.32</td>
<td></td>
</tr>
</tbody>
</table>

*Values obtained from El-Masri and Portier [140]. The volume fractions for the richly perfused and slowly perfused tissue compartments are scaled from the tissues included in each.

Obtained from Haddad et al. [149] (assumed to be the same as rat).
6.2.1. Calibration

Since the mass balance differential equations represent the concentration of substrate in each tissue compartment and the in vivo data that the simulations are fitting the equations to are average radiance measurements, the radiation measurements must first be converted to substrate concentration measurements. Thus, in order to most accurately convert in vivo average radiance (p/s/cm²/sr) measurements to substrate concentration (μM), we used the IVIS Imager to measure in a 96-well plate the average radiance over increasing concentrations of Pyro-PL ranging from 75 μM to 0 μM in 1% Intralipid tissue-simulating phantom solutions (Figure 65). A 1% Intralipid solution has been commonly used to simulate the scattering properties of biological tissue. Solutions were made up in volumes of 200 μL or 100 μL in order to investigate a possible difference in concentration measurement with tissue thickness.

Figure 65 Calibration assay to convert average radiance measurements to concentration.
Varying concentrations of Pyro mixed in 1% Intralipid tissue phantom solutions. Rows A through C contain 200 μL of solution and rows D through F contain 100 μL of solution. Concentrations in columns 1 through 12 are 75, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, and 0 μM.
The average radiance was measured over each well and plotted versus Pyro concentration (Figure 66). The data shows that average radiance is linear with Pyro concentration at concentrations under 25 μM. At higher concentrations, the average radiance measurements become saturated, probably due to self-quenching as Pyro forms aggregates. The difference in radiance measurements between the two solution volumes is greater at very high concentrations. However, this difference is minimal for concentrations under 10 μM, which is the concentration region of our in vivo measurements, where a maximum radiance measurement of $1 \times 10^9$ was observed. This is indicative that a variation in tissue thickness between tumor tissue and muscle tissue will not yield disproportionate concentration measurements. A linear trendline was drawn between both volume measurements over the linear portion of the data, yielding a conversion factor of $7 \times 10^7$. Using this factor, the tumor and muscle average radiance measurements were converted to Pyro concentration measurements.

![Graph showing average radiance vs. Pyro concentration](image)

**Figure 66 Determining conversion factor for average radiance measurements.**
Plot of average radiance versus Pyro concentration in 100 or 200 μL volumes of 1% Intralipid.
6.3. **MODEL SIMULATIONS**

The models were constructed using the Matlab software (The Mathworks Inc., MA, USA). The unknown parameters were optimized using the *lsqnonlin* curve fitting routine of the optimization toolbox in Matlab. The mass balance equations were solved using the *ode45* function in Matlab and the simulated concentrations for the tumor and periphery compartments were fit to the measured tumor and contralateral muscle concentrations (converted from average radiance measurements).

### 6.3.1. Pyro-PL Model Results

The purpose of this model was to characterize the pharmacokinetic profile of Pyro-PL. As the fluorescent analog to Pyro-PL-BHQ, the pharmacokinetics of this substrate offers a starting point for determining the pharmacokinetics of Pyro-PL-BHQ, which cannot be measured by fluorescence imaging since it is dark until activated.

In Figure 67, the simulated and calculated Pyro-PL concentrations for the tumor and periphery (contralateral muscle) compartments are shown for two subject mice. Figure 68 shows the representative simulated amounts of Pyro-PL over time in the blood, tumor, periphery, and liver/GI tissue compartments after fitting to the measurements from the first subject.
Figure 67 Simulated and measured concentrations of Pyro-PL in the tumor and periphery.

Representative model simulations fit to the measured Pyro-PL concentrations from two different subjects. Black dots represent actual measurements (average radiance converted to concentration) and red solid line represents simulated concentrations from the tissue blood and CM compartments combined.
Figure 68 Model simulations of Pyro-PL distribution in Pyro-PL PBPK model tissue compartments.
Representative simulated amounts of Pyro-PL for one subject. Solid red line represents the amount of Pyro-PL in the tissue (blood); dashed red line represents the amount of Pyro-PL in the tissue CM. In the case of the liver/GI compartment, the dashed red line represents the amount of Pyro-PL that has been excreted via biliary transport and no longer in circulation.

These simulated results are consistent with images of Pyro-PL distribution observed using the Maestro in vivo imaging system (CRI, Woburn, MA), which are shown in Figure 69. Images were acquired every hour up to 4 hours and the images were spectrally resolved by the Maestro software, such that red is the color of Pyro-PL and blue is the color of skin autofluorescence. Pyro-PL was clearly seen in the blood vessels at 10 min. At 2 h post-injection, Pyro-PL had started to diffuse from the blood into the tissues, primarily the tumor and the liver, with a faint glow in tissues surrounding blood vessels (periphery). By 3 h post-injection, Pyro-PL was beginning to dissipate from the
tumor tissue and periphery and pool in the liver. By 4 h Pyro-PL had cleared from the
tumor and periphery tissues and was completely stored in the liver.

![Image of tumor tissue and periphery](image)

**Figure 69** High-resolution *in vivo* imaging of Pyro-PL distribution in DU145 tumor-bearing mouse. Images are spectrally resolved to separate Pyro-PL fluorescence (red) from tissue autofluorescence (blue).

By fitting the simulated tumor and periphery compartment substrate
concentrations to the actual "concentration" measurements from the tumor and
contralateral muscle, respectively, several parameters were optimized. These parameters
and their optimized values are given in Table 4, along with expected values.

Note that in Figure 68, the initial (dose in blood compartment) and final (excreted
by liver/GI compartment) amount of substrate was calculated to be 200 nmol. The reason
for this is that the Dose was also optimized to allow for better fitting of the data. Since
the tissue volumes were found in the literature and fixed, the calculated amount in the
compartments \( A_t = (\text{Avg Radiance}/7 \times 10^7) \times V_t \) totaled together was greater than the
actual dose amount of 80 nmol. Therefore, the apparent Dose (\(Dose_{app}\)) was also
optimized by the model. A possibility for this discrepancy in dose amounts (actual versus
optimized) is that the conversion factor used to convert average radiance measurements
to concentration overestimates the actual substrate concentrations.
Table 4 Optimized parameters in Pyro-PL PBPK model.

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>OPTIMIZED VALUE (S.D.)</th>
<th>EXPECTED VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Q_c$ (mL/min)</td>
<td>17 (2.0)</td>
<td>3 - 20</td>
</tr>
<tr>
<td>$P_{tum}$</td>
<td>26 (20)</td>
<td></td>
</tr>
<tr>
<td>$P_p$</td>
<td>5.3 (5.7)</td>
<td>38&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>$P_{lg}$</td>
<td>5.8 (6.2)</td>
<td>36&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>$FPA_{tum}$ (mL/min)</td>
<td>0.23&lt;sup&gt;b&lt;/sup&gt; (0.20)</td>
<td></td>
</tr>
<tr>
<td>$FPA_p$ (mL/min)</td>
<td>0.15&lt;sup&gt;b&lt;/sup&gt; (0.09)</td>
<td></td>
</tr>
<tr>
<td>$FV_{vtum}$ (fraction, unitless)</td>
<td>0.78&lt;sup&gt;c&lt;/sup&gt; (0.14)</td>
<td></td>
</tr>
<tr>
<td>$FV_{vp}$ (fraction, unitless)</td>
<td>0.13&lt;sup&gt;c&lt;/sup&gt; (0.06)</td>
<td>0.04&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>$V_{maxB}$ (nmol/min)</td>
<td>1.6 (1.3)</td>
<td></td>
</tr>
<tr>
<td>$K_{mb}$ (µM)</td>
<td>18 (19)</td>
<td></td>
</tr>
<tr>
<td>$Dose_{app}$ (nmol)</td>
<td>150 (80)</td>
<td>80</td>
</tr>
</tbody>
</table>

<sup>a</sup> Estimated from Equation 17.
<sup>b</sup> Given as fraction of $Q_{tum}$ and $Q_p$, respectively.
<sup>c</sup> Given as fraction of $V_{tum}$ and $V_p$, respectively.
<sup>d</sup> Obtained from International Life Sciences Institute (1994) [144] for muscle.

As shown in Table 4, the model results yielded much variability in the optimized parameters among the 5 subject mice analyzed. The value for $Q_c$ had the smallest standard deviation, so this value was held constant in the Pyro-PL-BHQ PBPK model. The tissue blood volume fraction values ($FV_{vtum}$ and $FV_{vp}$) were also fixed in the Pyro-PL-BHQ model. All of the other parameter average values were used as initial inputs for the parent Pyro-PL-BHQ parameters in the Pyro-PL-BHQ model.

6.3.2. Pyro-PL-BHQ Model Results

The main objective in performing simulations to characterize the pharmacokinetic behavior of Pyro-PL-BHQ and its resulting metabolite was to be able to quantify the
amount of substrate that is hydrolyzed \textit{in vivo} and to be able to deconvolve newly 
activated probe in the tumor tissue from the fluorescent metabolite re-circulating into the 
tumor from the blood. This type of pharmacokinetic analysis allows for a better 
understanding of the mechanism of activation. This information also allows future 
synthesized probes to be compared for their efficiency as \textit{in vivo} molecular beacons for 
specific enzyme activities.

The fluorescent images of a tumor-bearing mouse after being injected with Pyro-
PL-BHQ in Figure 70 offer a reference to the expected degree of accumulation that 
should be found by the model simulations. Notice, in the supine image at 6 – 7 h post-
injection, that much of the metabolite is visible in the liver and intestines.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure70.png}
\caption{\textit{In vivo} imaging of Pyro metabolite distribution in DU145 tumor-bearing nude mouse.}
\end{figure}

Images acquired at prescan and 1.5 – 2 h and 6 – 7 h after injection of Pyro-PL-BHQ. The top row shows 
images taken from the top of the mouse, and the bottom row shows the mouse in the supine position in 
order to get a clearer view of liver/GI accumulation.
Figure 71 shows a representative set of simulated and actual tumor and periphery (contralateral muscle) concentration measurements of the Pyro metabolite from one subject. The simulated amounts of substrate in all compartments from this subject are shown in Figure 72, with Pyro-PL-BHQ represented in black and the Pyro metabolite represented in red (solid = tissue blood, dashed = tissue CM).

![Graphs showing concentration over time](image)

**Figure 71** Simulated and measured of concentrations of Pyro metabolite of Pyro-PL-BHQ in the tumor and periphery. Representative model simulations fit to the measured Pyro metabolite concentrations from one subject. Black dots represent actual measurements (average radiance converted to concentration) and red solid line represents simulated concentrations from the tissue blood and CM compartments combined.
Figure 72 Model simulations of Pyro-PL-BHQ and Pyro metabolite distribution in Pyro-PL-BHQ PBPK model tissue compartments.

Representative simulated amounts of Pyro-PL for one subject. Solid black line represents the amount of Pyro-PL-BHQ in the tissue (blood); dashed black line represents the amount of Pyro-PL-BHQ in the tissue CM; solid red line represents the amount of Pyro metabolite in the tissue (blood); and dashed red line represents the amount of Pyro metabolite in the tissue CM. In the case of the liver/GI compartment, the dashed black/red lines represent the amount of Pyro-PL-BHQ/Pyro metabolite that has been excreted via biliary transport and no longer in circulation. (A) Viewed with maximal y-scale and (B) viewed with smaller y-scale in order to observe Pyro metabolite distribution.
The simulations show that Pyro-PL-BHQ is absorbed by the tumor CM, where most of the substrate is rapidly hydrolyzed and quickly fed back into the system. However, only a minimal amount of Pyro metabolite is reabsorbed by the tumor CM and the majority is absorbed by the periphery CM. Thus, the fluorescence observed in the tumor, according to these simulations, is largely due to the concentration of the Pyro metabolite in the tumor blood compartment. By 24 h, the majority of the Pyro metabolite was in the process of being excreted by the liver/GI compartment.

Table 5 Optimized parameters in Pyro-PL-BHQ PBPK model.

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>OPTIMIZED VALUE (S.D.)</th>
<th>EXPECTED VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>( p_{tumP} )</td>
<td>67 (1.0)</td>
<td>26</td>
</tr>
<tr>
<td>( p_p )</td>
<td>4.6 (1.1)</td>
<td>5.3</td>
</tr>
<tr>
<td>( p_{lgP} )</td>
<td>0.69 (0.81)</td>
<td>5.8</td>
</tr>
<tr>
<td>( p_{tumM} )</td>
<td>0.73 (0.27)</td>
<td>–</td>
</tr>
<tr>
<td>( p_{pM} )</td>
<td>0.58 (0.091)</td>
<td>–</td>
</tr>
<tr>
<td>( p_{lgM} )</td>
<td>0.55 (0.17)</td>
<td>–</td>
</tr>
<tr>
<td>( FPA_{tumP} )</td>
<td>0.45 (^b) (0.0096)</td>
<td>0.23</td>
</tr>
<tr>
<td>( FPA_{pP} )</td>
<td>0.015 (^b) (0.003)</td>
<td>0.15</td>
</tr>
<tr>
<td>( FPA_{tumM} )</td>
<td>0.53 (^b) (0.42)</td>
<td>–</td>
</tr>
<tr>
<td>( FPA_{pM} )</td>
<td>0.78 (^b) (0.075)</td>
<td>–</td>
</tr>
<tr>
<td>( V_{max,M} ) (nmol/min)</td>
<td>0.14 (0.03)</td>
<td>–</td>
</tr>
<tr>
<td>( K_{mol} ) (µM)</td>
<td>7.0 (11)</td>
<td>–</td>
</tr>
<tr>
<td>( V_{max,BP} ) (nmol/min)</td>
<td>0.001 (0.0004)</td>
<td>1.6</td>
</tr>
<tr>
<td>( K_{mbP} ) (µM)</td>
<td>35.5 (25.7))</td>
<td>18</td>
</tr>
<tr>
<td>( V_{max,BM} ) (nmol/min)</td>
<td>0.14 (0.057)</td>
<td>–</td>
</tr>
<tr>
<td>( K_{mMB} ) (µM)</td>
<td>1.7 (1.6)</td>
<td>–</td>
</tr>
<tr>
<td>( Dose_{app} ) (nmol)</td>
<td>230 (19)</td>
<td>150</td>
</tr>
</tbody>
</table>

\(^a\) Obtained from the results of the Pyro-PL PBPK model results.
\(^b\) Given as fraction of \( Q_{tum} \) and \( Q_p \), respectively.
– No expected value available.
A list of the average values for the parameters that were optimized in the Pyro-PL-BHQ/metabolite PBPK model is found in Table 5 (n=3). The parameters describing the pharmacokinetics of the parent compound are listed separately from those describing metabolite (M) pharmacokinetics. The expected parameter values are shown for the parent compound based on the results from the Pyro-PL PBPK model. Overall, less variability between the optimized parameters from each subject was found in this model. The parameters that had the most variability between subjects were the Michaelis constants ($K_{mB(PM)}$ and $K_{mM}$), so, unfortunately, the in vivo enzyme activity cannot be characterized at this time.

6.4. DISCUSSION

The main benefit of performing PBPK models is that they offer some insight into how a drug, or in this case, a molecular beacon, is distributed, absorbed, metabolized, and excreted in the body. The more information investigators have in this regard, the easier it is to optimize beacons for achieving the desired molecular targeting and activation. In the particular case of enzyme-activated molecular beacons, the pharmacokinetics of the parent probe is especially difficult to determine, since they are initially dark. With a non-fluorescent compound, blood samples and tissue samples must be harvested and concentrations measured using traditional more time-consuming methods. Here, we have developed a PBPK model to determine the pharmacokinetics of the PC-PLC-sensitive probe, Pyro-PL-BHQ. We did this by first building a PBPK model to determine the pharmacokinetics of Pyro-PL, the fluorescent analog of Pyro-PL-BHQ, and fitting simulated tumor and periphery tissue substrate concentration measurements to actual tumor and contralateral muscle tissue concentration measurements. Actual substrate
concentrations were determined by using a calibration factor to convert average radiance measurements to concentration. Using these results as an estimate for the pharmacokinetic profile of Pyro-PL-BHQ, we were able to build a metabolite submodel into the PBPK model in order to determine the pharmacokinetics of the Pyro metabolite.

Many of the physiological parameter values could be found in the literature. However, those that were not available were optimized by the PBPK models as the simulated tissue concentration measurements were fit to the actual data. The parameters optimized by the Pyro-PL PBPK model were used as initial inputs for the unknown parameters in the Pyro-PL-BHQ/Pyro metabolite PBPK model. We attempted to estimate the tissue: blood partition coefficients for the Pyro-PL PBPK model using a method developed by Huang et al. [145] to determine $K_{mw}$ for fluorescent probes, in conjunction with an algorithm developed by Dulfer et al. [146], which draws a relationship between $\log K_{mw}$ and $\log K_{ow}$, and an algorithm developed by Poulin and Krishnan [147] that calculates a tissue: blood $P_t$ from the compound $K_{ow}$ and known tissue lipid and water contents. We found that this method overestimated the average values for the optimized Pyro-PL periphery and liver/GI partition coefficients ($P_p$ and $P_{ig}$). Interestingly, we found that $P_{tum}$ fell in the range of these calculated values. A possible reason for this discrepancy is that the Dulfer et al. method was done using DAPC as the phospholipid carrier whereas, in our experiments, egg-PtdCho was utilized. Perhaps, more accurate values for the partition coefficients could be found using DAPC in a future study. It is worthwhile to be able to calculate these parameters a priori, so that they can be held constant during the simulations. The parameters that we were most interested in
determining were the Michaelis-Menten maximal velocities and constants ($V_{\text{max}}$ and $K_m$) in order to characterize the metabolism of Pyro-PL-BHQ. Unfortunately, due to the high degree of variability between subjects, this value could not be determined. It is unclear at this time whether the discrepancy between values is a real effect of differences in enzyme activities among subjects, or whether, with more actual data, the parameters could be better optimized.

The actual tissue concentrations were calculated by converting fluorescence to concentration using a calibration factor. The calibration factor was determined by measuring the fluorescence of known Pyro concentrations dispersed in 1% Intralipid, a commonly used solution for tissue phantoms. However, in performing the simulations, we found that the tissue concentrations were overestimated and that, in order to compensate for this, it was necessary to optimize the apparent Dose ($Dose_{\text{app}}$), which was 2 – 3-fold higher than the actual Dose. The 1% Intralipid solution accounts for tissue scattering, however tissue absorption by blood was ignored. The addition of black india ink is often used in optical experiments to match the absorption coefficients of biological tissue. It is obvious by these results that this factor cannot be ignored and should be included in the future.

The most interesting observation from these results is that much more variability was found in the parameters optimized in the Pyro-PL PBPK model than in the Pyro-PL-BHQ PBPK model. This was unexpected since the Pyro-PL-BHQ model system is more complex. However, this is likely due to pathways of Pyro-PL metabolism that were not considered by the Pyro-PL model. Without BHQ-3 at its head group (Pyro-PL has
ethanolamine at its head group), the molecule is more susceptible to hydrolysis, including hydrolysis by phospholipases other than PC-PLC. Thus, Pyro-PL is not a true analog for Pyro-PL-BHQ, but serves a purpose in estimating initial starting points for parameters to be optimized in the Pyro-PL-BHQ model.

Although the models only utilized four compartments (blood, tumor, periphery, and liver/GI), the simulations yielded reassuring results, since the amounts of substrate in each tissue compartment were consistent with the tissue fluorescence observed using *in vivo* fluorescence imaging. It was particularly interesting to see that, in the Pyro-PL-BHQ PBPK simulated results, very little Pyro-PL-BHQ was shown to be excreted by the liver/GI compartment, indicating that nearly all of the substrate was metabolized in the tumor and distributed throughout the body. This was unexpected, since it was previously assumed that only a small fraction of the substrate was activated, since the maximum average radiance measured in the tumor is 4-fold higher in mice injected with Pyro-PL than in mice injected with Pyro-PL-BHQ. The addition of fluorescence measurements from other tissues would greatly improve the accuracy of the PBPK models, such as the incorporation of actual plasma substrate concentration measurements from blood samples. This would likely decrease the variability found in the optimized parameters.

Overall, this is the first attempt to determine the pharmacokinetics of a molecular beacon using *in vivo* fluorescence imaging and there is much room for improvement in this area. However, this work offers a significant advance to the field of molecular imaging in that it offers a method that can be used universally to describe activatable contrast agents.
CHAPTER 7: METHODS

7.1. DESIGN AND SYNTHESIS

The self-quenching NIR molecular beacons characterized in this work were designed and synthesized by our collaborator, A.V. Popov, Ph.D. The fluorochrome we have chosen for these probes is pyropheophorbide $a$ (Pyro), a neutral NIR photosensitizer derived from chlorophyll $a$ ($\lambda_{\text{ex}} = 418, 675 \text{ nm}; \lambda_{\text{em}} = 670-730 \text{ nm}$). An uncharged fluorophore is necessary for incorporation into the hydrophobic portion of the lipid molecule. This, in turn, is necessary for the incorporation of the probes into liposomes for in vitro and in vivo delivery. The quencher being implemented is the Black Hole Quencher-3 (BHQ-3, quenching range: 620-730 nm).

7.1.1. Pyro-PL and Pyro-PL-BHQ

Briefly, Pyro-PL-BHQ was synthesized by first acylating an N-Boc protected 1-palmitoyl-sn-glycero-3-lysophosphoethanolamine (N-Boc Lyso-PE) with pyropheophorbide acid (Pyro-acid) in the presence of N-(3-dimethylaminopropyl-N'-ethyl carbodiimide hydrochloride (EDC) and 4-dimethylaminopyridine (DMAP). At this point, N-Boc deprotection with trifluoroacetic acid (TFA) results in the permanently fluorescent phospholipid analog, Pyro-PL, at 30% yield (50 mg). Further N-acylation of Pyro-PL with BHQ-succinimidyl ester (BHQ-NHS) in the presence of pyridine (Py) or triethylamine (TEA) gives rise to the desired probe, 1-palmitoyl-2-pyropheophorbide-sn-glyceryl-3-phosphoethanolamide of BHQ-3 acid (Pyro-PL-BHQ), at 15% yield (20 mg).

7.1.2. Pyro-C12-PL-BHQ

The preparation of Pyro-C12-PL-BHQ can be concisely described as follows. Initially, Pyro-C12-acid was synthesized by the reaction of Pyro-acid with N-
hydroxysuccinimide (NHS) in the presence of EDC and DMAP followed by treatment with ω-aminolaeric acid at 70% yield (500 mg). Then Pyro-C12-acid was coupled with N-Boc Lyso-PE with EDC and DMAP present. Next, the continuously fluorescent phosphoethanolamine derivative Pyro-C12-PL was prepared by N-Boc deprotection with TFA at 20% (10 mg). The final acylation of Pyro-C12-PL with BHQ-NHS in the presence of TEA resulted in the target 1-palmitoyl-2-(pyroepheophorbide-ω-amidolauryl)-sn-glyceryl-3-phosphoethanolamide of BHQ-3 acid (Pyro-C12-PL-BHQ), at 5% yield (8 mg).

7.2. PHOSPHOLIPIDS

PtdCho (chicken egg), L-α-phosphatidyl-DL-glycerol (PtdGro, chicken egg, sodium salt), and cholesterol were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). L-α-phosphatidylinositol (PtdIns, bovine liver, ammonium salt), L-α-phosphatidylethanolamine (PtdEtn, chicken egg) and sphingomyelin (SM, bovine brain) were obtained from Sigma-Aldrich (St. Louis, MO). All phospholipids were stored as chloroform solutions at -20°C.

7.3. LIPID DISPERSIONS AND MICELLES

Lipid dispersions were freshly made on the day of the experiment. Pyro-PL-BHQ was solubilized in chloroform and concentration was determined by measuring the absorbance (Molecular Devices SpectraMax M5, ε = 110,000 M⁻¹ cm⁻¹ at 418 nm). Pyro-PL-BHQ was combined with natural phospholipids at the appropriate MF in chloroform and dried under a stream of nitrogen until completely dry. Lipid films were rehydrated in buffer (50 mM Tris-HCL, pH 7.4) at the volume needed to yield the desired molar concentration, vortexed briefly and sonicated for 15 – 30 min until optically clear.
7.4. PHOSPHOLIPASES

PC-PLC (*Bacillus cereus*), PI-PLC (*B. cereus*), SMase (*B. cereus*), PC-PLD (*Streptomyces chromofuscus*), type IA sPLA₂ (*naja mossambica mossambica*), type IB sPLA₂ (porcine pancreas), and type IB sPLA₂ (bovine pancreas) were obtained from Sigma (St. Louis, MO, USA). PC-PLC (*Listeria monocytogenes*) was provided to us as a gift from Dr. Howard Goldfine (University of Pennsylvania). Type V human recombinant sPLA₂ and type III sPLA₂ were obtained from Cayman Chemical (Ann Arbor, Michigan, USA).

7.5. TLC ASSAY

Pyro-PL-BHQ (10 μM) was prepared in lipid dispersions (MF 0.05) as described above. Vesicles were composed of various phospholipids: PtdCho, SM, PtdGro, PtdIns, and cholesterol (5:5:0.5:0.5:2); PtdCho, PtdEtn, cholesterol (2:1:1), or PtdCho alone. The Pyro-PL-BHQ/lipid dispersions were divided into aliquots of 200 μL and incubated at 37°C with 1 unit of enzyme: PC-PLC, PI-PLC, SMase, PC-PLD, type IA sPLA₂ and/or type IB sPLA₂. A UV Silica Gel TLC plate was washed with chloroform:methanol (5:1). After 24 h, 100 pmoles from each aliquot was loaded onto the washed UV Silica Gel TLC plate. A total of 10 μL from each aliquot was loaded onto the plate by pipetting 1 μL from each aliquot onto the plate at a time and waiting for the spot to be dry before pipetting the next amount to the spot. When 10 μL from each aliquot was loaded and completely dry, the TLC plate was placed in chloroform:methanol (5:1) in order to separate the metabolites from each aliquot. Fluorescent bands were detected using a UV lamp at 385 nm.
7.6. HPLC AND MALDI-TOF MASS SPECTROMETRY

Enzyme-treated solutions were separated by HPLC (Waters Corp., Milford – 600 Controller with a 2996 Photodiode Array Detector) using the following eluents: A=0.1M TEAA (pH 7, triethylamine + acetic acid), B=acetonitrile and C=MeOH, and the method:

20% of A and 80% of B → 10% of A and 90% of B in 10 min → 90% of B and 10% of C in 10 min → 100% of C in 60min; with flow 1.5 mL/min on a Zorbax 300SB-C3 column. The identity of isolated fragments was confirmed using MALDI-TOF obtained from an Applied Biosystems Voyager DE Mass Spectrometer using a positive mode ionization and CHCA (α-cyano-4-hydroxycinnamic acid) or HABA (2-(4-hydroxyphenylazo)benzoic acid) matrix.

7.7. CELL CULTURE

The human prostate carcinoma cell line, DU145, was maintained in MEM Eagle culture medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 1% penicillin/streptomycin and buffered with 20 mM sodium bicarbonate. Cultures were routinely grown in 150 cm$^2$ filter cap tissue culture flasks, passaging every other day 1:3 at a seeding density of 1×10$^5$ cells/mL and using standard culture conditions of 37°C and 5% CO$_2$ in air. Fresh cultures were revived from frozen stocks every three months.

7.8. FLUORESCENCE SPECTROSCOPY

Two microplate spectrofluorometers were used in the experiments presented here: a Molecular Devices SpectraMax XPS, generously provided by Dr. S. Walter Englander (University of Pennsylvania) and a Molecular Devices SpectraMax M5, later purchased by our lab. The Softmax Pro Software was used to adjust microplate reader settings. The kinetics mode was utilized to measure enzyme reactions. The excitation and emission
wavelengths for detecting Pyro fluorescence were set to 418 nm and 675 nm, respectively. A 495 nm cut-off filter was selected to reduce excitation artifacts. The reactions were aliquotted into 96-well plates and inserted into the microplate reader. The wells containing the reactions were selected for detection. The plate was automixed (shaken) for 5 seconds prior to measurement in order to ensure that the reaction was well-mixed. The temperature was set to 37°C. The time interval between reads was set to 11 sec and the total time of detection was set to 30 min. After reactions were started, the microplate reader was set to begin reading. The SoftMax Pro Software was used to measure initial rate and determine enzyme activity.

7.9. ENZYME ASSAYS

Enzymatic activation of Pyro-PL-BHQ was determined by measuring the release of fluorescence emission in real time by fluorescence spectroscopy. Solutions were prepared with 1 μM Pyro-labeled substrate in lipid dispersions as described above and aliquotted into 96-well plates at volumes of 100 μL. Reaction mixtures were incubated for 10 minutes in order to allow the reactions to equilibrate to 37°C. Phospholipase was added to samples to begin reactions.

7.10. SURFACE DILUTION KINETICS

The kinetic equation has been derived previously [107, 150] and is shown below. The first $E$ and $A$ shown in the bulk step represent the molar concentrations of the free enzyme and the free substrate, respectively, and should be represented in terms of bulk units (mole/volume), where as all other terms should be represented in surface units (mole fraction, unitless). Thus, for ease and simplicity in the derivation, these terms will be denoted $E_b$ and $A_b$, and the reaction scheme rewritten as follows.
\[
E_b + A_b \xrightarrow{k_1} EA + B \xrightarrow{k_2} EAB \xrightarrow{k_3} EA + P
\]  

(19)

From Equation 19, the equations for the initial velocity and the steady state conditions can be deduced. The term \( L_0 \) is used to convert surface concentration units to bulk concentration units, where \( L_0 \) represents the total molar concentration of the interface (mole/volume).

\[
v = L_0 \frac{d[P]}{dt} = L_0 k_3 [EAB]
\]  

(20)

\[
L_0 \frac{d[EA]}{dt} = k_1 [E_b] [A_b] + (k_{-2} + k_3) L_0 [EAB] - k_{-1} L_0 [EA] - k_2 L_0 [EA][B] = 0
\]  

(21)

\[
\frac{d[EAB]}{dt} = k_2 [EA][B] - (k_{-2} + k_3) [EAB] = 0
\]  

(22)

The total enzyme concentration, \( E_0 \), is given in Equation 23.

\[
E_0 = [E_b] + L_0 [EA] + L_0 [EAB]
\]  

(23)

An expression for \([EAB]\) can be obtained by combining Equation 21 - 23.

\[
[EAB] = E_0 [B][A_b]/L_0 \left( K_s K_m + K_m [A_b] + [B][A_b] \right)
\]  

(24)

where \( K_s = k_{-1}/k_1 \) and is the dissociation constant for enzyme binding to the vesicle surface (mole/volume), and \( K_m = (k_{-2} + k_3)/k_2 \) and is the interfacial Michaelis constant (mole fraction, unitless).
By substituting Equation 24 into Equation 20, the velocity at steady state (v) can be written in terms of the total initial bulk phospholipid substrate \( S_0 \), where \( S_0 = [A_b] \) (mole/volume), and the total initial surface concentration of substrate \( X_s \), where \( X_s = [B] \) (mole fraction, unitless) and is shown in Equation 25.

\[
v = \frac{V_{\text{max}} X_s S_0}{K_m K_s + K_m S_0 + X_s S_0}
\]

Thus, in order to carry out a surface dilution kinetic analysis and determine the kinetic parameters, \( X_s \) must be varied independently of \( S_0 \). In Case I, \( X_s \) is held constant while \( S_0 \) is varied. In Case II, \( S_0 \) is held constant while \( X_s \) is varied. For Case I, the plot of \( v \) versus \( S_0 \) can be fit to the Michaelis-Menten form of Equation 25 with \( S_0 \) as the dependent variable, as shown in Equation 26.

\[
v = \frac{V_{\text{max(app)}} S_0}{K_m(app) + S_0}
\]

where

\[
V_{\text{max(app)}} = \frac{V_{\text{max}} X_s}{K_m + X_s}
\]

and

\[
K_{m(app)} = \frac{K_m K_s}{K_m + X_s}
\]
Thus, \( V_{\text{max(app)}} \) is the apparent \( V_{\text{max}} \), i.e., the maximal rate achieved at some constant value of \( X_s \), and \( K_{m(app)} \) is the apparent \( K_m \), or the bulk concentration of \( S_0 \) at \( V_{\text{max(app)}}/2 \).

In Case II, Equation 25 can be rearranged in Michaelis-Menten form with dependence on \( X_s \), as shown in Equation 29.

\[
\frac{v}{V_{\text{max}} X_s} = \frac{1}{K_{m(app)} + X_s}
\]  

(29)

The plot of \( v \) versus \( X_s \) can be fitted to a hyperbolic saturation curve and, in this case, \( V_{\text{max}} \) is the true \( V_{\text{max}} \) at infinite \( S_0 \) and infinite \( X_s \). The \( K_{m(app)} \) found in this case is different from that found in Case I, and is given by Equation 30.

\[
K_{m(app)} = K_m \left( \frac{K_s}{S_0} + 1 \right)
\]

(30)

7.11. CONFOCAL IMAGING

For confocal imaging, cells (1 x 10^5 cells/mL) were seeded into Nunc chambered culture slides and incubated for 24 h. PB (10 mM) in culture medium (or control PBS) was added and the cells were incubated for 4-16 h. The cells were washed in PBS, and overlaid with 10 \( \mu \)L of 4.5 \( \mu \)M Pyro-PL-BHQ or PED6 in 0.1% Tween-80 or 0.1% DMSO, respectively, and incubated for 1 h at 37 °C. For PLA2 inhibition, cells were pre-incubated with 10 or 100 \( \mu \)M AACOCF3 for 1 h prior to probe delivery and in conjunction with probe delivery. The cells were washed and mounted for microscopy on a Zeiss LSM 510 META confocal microscope.
7.12. **SUBCUTANEOUS TUMOR GROWTH**

DU145 cells ($5 \times 10^6$) were injected subcutaneously into the flanks of 4-6 week old athymic nude mice (NCI, Fort Dietrich, MD). Tumors were studied when they reached a volume of about $200 \mu m^3$, which is 4-6 weeks after tumor implantation. Volumes were calculated using the formula, $V_{tum} = lw^2/2$, where $l$ (mm) is the length of the tumor diameter at the broadest lateral point and $w$ (mm) length of the tumor diameter perpendicular to $l$.

7.13. **NIR OPTICAL IMAGING**

Mice were anesthetized with 100 µL ketamine/acepromazine. Mice were imaged using a Xenogen IVIS system with settings of Cy5.5 fluorescence filters, and an exposure time of 1 s. A black background sheet was placed in the IVIS and a fluorescence background image was taken. Mice were placed on the black background sheet and pre-scan images were taken. The fluorophore (80 nmol in 200 µL of 50 mM Tris-HCl, pH 7.4, 0.1% Tween-80) was injected via the tail vein, the catheter flushed with saline and the mouse returned to the Xenogen. Images were acquired over the first 1.5 h, and hourly up to 6 h and followed up the next day at 24 h. A region of interest (ROI) was drawn around the tumor and contralateral muscle and the average radiance (p/s/cm²/sr) was measured. The mice were sacrificed at varying times up to 28 h. The organs were harvested, washed and placed in a 24-well plate for optical imaging in the Xenogen. Using the same settings that were used for in vivo imaging, the total flux (p/s) was measured for each organ and compared to autofluorescence signals from mice not injected with fluorophore.
7.14. **Whole Cell Extracts of DU145 Cells**

Cells were washed and scraped from confluent 150 cm$^2$ filter cap tissue culture flasks and suspended in 2 mL of PBS. Samples were centrifuged at 1000 rpm for 5 min at 4°C. Cells were resuspended in 200 μL PBS, transferred to Eppendorf tubes on ice, and centrifuged again at 1000 rpm for 5 min. While on ice, samples were then homogenized using a hand-held micro-homogenizer. Samples were stored frozen at -80°C until needed.

7.15. **Extracts of DU145 Tumor Xenografts**

DU145 tumors were excised from euthanized mice and weighed. Tumors were sliced, minced and transferred to 15 mL centrifuge tubes in 7 mL of cold PBS (without Ca, without Mg). Samples were centrifuged at 7000 rpm for 8 min at 4°C. PBS was removed and pellets were lysed on ice for 30 min with 0.5 mL of ice-cold lysis buffer (10 mL of 50 mM Tris-HCl at pH 7.4, 1% Triton X-100, 1 Complete Mini protease inhibitor, 1 mM EGTA, 2 mM EDTA, 150 mM NaCl). The lysed suspensions were homogenized for 5 min on ice, centrifuged at 13,500 rpm for 10 min at 4°C, and the supernatant fraction was collected and stored at -80°C for PC-PLC assay.

7.16. **Amplex Red Assay of PC-PLC Activity**

The Amplex Red assay was performed according to Zhou et al. (49). PC-PLC activity was determined by adding the thawed supernatant fraction to 100 μL of an Amplex Red reaction mixture containing 0.4 mM Amplex Red, 1 unit/mL horseradish peroxidase, 4 unit/mL alkaline phosphatase, 0.1 unit/mL choline oxidase, and 0.5 mM PtdCho in 1X Reaction Buffer (50 mM Tris-HCl, pH 7.4, 0.14 M NaCl, 10 mM dimethylglutarate, 2 mM CaCl$_2$) at 37°C. PC released from PtdCho by PC-PLC is
converted to choline by alkaline phosphatase, which is further oxidized to form H$_2$O$_2$. In the presence of horseradish peroxidase, the H$_2$O$_2$ reacts with Amplex Red to generate the fluorophore, resorufin, which was detected using a Molecular Devices SpectraMax M5 microplate reader ($\lambda_{ex} = 560$ nm and $\lambda_{em} = 590$ nm).

### 7.17. $^3$P-MRS ASSAY FOR PC-PLC ACTIVITY

Sonicated egg-PtdCho dispersions (10 mM) were prepared in 4 mL of 50 mM Tris-HCl at pH 7.4. DU145 whole cell extracts from two confluent 150 cm$^2$ filter cap tissue culture flasks were prepared. Egg-PtdCho (4mL) was incubated at 37°C with whole cell DU145 extracts (20 µL) ± 100 µM D609, or with 1 U PC-PLC in a 10 mm NMR tube. $^3$P-MR spectra were obtained over 2 h on a Varian INOVA 9.4T (256 scans, 45° pulse, TR=30 s, data size 2K, SW=5 kHz).

### 7.18. STATISTICAL ANALYSIS

All data are presented as mean ± s.d or ± s.e. Statistical analysis was conducted using Student’s t-test. A p value ≤ 0.05 was considered to be significant.
REFERENCES


