Biochemical characterization of a *Pseudomonas aeruginosa* phospholipase D

By

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AHL	N-acyl homoserine lactone
BMP	bis(monoacylglycero)phosphate
BuOH	butanol
CF	cystic fibrosis
CO	choline oxidase
CRC	concentration response curve
CTD	C-terminal domain
DAG	diacylglycerol
DGPP	diacylglycerol pyrophospate
DMPK	drug metabolism and pharmacokinetics
DO	dioleoyl
DP	dipalmitoyl
EB	elementary body
EtOH	ethanol
GlcNac	*N*-acetylglucosamine
hPLD	human PLD
HRP	horseradish peroxidase
HSL	homoserine lactone
IPTG	isopropyl β-D-1-thiogalactopyranoside
LC-MS	liquid chromatography-mass spectrometry
LCV	*Legionella* containing vacuoles
LPC	lysophosphatidylcholine

LPE	lysophosphatidylethanolamine
LPG	lysophosphatidylglycerol
LPI	lysophosphatidylinositol
LPS	lysophosphatidylserine
MAFP	methyl arachidonyl fluorophosphates
mDAP	*meso*-diaminopimelic acid
MeOH	methanol
MGNC	multinucleated giant cell
MurNac	*N*-acetylmuramic acid
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PH	pleckstrin homology
PI	phosphatidylinositol
$PI(3,4,5)P_3$	phosphatidylinositol 3,4,5-triphosphate
$PI(3,4)P_2$	phosphatidylinositol 3,4-bisphosphate
$PI(3,5)P_2$	phosphatidylinositol 3,5-bisphosphate
$PI(3)P_2$	phosphatidylinositol 3-monophosphate
$PI(4,5)P_2$	phosphatidylinositol 4,5-bisphosphate
PI(4)P	phosphatidylinositol 4-monophosphate
PI(5)P	phosphatidylinositol 5-monophosphate
PI3K	phosphatidylinositide 3-kinase
PIP_2	phosphatidylinositol bisphosphate

PIP3	phosphatidylinositol triphosphate
PLA1	phospholipase A1
PLA2	phospholipase A2
PLC	phospholipase C
PLD	phospholipase D
PS	phosphatidylserine
PtdBuOH	phosphatidylbutanol
PtdOH	phosphatidic acid
PX	phox
PZ	plasticity zone
QS	quorum sensing
RB	reticulate body
SAR	structure activity relationship
SERM	selective estrogen receptor modulator
SM	sphingomyelin
T3SS	Type III secretion system
T6SS	Type VI secretion system
Ymt	Yersinia murine toxin

CHAPTER I

INTRODUCTION

Phospholipase D

Phospholipase D, or PLD, is a ubiquitous enzyme found in bacteria, fungi, plants, and mammals. PLD is a member of the PLD superfamily that consists of phosphodiesterases that hydrolyzes lipids or nucleic acids. There are several classifications for phospholipases, depending on the type of bond cleaved on phospholipids (Figure 1). Phospholipase A1 and A2 (PLA1 and PLA2) generate a lysophospholipid and a fatty acid by cleaving the ester bond linking the fatty acyl and glycerol backbone at the S_N1 and S_N2 positions, respectively. Phospholipase B (PLB) cleaves both ester bonds at S_N1 and S_N2 positions. Phospholipase C (PLC) cleaves the phosphodiester bond between the glycerol backbone and the phosphate to generate diacylglycerol (DAG) and a phosphate -containing headgroup. Finally, PLD cleaves the bond between the phosphate and the headgroup to generate phosphatidic acid and a free headgroup.

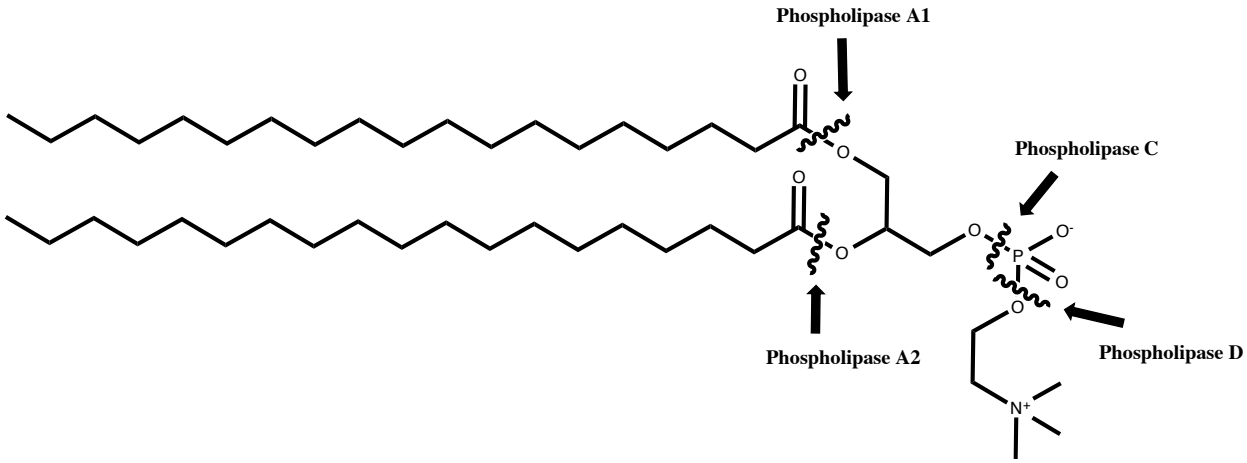

Figure 1. Comparison of phospholipase cleavage sites.

Enzymes were initially designated as PLD superfamily members based on their ability to hydrolyze phosphatidylcholine (PC), however with advancements in genome sequencing and bioinformatics, the PLD superfamily has grown to include more than just enzymes that hydrolyze PC. Now, classification as a PLD family member is based on conservation of a characteristic $HxKx_4Dx_6G(G/S)$ catalytic motif known simply as an HKD motif. HKD-containing family members include phospholipases but also cardiolipin synthase, phosphatidylserine synthases, and endonucleases.[1] Several non-HKD containing enzymes are also included in the family based on their ability to hydrolyze lipids to generate phosphatidic acid (PtdOH). Non-HKD family members include *N*-acyl phosphatidylethanolamine PLD, or NAPE-PLD[2], sphingomyelinases[3], glycosylphosphatidylinositol PLD[4], or GPI-PLD, and autotaxin[5].

Most HKD-containing PLDs possess two HKD motifs that, based on crystal structures of PLD superfamily members, come together to form one active site. Crystal structures of several endonucleases and bacterial PLDs including *Streptomyces* sp. PMF[6, 7], *Streptomyces antibioticus* (PDB deposited, but unpublished), *Yersinia* murine toxin[8], and Nuc endonuclease from *Salmonella typhimurium*[9] have been determined. Bacterial PLDs containing 2 HKD motifs have a bi-lobal structure with a pseudo 2-fold axis of symmetry, while the 1 HKD-containing Nuc protein crystallized as a homodimer. Based on structural and biochemical data, one histidine residue serves as the nucleophile to attack and cleave the phosphodiester bond in the substrate forming a phosphatidyl histidine covalent intermediate.[7, 8, 10] The second histidine hydrogen bonds to and activates water, which facilitates a nucleophile attack on the phosphatidyl histidine intermediate to generate PtdOH. Although PLDs primarily generate phosphatidic acid; many enzymes are also capable of using primary alcohols preferentially over water as a nucleophile to

produce a transphosphatidylation product. The transphosphatidylation reaction is commonly exploited to measure PLD generated PtdOH in cells. Phosphatidylalcohols are significantly more stable in cells than phosphatidic acid, which undergoes rapid turnover. Unfortunately no crystal structures have been solved for the larger and more complex PLDs from higher order eukaryotic organisms.

Eukaryotic PLDs are often much larger than their bacterial counterparts with more regulatory domains. Plant PLDs contain either a C2 domain or pair of PX and PH domains. C2 domains are only found in plants while mammalian PLDs contain PX and PH domains. C2 domains are lipid binding domains found in a variety of eukaryotic proteins, but most wide studied in Protein kinase C (PKC), with membrane recruitment often mediated in a calcium-dependent manner.[11] PX, or phox, domains bind phosphoinositides, primarily PI(3)P, but also $PI(3,4)P_2$, $PI(3,5)P_2$ and $PI(3,4,5)P_3$ and are found in proteins that mediate vesicular trafficking and protein sorting.[12] PH, or pleckstrin homology, domains bind phosphoinositides including $PI(3,4)P_2$, $PI(4,5)P_2$ and $PI(3,4,5)P_3$ and mediate protein-protein interactions.[13]

Human PLD isoforms 1 and 2 are the most studied HKD-containing PLD enzymes. Human PLD plays critical roles in cell signaling pathways that modulate vesicular trafficking, cell proliferation, and cell survival.[14-16] As such PLD has been studied in a variety of pathophysiological conditions including, cancer, inflammation, neurodegeneration, and pathogenic infections.[17] Here I will focus on the roles identified for PLD in infection.

PLD is expressed in a variety of mammalian tissues, including leukocytes. At least one PLD isoform is expressed in monocytes[18], macrophages[19], eosinophils[20], basophils[21], dendritic cells[18, 22], and lymphocytes[23, 24]. PLD expressed in human leukocytes is associated with antimicrobial mechanisms such as phagocytosis[19, 22, 25, 26], degranulation[27-29], respiratory burst[30, 31], microbial

killing[32-34], and chemotaxis[35, 36]. PLD activity is highly correlated with these antimicrobial functions however the intracellular mechanism by which PLD mediates these processes is poorly understood. Phagocytosis, chemotaxis, degranulation, and microbial killing involve actin polymerization and membrane trafficking, processes known to be regulated by PLD. PtdOH has also been shown to directly stimulate NADPH oxidase[31, 37] and may be a mechanism by which reactive oxygen species generation is stimulated.

Bacterial phospholipase D proteins

Although human PLD is associated with anti-microbial processes, several intracellular pathogens are known to secrete their own PLD enzymes to promote virulence through increased internalization or intracellular survival.[38-45] Virulence determinants are factors produced by a pathogen that serve to increase fitness during infection of a host. They are non-essential to cell viability, but are essential for pathogenesis, or the ability to cause disease. Virulence mechanisms may function to mediate entry into or colonization of the host, evasion or inhibition of the host immune defenses, and acquisition of nutrients from the host. Generally the processes mediated by bacterial phospholipases during infection are either (1) cytolytic by sensitizing or degrading host cell membranes or (2) via modulation of host signaling networks. PLD virulence factors are usually produced by obligate or facultative intracellular Gram-negative bacteria and they are less common than PLA or PLC virulence factors. Only a handful of bacterial PLDs have been characterized in terms of their role in pathogenesis. Table 1 summarizes the bacterial PLDs highlighted below.

Phospholipase D	Bacterium	Function
Ymt	*Yersinia pestis*	Flea colonization
NgPLD	*Neiserria gonorroheae*	Cell adhesion and invasion Akt signaling
LpdA	*Legionella monocytogenes*	Exacerbate cell toxicity of LecE Increasing DAG accumulation
PZ-PLDs	*Chlamydia* genus	Intracellular survival
A1S_2989	*Acinetobacter baumannii*	Serum survival Persistent colonization
PldA	*Pseudomonas aeruginosa*	Cell invasion Chronic infection Akt signaling Bacterial competition
PldB	*Pseudomonas aeruginosa*	Cell internalization Akt signaling Bacterial competition

Table 1. Bacterial PLD virulence factors and their associated functions

Yersinia pestis

Yersinia pestis is a Gram-negative bacterium that is the causative agent of bubonic plague. *Yersinia* murine toxin (Ymt) is a PLD that contains 2 HKD motifs. Recombinant Ymt was purified from *E. coli* and found to hydrolyze phosphatidylethanolamine (PE) and to lesser extents phosphatidylcholine (PC) and phosphatidylserine (PS), however it was not stimulated by canonical human PLD activators, phosphatidylinositol-4,5-bisphosphate (PIP_2) or the small GTPase, ADP-ribosylation factor 1, or Arf1.[8] Ymt is one of a few bacterial PLDs for which *in vitro* substrates have been characterized, however the *in vivo* substrates have not been identified. Purified Ymt was highly toxic to mice when directly injected but not required for virulence of *Y.*

pestis in mice.[46] *Y. pestis* is transmitted to its host by the flea vector and Ymt expression was higher at 26 °C than 37 °C, suggesting a role in flea transmission. Ymt was found to be important for colonization of the flea digestive tract.[47] *Y. pestis* colonizes the midgut and forms cohesive aggregates that eventually block normal feeding. With continued attempts to feed, bacteria are dislodged into the bite site and transmitted to the host. By increasing survival in the flea midgut, Ymt promotes vector colonization required for transmission to the host. Interestingly, heterologous expression of Ymt was able to increase colonization of the flea midgut by *Yersinia pseudotuberculosis* and *Escherichia coli.*[47] Wild type *Y. pestis* cells expressing Ymt could not rescue survival of Ymt mutant cells in fleas even with co-inoculation of both strains. This coupled with the lack of detectable secretion of Ymt, suggests that the effects of Ymt are mediated intracellularly, in constrast to most other bacterial PLDs studied to date which are secreted.[47]

Neisseria gonorrhoeae

Neisseria gonorrhoeae, an exclusively human pathogen, is a Gram-negative bacterium that is the causative agent of the sexually transmitted disease gonorrhea. *N. gonorrhoeae* invades human cervical epithelial cells by binding the complement CR3 receptor.[48] Engagement of the CR3 receptor triggers membrane ruffling to mediate endocytosis.[48] Upon cervical cell infection by *N. gonorrhoeae,* a subset of proteins are released. Proteomic analysis of the culture supernatant identified a secreted 55 kDa PLD homolog termed NgPLD, however the mechanism of secretion has not been identified.[44] Loss of NgPLD resulted in decreased cell association and invasion by *N. gonorrhoeae*. The exogenous addition of an unrelated PLD from *Streptomyces* sp. did not rescue the decrease in association and invasion, suggesting function specific to NgPLD. This PLD was found to increase recruitment of the CR3 receptor to the cervical cell surface and

promote cytoskeletal rearrangement to induce membrane ruffling. Upon cell infection, human Akt, a serine/threonine kinase important for mediating a number of different cellular processes including cell proliferation and survival, nutrient metabolism, endocytosis and vesicular trafficking, is activated in a phosphatidylinositol-3-phosphate kinase (PI3K) independent manner. PI3K is the canonical upstream activator of Akt signaling. PI3K converts phosphatidylinositol-4,5-bisphosphate (PIP_2) to phosphatidylinositol-3,4,5-triphosphate (PIP_3). Akt binds PIP_3 with high affinity within its PH domain allowing for recruitment of Akt to the plasma membrane. Upon translocation, phosphoinositide dependent kinase 1 (PDK1) phosphorylates Akt at T308. Akt is subsequently phosphorylated at S437 by mammalian target of rapamycin complex 2 (mTORC2) leading to full activation of Akt kinase activity. Akt signaling is also known to be activated during infection by a number of pathogens.[43, 49-53] NgPLD was found to interact with Akt in infected cell lysates and directly bind the PH domain of Akt *in vitro*.[43] Akt was the first host factor demonstrated to interact with a bacterial PLD during infection.

Legionella pneumophila

Legionella pnemuophila, a gram-negative facultative intracellular pathogen, is the cause of Legionnaires' disease, a severe form of pneumonia. Bacteria gain access to the lungs through contaminated water droplets and invade lung macrophages and epithelial cells to replicate. This leads to lung damage and subsequently pneumonia. *L. pneumophila* virulence is associated with a Type IV secretion system for which over 300 effectors have been identified.[54] Many effectors have redundant functions and target similar pathways in the host to evade phagosomal degradation and redirection of endoplasmic reticulum and mitochondria derived vesicles to *Legionella*-containing vacuoles (LCV) for LCV biogenesis and maturation.[54] *L. pneumophila*

replicates inside LCVs until membrane rupture and release of bacteria into the cytosol for further replication. Functional redundancy of these effectors makes identification of effectors and their mechanism difficult using tradition single gene manipulations. To expand identification of the Type IV effectors, a screen was performed to detect translocation of all open reading frames genes larger than 300 base pairs using a β–lactamase reporter assay.[39] Almost 800 gene products were analyzed for translocation and 70 new genes were identified as effectors. Several phospholipase genes were identified in this screen, including a PLD named LpdA for which the function later characterized. Unlike most of the phospholipase A effectors identified, LpdA did not induce cell toxicity in wild type yeast when overexpressed, however deletion of the host diacylglycerol kinase 1, *dgk1*, gene with LpdA expression was lethal to cells.[38] Co-expression of another Type IV effector, LecE, also promoted lethality in yeast. Although LecE was lethal when expressed alone, LpdA was able to exacerbate the toxicity suggesting synergy. LecE was found to stimulate the yeast phosphatidic acid phosphatase, Pah1, the enzyme that converts PtdOH to diacylglycerol (DAG). The increase in PtdOH by LpdA promoted an increase in DAG mediated by LecE activation of Pah1.[38] DAG generation is thought to promote recruitment of Protein Kinase D (PKD) and PKC to LCV to modulate phosphoinositides on the LCV membrane, which serve to anchor Type IV secretion system effectors to the LCV.

Chlamydia trachomatis

Chlamydia trachomatis is an obligate intracellular Gram-negative bacterium that infects ocular and genital tract epithelial cells causing conjunctivitis and sexually transmitted infections. *C. trachomatis* has a biphasic developmental cycle.[55] Elementary bodies (EB) are metabolically inactive and infectious. EBs invade host cells and remain within inclusions, or membrane bound vacuoles. EBs then differentiate into reticulate bodies (RBs) which divide by binary fission but

are not infectious. *C. trachomatis* is able to inhibit degradation within host cells by prohibiting maturation of inclusions into lysosomes. After replication, RBs differentiate back into EBs, which exit from host cells to repeat the cycle of infection. The core genome of *Chlamydia* strains is highly conserved, despite the range of hosts and tissue types infected. The plasticity zone (PZ) is a region of genetic diversity thought to be important for mediating the host, tissue, and disease diversity observed for *Chlamydia* species. *C. trachomatis* encodes several PLD homologs within the PZ (PZ-PLDs) and two PLDs found outside of the PZ, referred to as chromosomal PLDs. One chromosomal PLD, CT284, was found expressed early during the developmental cycle in EBs. While the other chromosomal PLD (CT084) and all PZ-PLDs were upregulated in mid- to late-cycle (CT154, CT155, CT156 – a protein truncated before the first HKD motif, CT157, CT158).[55] Protein expression of one PZ-PLD, CT155, was confirmed in the mid- to late-developmental cycle. CT155 was also found selectively localized to RBs adjacent to the inclusion membrane.[55] Primary alcohols have been used for years to investigate the roles of PLD in cellular processes. PLD utilizes primary alcohols such as ethanol or butanol thereby generating phosphatidylalcohol products and inhibiting PtdOH production. Viability of *Chlamydia* strains that encoded PZ-PLDs was decreased with primary alcohol treatment, while a strain of *Chlamydia caviae* that does not contain PZ-PLDs were not sensitive to primary alcohols.[55] There are caveats to this study. Primary alcohols would have inhibited the host PLD also, but no control experiments were performed to analyze the contribution of host PLD in *C. trachomatis* viability. Also no direct confirmation of PLD activity was performed for any PZ-PLDs and consequently no direct confirmation of primary alcohol inhibition of PtdOH production.

Acinetobacter baumannii

Acinetobacter baumannii is an emerging opportunistic pathogen for which multi-drug resistance has emerged. *A. baumannii* primarily infects the immunocompromised causing skin infections, ventilator-associated pneumonia, and bacteremia. A PLD gene, A1S_2989, has been implicated in modulation of serum survival of *A. baumannii*.[45] In agreement with its ability to contribute to serum survival, in a murine model of pneumonia, *pld* mutant strains exhibited decreased bacteremia. *In vitro* cell invasion and colonization of visceral organs were also decreased with genetic ablation of A1S_2989. Although lung bacterial burden and colonization of heart and liver tissue was not different between wild type and *pld* mutant strains 24 h after infection, colonization after 48 h was significantly lower for mutant strains, suggesting a role in persistence but not colonization for A1S_2989.[45]

Pseudomonas aeruginosa

Bacterial PLD enzymes produced by human pathogens generally have poor sequence homology to the mammalian host PLD, but one exception is PldA produced by *Pseudomonas aeruginosa* which contains up to 23-31% sequence identity and 39-50% sequence similarity, in the regions including and surrounding both HKD motifs.[56] Early characterization of PldA demonstrated that PldA could hydrolyze PC to generate PtdOH and perform the characteristic PLD reaction, transphosphatidylation, using ethanol and butanol, although weakly.[56] PldA was found localized to the periplasm, and its activity was stimulated by millimolar concentrations of calcium.[56] Genomic analysis of several environmental and clinical isolates revealed that only 30% of the examined strains encoded the *plda* gene. One important observation was that an upstream *vgr* gene and downstream open reading frame 4 (*ORF4*) were invariably present in only strains that also contained *plda*.[56] This tight genetic association suggests possible functional

association that was later confirmed by other groups.[57] Using a murine pulmonary infection model, *plda* deficient cells were outcompeted by the wild type cells after two weeks but not initially when co-infected, suggesting that PldA was important for persistent infections.[56] The mechanism for this was at the time unclear.

In 2006, the function of VgrG proteins was identified as a required, secreted component of a newly identified protein secretion system, referred to as the Type VI secretion system (T6SS) in *P. aeruginosa*.[58, 59] PldA, also named $Tle5^{PA}$, has since been confirmed as a secreted factor of the T6SS that facilitates bacterial killing and cell invasion by *P. aeruginosa*.[52, 57] *P. aeruginosa* encodes three distinct T6SS loci, H1, H2, and H3. The PldA-mediated interspecies growth advantage of *P. aeruginosa* with *Pseudomonas putida* required a functional H2-T6SS, suggesting that the H2-T6SS mediates PldA secretion into bacterial target cells.[57] The downstream *ORF4* gene was also identified to encode an immunity protein, termed $Tli5^{PA}$ that protects *P. aeruginosa* from the toxicity associated with PldA.[57] The exact mechanism has not been elucidated, but is hypothesized to be mediated by a direct protein-protein interaction.

Subsequent analysis confirmed PldA translocation of PldA into human epithelial cells and induction of cell internalization of *P. aeruginosa*.[52] Another PLD, PldB, was established as an H3-T6SS effector that also contributed to bacterial killing and cell invasion, in a similar manner to PldA.[52] Although only H2-T6SS and PldA were upregulated and expressed in exponential phase bacteria, stationary phase bacteria expressed both H2- and H3-T6SS gene clusters and both PLDs resulting in redundancy requiring gene ablation of both T6SS or both PLD for decreased cell invasion. This correlates well with previously described roles and redundancy of H2- and H3-T6SS in modulation of eukaryotic infection by *P. aeruginosa*.[60, 61] Both PldA and PldB directly interacted with Akt isoforms 1 and 2, but not 3 and induced activation of Akt as

evidenced by increased phosphorylation. Unlike NgPLD described earlier[43], PldA/PldB activation of Akt was PI3K-dependent[52]. PldA and PldB are two of only three T6SS effectors identified so far with dual functions modulating bacterial and eukaryotic cells.

Type Six Secretion system

The Type Six secretion system, T6SS, is the most recently discovered protein secretion system of Gram-negative bacteria. T6SS gene clusters have been identified in over 25% of bacterial genomes sequenced and are primarily concentrated in Proteobacteria.[62] About one third of bacteria encoding a T6SS have more than one T6SS locus with *Y. pestis* and *Burkholderia pseudomallei* having the most with 6 loci.[63] Most of these do not appear to be derived from recent duplication events. The evolutionarily distinct and conserved nature of these gene clusters suggests that they have evolved to serve particular niche functions.

A functional T6SS locus contains at minimum of 13 genes and upwards of 38 genes within the cluster.[64] These 13 genes comprise the core components. Nine genes encode proteins that are structurally similar to the bacteriophage tail, a needle-like injection complex. The T6SS is proposed to function like an inverted phage tail. Shown in Figure 2 is a depiction of structural components of the T6SS in comparison to a phage tail. The Hcp protein is a structural homolog of the phage lambda tail tube protein and forms hexameric rings thought to generate a tube-like structure serving as a conduit by which effectors may be translocated.[58, 65] The VgrG protein forms a trimer and is thought to serve as the cap of the Hcp tube that functions as the tail spike-like puncturing device.[66] VipA and VipB oligomerize to assemble long tubes thought to form a contractile tail sheath-like structure in the cytoplasm.[67, 68] VipA/B contraction may propel the Hcp/VgrG tube out the bacterial cell and into the target cell.[68] ClpV ATPase activity regulates the assembly and disassembly of the VipA/B contractile sheath.[67, 68] A four-gene cluster, *tssJ*,

tssK, *tssL*, and *tssM*, are thought to form a membrane-bound complex that anchors the phage-related complex to the cell envelope. The role of other core components has not been clearly defined but may serve a function similar to the phage baseplate.

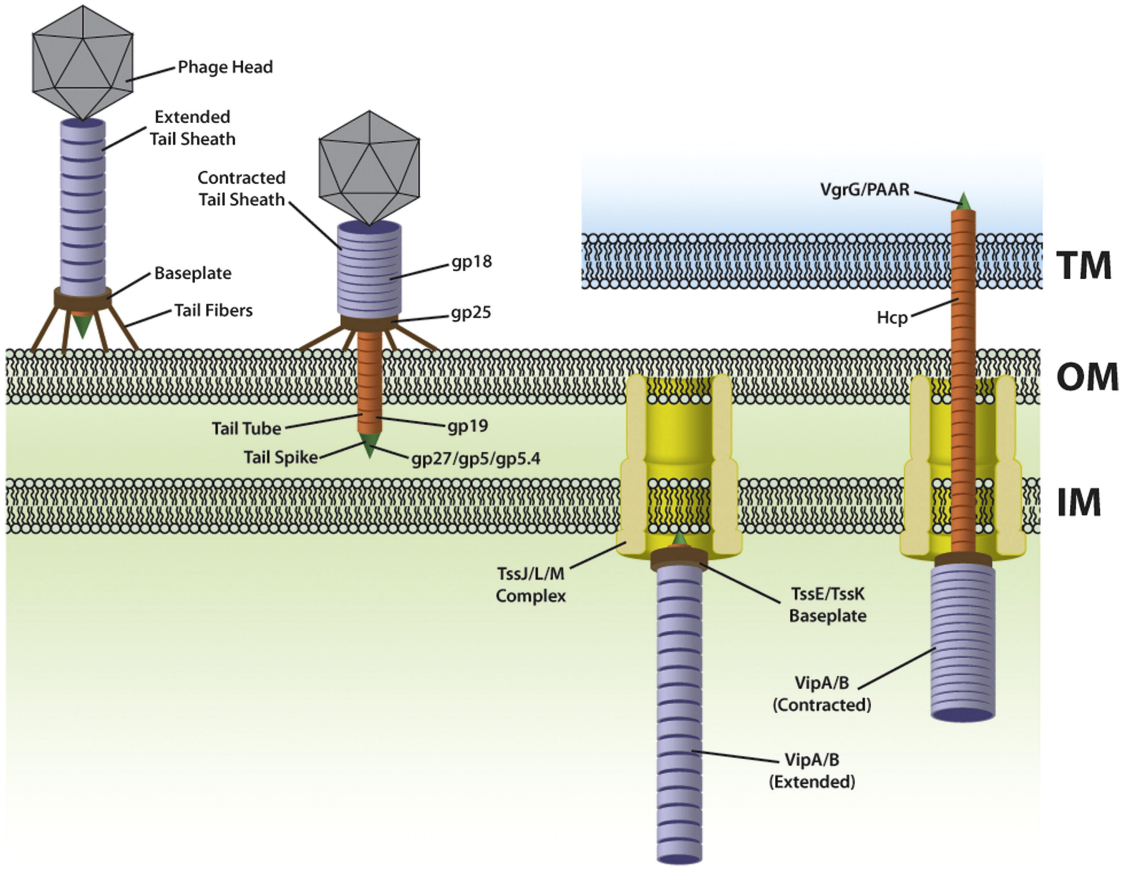

Figure 2. Comparison of bacteriophage tail structure and proposed T6SS structure. (Reproduced with permission from Ho, B. et al.[69])

Besides these core components, T6SS loci also may encode accessory proteins that post-translationally regulate T6SS activity. Some loci encode a threonine kinase (PpkA) and phosphatase (PppA) pair. PpkA phosphorylates another accessory protein Fha to trigger ClpV recruitment and T6SS assembly.[70, 71] The specific signal detected for PpkA activation is

unknown. Post-translational regulation allows for controlled and productive T6SS activity. The presence of various accessory proteins is highly variable across species suggesting that they have evolved to suit the particular needs of the organism.

P. aeruginosa encodes three evolutionarily distinct T6SS loci referred to as H1, H2, and H3. Like other bacteria with multiple T6SS gene clusters, they differ in transcriptional regulation, biological function, and associated effectors. In some bacteria T6SS are constitutively active,[59, 72, 73] while in other bacteria, such as *P. aeruginosa*, expression is tightly regulated. Various environmental cues and transcription factors modulate T6SS activity. In *P. aeruginosa*, the three T6SS gene clusters are differentially regulated by quorum sensing (QS), a cell-to-cell means of communication between bacteria discussed later. The H1-T6SS expression is suppressed by the LasR/LasI signaling, while H2- and H3-T6SS are upregulated.[60, 74] The RetS and LadS, two orphan hybrid sensor kinases, also regulate H1-T6SS expression.[75-77] RetS suppresses H1-T6SS and biofilm expression, whereas LadS counteracts these effects. RetS deletion is a commonly used method of inducing constitutive H1-T6SS activation. QS and two component systems are commonly used regulatory mechanism of T6SS expression for most bacteria.[78] Environmental conditions such as pH, temperature, osmolarity, phosphate, and iron have been shown to regulate T6SS expression.[78]

T6SS biological function

Initial characterization identified roles for the T6SS in virulence, however a growing body of research clearly demonstrates a role in intra- and inter-species bacterial competition. In some cases, the same T6SS can deliver both anti-eukaryotic and anti-bacterial effectors, while in other cases, a particular T6SS locus is associated with only one function. The T6SS of *V. cholerae* promotes killing of *Dictyostelium discoideum* amoebae and human macrophages and impaired

phagocytosis,[59, 79, 80] as well as cell-to-cell contact dependent bacterial killing[81]. Cell invasion of epithelial cells and virulence in *Arabidopsis thaliana, C. elegans,* and mouse infection models are mediated by the H2- and H3-T6SS of *P. aeruginosa,*[52, 60, 61] while H1-, H2-, and H3-T6SS all secrete toxins that facilitate bacterial competition. The H1-T6SS is also important for infection based on a rat model of chronic infections.[82] Loss of T6SS activity of avian pathogenic *E. coli* and *Edwardsiella tarda* resulted in reduced pathogenesis in chicks and fish, respectively.[83, 84] The killing of red blood cells, but not macrophages by *Campylobacter jejuni*, a major cause of food-borne infection, was mediated by the T6SS.[85] The T6SS-5 gene cluster in *Burkholderia mallei, B. pseudomallei, and B. thailandensis* is required for virulence in a hamster model of infection,[86] normal intracellular growth, proper actin cytoskeleton based motility after internalization and formation of multinucleated giant cells (MNGC) which are proposed to enable host immune system evasion and persistence.[86-88] The T6SS-1 cluster in *B. thailandensis* mediates bacterial competition in mixed bacterial cultures by protecting *B. thailandensis* from killing or stasis by other bacteria.[73] The T6SS of *Aeromonas hydrophila*, a cause of gastroenteritis, skin infections, and septicemia, modulates macrophage and epithelial toxicity, macrophage endocytosis, and septicemia in mice.[89, 90]

T6SS VgrG effectors

Although Hcp and VgrG were the first identified secreted proteins of the T6SS, these primarily function as structural proteins and are not secreted substrates in the truest sense. Because the Hcp/VgrG components are propelled out of the bacterial cell with T6SS activation, these proteins can be broken off into the culture supernatant or into target cells. There may be more than one VgrG protein associated with a particular T6SS locus. All T6SS have at least one associated 'core' VgrG, or VgrG proteins without an effector domain. A limited subset of T6SS

are also associated with an 'evolved' VgrG protein that has dual functions as a structural element as well as containing an effector domain.[89, 91-94] A particular T6SS may also be associated with orphan Hcp and VgrG genes distally located in the genome separate from the T6SS locus. *P. aeruginosa* encodes 10 different VgrG proteins, but only 3 T6SS loci. All 10 *vgr* genes are transcriptionally co-regulated with at least one T6SS locus.[95] Genes downstream of orphan Hcp and Vgr genes are hypothesized to encode for effectors.[95, 96] Some genes downstream of orphan VgrG genes have been confirmed as T6SS effectors, including PldA.[57, 97, 98]

T6SS bacteria targeting effectors

Identification of T6SS effectors has been a difficult and slow process, as effector secretion often requires cell-to-cell contact. Only then are proteins translocated and often at very low detectable levels. The wide range of functions displayed by T6SS effectors in part mediates the versatility of the T6SS. Confirmed effectors identified to date that mediate bacterial competition modulate one of three cellular components: (1) cell walls (2) membrane lipids, or (3) nucleic acids. Bacteria that encode bacteria-targeting effectors are also susceptible to the effectors they produce. To avoid this problem, cells concomitantly produce an 'immunity protein' that serves to neutralize the activity of the effect preventing autotoxicity, through a direct binding interaction.[99-101]

Cell wall targeting effectors have been confirmed as peptidoglycan amidases and muramidases. Peptidoglycan amidases were first identified in *P. aeruginosa* as Tse1 and Tse3.[102] Tse1, renamed Tae1, is a peptidoglycan amidase whereas Tse3, renamed Tgi1, is a muramidase. Peptidoglycan is an integral component of the cell wall of most bacteria. Peptidoglycan provides structural integrity and acts as an anchoring scaffold for cell envelope components. Peptidoglycan consists of linear glycan strands composed of alternating *N*-acetylglucosamine

(GlcNAc) and *N*-acetylmuramic acid (MurNAc) cross-linked by short peptides, which may include L- and D- amino acids and non-protein amino acid, commonly mDAP, or *meso*-diaminopimelic acid.[103] (Figure 3)

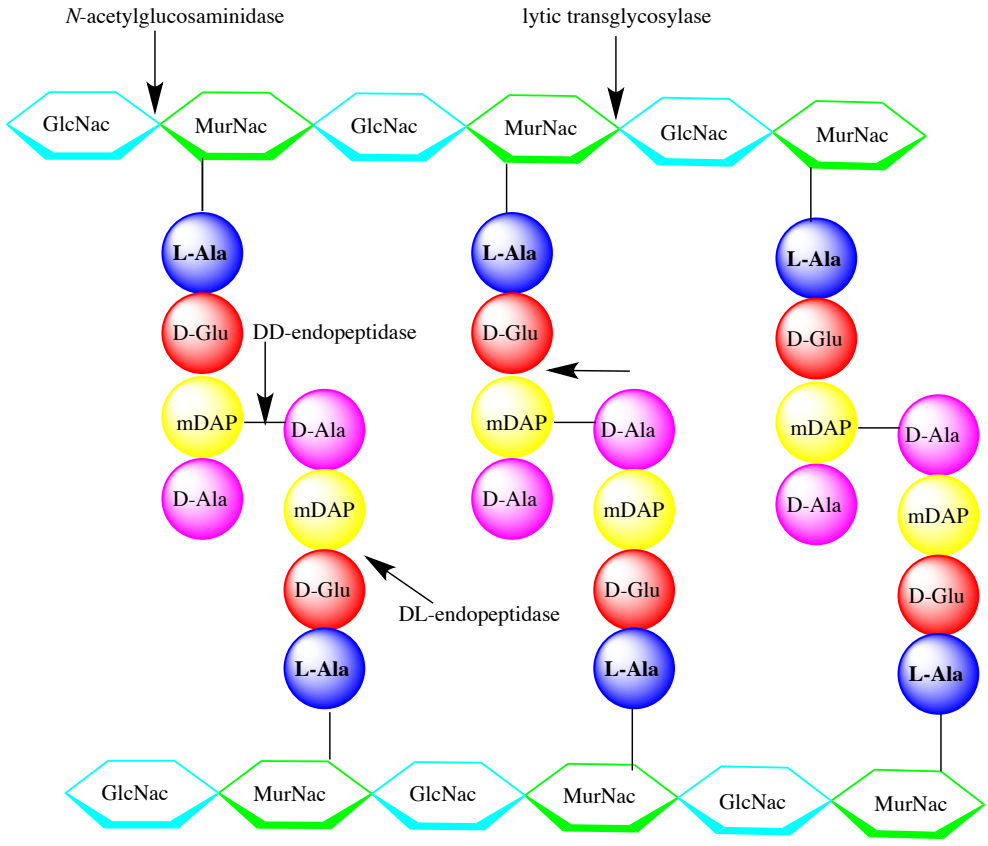

Figure 3. Structure of peptidoglycan and sites of cleavage by amidases and glycoside hydrolases. GlcNAc, *N*-acetylglucosamine; MurNAc, *N*-acetylmuramic acid; mDAP, *meso*-diaminopimelic acid; D-Ala, D-alanine; L-Ala, L-alanine; D-Glu, γ-D-glutamic acid.

Using bioinformatics, 51 total amidase-immunity protein pairs were identified in different bacteria and grouped into four clades, Tae1-4. Based on biochemical characterization, Tae1 and Tae4 members function as DL-endopeptidases, cleaving the bond between D-Glu and –mDAP,

whereas Tae2 and Tae3 are DD-endopeptidases, which hydrolyze the mDAP/D-Ala cross-bridges.[104] Muramidases, or Tge proteins, have peptidoglycan glycoside hydrolase activity meaning they cleave the glycan strand. Based on bioinformatics analysis of $T6SS^{+}$ bacterial genomes, three phylogenetically distinct families of predicted glycoside hydrolase effectors were identified.[105] Tge1 of *P. aeruginosa* was found to have lytic transglycosylase activity (cleavage of β-1,4-glycosidic bond between MurNAC, and GlcNAc) similar to lysozyme. Tge3 family members possess a Glu-Asp-Thr catalytic triads found in T4 lysozyme suggesting lytic transglycosylase activity as well. Based on crystallographic analysis, Tge2 family members in contrast are more structurally related the muramidases with *N*-acetylglucosaminidase activity (cleavage of β-1,4-glycosidic bond between GlcNac and MurNAC).

Lipases and phospholipases are confirmed effectors that target bacterial cell membranes. These proteins were separated into five families (tle1-5) depending on sequence homology and phylogenetic distribution.[57] Tle1-4 contained a GxSxG catalytic motif commonly found in esterase, lipases, and phospholipases. The Tle5 family contained $HxKx_4D$ catalytic motifs found in PLD superfamily members. The presence of immunity genes adjacent to *tle* genes suggested that they also target bacterial cells. However some Tle members facilitate bacterial competition while others are associated with virulence. Only members of the Tle1, Tle2, and Tle5 family have been enzymatically characterized to date. Tle1 from *Burkholderia thailandensis* ($Tle1^{BT}$) was found to have phospholipase A2 activity, while a Tle2 from *Vibrio cholerae* ($Tle2^{VC}$) possessed phospholipase A1 activity *in vitro*.[57] PldA, or $Tle5^{PA}$, and PldB of *P. aeruginosa* have been demonstrated to have PLD activity.[52, 56, 57] Genetic oblation of the PldA immunity protein resulted in decreased PE levels in *P. aeruginosa*, suggesting PE is the predominant substrate in bacterial cells.[57]

Interestingly, PldA and PldB have dual roles in mediating eukaryotic virulence and bacterial competition. PldA and PldB have been shown to promote cell internalization of *P. aeruginosa* and chronic infection.[52, 56] PldA as noted earlier is more related to eukaryotic PLDs and is believed to have been acquired through horizontal gene transfer from a eukaryotic source[56], while PldB is evolutionarily more similar to other bacterial PLDs[52]. PldA is specifically secreted by the H2-T6SS, while PldB is secreted by the H3-T6SS of *P. aeruginosa*.[52] The H2- and H3-T6SS are transcriptionally regulated in a similar manner and appear to have redundant functions.[60, 61, 106] No role in bacterial killing has been shown for $Tle2^{VC}$, but it is required for amoeba killing and to escape predation.[107] Deletion of $Tle5^{KP}$ of *Klebsiella pneumoniae* rendered the bacterium avirulent in a mouse model of acute pneumonia.[108]

Some predicted T6SS effectors contain a predicted nuclease domain linked to an Rhs (rearrangement host-spot) domain. Rhs domains have been found fused to a variety of predicted domains. RhsA and RhsB of *Dickeya dadantii* when overexpressed in *E. coli* caused degradation of cellular DNA and data suggest a T6SS-dependent secretion mechanism.[109] Tse2 of *P. aeruginosa* induces quiescence of targeted bacterial cells and inhibited growth of yeast cells, suggesting a conserved target.[99, 102] Tse2 is predicted to be an endonuclease, however no experimental confirmation of this is available. Other predicted endonuclease T6SS effector genes include PA0099 of *P. aeruginosa* and VP1415 and VP1263 of *Vibrio parahaemolyticus*.[97, 110] Downstream of two different VgrG proteins in *Agrobacterium tumefaciens*, two confirmed nuclease effectors were identified, Tde1 and Tde2.[111] Purified Tde1 demonstrated Mg^{2+} dependent DNase activity. Intracellular DNA fragmentation by Tde1 was confirmed using TUNEL staining. Tde1- and Tde2-mediated competition is the first case of an environmentally specific T6SS-associated competition between different species.[111] Interspecies competition with

E. coli on agar plates was Tde1-, Tde2- or T6SS-dependent, however no intraspecies competitive advantage was observed between wild type and T6SS deficient *A. tumefaciens* cells. However when bacterial competition was assessed using a plant co-infection model system, which more closely mimics a relevant *in vivo* environment than commonly used *in vitro* bacterial competition assays on agar, intraspecies competition was dependent on a functional T6SS and the Tde1 and Tde2 effectors. Interestingly, when bacterial competition with *P. aeruginosa* was assessed using the *in vitro* competition assay, *P. aeruginosa* outcompeted *A. tumefaciens* and the presence of a functional T6SS of *A. tumefaciens* actually induced increased killing by *P. aeruginosa* suggesting that a T6SS strike by *A. tumefaciens* could induce a toxic T6SS counterattack by *P. aeruginosa* under these conditions. This phenomenon, described as T6SS-dueling behavior, has been previously seen between *P. aeruginosa* and *V. cholerae or Acinetobacter baylyi*.[112] When bacterial competition of *A. tumefaciens* and *P. aeruginosa* was examined in the plant co-infection model, *P. aeruginosa* survival was reduced in the presence of wild type *A. tumefaciens* in a Tde1-, Tde2-, and T6SS-dependent manner.

For years, because of the large variability in regulation, biological function, and effector activities, context and niche specific functionality has been proposed for the T6SS. This study with *A. tumefaciens* highlights and confirms the role environmental settings have on observed bacterial competition mediated by the T6SS. This work also shows that T6SS-mediated bacterial competition can directly modulate populations of other bacterial species during infection. Bacterial competition may be an indirect virulence mechanism to promote colonization by eliminating competitors during infection. This is particularly interesting in the context of *P. aeruginosa* infections in cystic fibrosis (CF) patients. *Staphylococcus aureus* is an earlier colonizer of CF patients, however over time and with development of chronic infections, *P.*

aeruginosa becomes the primary colonizer outcompeting other bacteria, however the exact mechanisms by which this occurs are unclear.

T6SS eukaryote targeting effectors

Most T6SS effectors currently known to target eukaryotic cells are evolved VgrG proteins with catalytic activity via their CTD, or C-terminal domains. VgrG1 of *V. cholerae* induces G-actin cross-linking both *in vitro* and within host cells thereby disrupting actin filament formation and host cell morphology.[92, 113] When acting upon phagocytic cells, VgrG1 disruption of actin dynamics inhibits phagocytosis to promote bacterial evasion of immune cells.[79, 114] A vegetative insecticidal domain comprises the CTD of VgrG1 of *A. hydrophila. In vitro,* this CTD possesses ADP-ribosyltransferase (ADPRT) activity against actin. VgrG1 overexpressed in HeLa cells inhibited actin polymerization and induced cell rounding and apoptosis.[89] VgrG5 of *B. pseudomallei* and *B. thailandensis* is required for host cell fusion and MNGC formation to mediate cell-to-cell spreading, however the specific mechanism by which VgrG5 mediates fusion is unknown.[93, 94]

T6SS and effectors such as PldA are just one mechanism of virulence utilized by *P. aeruginosa*. Antibiotic resistance and disease severity associated with *P. aeruginosa* infections coupled with a lack of novel anti-bactericidal therapeutics highlights the importance of identifying and understanding mechanisms of pathogenesis in order to develop alternative therapeutic targets.

Pseudomonas aeruginosa pathogenesis

Pseudomonas aeruginosa causes nosocomial, or hospital-acquired infections, in primarily immunocompromised individuals, such as those with CF, severe burns, open wounds, pre-existing infections, or patients on ventilators. *P. aeruginosa* causes most commonly respiratory

infections, but also soft tissue infections, keratitis, and bacteremia.[115] Severe *P. aeruginosa* infections are associated with high morbidity and mortality.[116] The genetic diversity and plasticity afford *P. aeruginosa* affords the ability to survive in an array of natural and artificial environments.[117]

Respiratory infections by *P. aeruginosa* can be classified as either acute or chronic. A common acute infection is ventilator-associated pneumonia. Acute lung infections are also seen in patients in hospitals with impaired immune response, such as patients taking immunosuppression medications for surgery, or chemotherapy for cancer, or the elderly.[116] If acute infections are not properly cleared, chronic infections can develop. This is most often seen in CF patients. CF is a genetic disorder in which a mutation in a chloride channel, the cystic fibrosis transmembrane receptor (CFTR), results in improper chloride, sodium, and water transport in the lungs resulting in thickened mucous secretion and aberrant lung function.[118] *P. aeruginosa* colonizes the lung and causes acute infections that are initially cleared using antibiotic therapies. Over time however, therapeutics become ineffective and chronic infection develops. A persistent immunological response leads to excessive inflammation in the lung and in conjunction with secreted bacterial toxins results in deterioration of lung tissue and function, ultimately leading to respiratory failure.[117] The transition from acute to chronic infections is associated with phenotypic and genetic changes in *P. aeruginosa* over time.[119-122] Strains isolated during chronic infection are less cytotoxic and inflammatory than bacteria found in early acute infections of CF patients. This has been attributed to factors such as the downregulation of the Type III secretion system (T3SS), loss of flagellum and pili, increased biofilm and alginate production, and development of antibiotic resistance.[123]

P. aeruginosa employs a plethora of virulence determinants that mediate both acute and chronic infections. Flagella and Type 4 pili are important for mediating cell adherence and motility. Upon cell contact the T3SS is activated and can translocate various effectors that cause host tissue damage as well as modulate host signaling networks. Quorum sensing (QS) regulates the expression of a number of virulence determinants. Biofilm and alginate production is associated with increased antibiotic resistance and chronic infections. This list is by no means exhaustive. The focus here is on the most characterized mechanisms of virulence of *P. aeruginosa* and those for which small-molecule modulators have been developed to abrogate pathogenesis. Several studies have shown proof-of-concept that modulation of virulence is an alternative strategy to attenuate bacterial infections. Progress is in the very preliminary stages and far more work is needed to fully vet the potential of these strategies.

Cell adherence

P. aeruginosa possesses a single polar flagellum and several Type 4 pili at each cell pole. These structures mediate swimming and twitching motility, respectively, and are also stimulators of the immune system. [124] Flagellum is a whip-like protrusion composed of over 30 proteins that rotates to facilitate motility. Pili, through a process of extension and retraction of long protein fibers, facilitate twitching motility across solid surfaces.[125] In concerted movement with flagella, pili also facilitate swarming motility.[126] These are also the major cell adhesins of *P. aeruginosa.* Flagella bind the host asialyated glycolipid asialoGM1 on epithelial cells and stimulate NF-κB signaling.[127] Flagella are important for initial colonization and inducing acute infections, however flagella are downregulated in chronic infection isolates. Pili promote aggregation and microcolony formation, early required stages for biofilm formation.[128]

Type III secretion system

The T3SS of *P. aeruginosa* is a primary mechanism of protein toxin secretion. The T3SS is primarily associated with acute infections and cytotoxicity.[116, 129] Several Gram-negative bacterial pathogens encode a T3SS. The T3SS is a needle-like projection that directly translocates effectors into the host cytosol. Four T3SS effectors have been identified for *P. aeruginosa,* ExoS, ExoU, ExoT, and ExoY. ExoS and ExoU are the major exotoxins and are usually mutually exclusive. Cells either encode ExoS or ExoU, but rarely both. ExoS modulates host actin cytoskeletal organization through N-terminal GTPase-activating protein (GAP) activity targeting host small GTPases Rho, Rac, and Cdc42,[130, 131] and C-terminal ADP-ribosyltransferase activity targeting several host proteins[129]. ExoU is a phospholipase A2 that causes significant phospholipid, lysophospholipids, and neutral lipid degradation. ExoU is highly cytotoxic to host cells due to extensive membrane degradation resulting a necrotic-like phenotype[132], whereas ExoS cytotoxicity is slower and induces an apoptotic-like phenotype.[133]

Quorum sensing

QS is a mechanism of cell-to-cell communication between bacteria that is mediated by synthesized small molecules called autoinducers.[134] Each bacterium constitutively produces these autoinducers, but at low cell density the autoinducer remains at insufficient concentrations to induce receptor binding and activation. When the bacterial population increases, autoinducer concentrations increase above a threshold level and autoinducers bind their transcription activator receptors inducing changes in gene expression of many virulence determinants.

N-Acyl homoserine lactones (AHLs) and 4-quinolone autoinducers are both produced by *P. aeruginosa*. AHLs 3-oxo-dodecanoyl homoserine lactone (3-oxo-C12 HSL) and butyryl homoserine lactone (C4-HSL) are synthesized by LasI and RhlI, respectively and activate their

particular receptors, LasR and RhlR. The 4-quinolone signal 2-heptyl-3-hydroxy-4-quinolone (PQS) is synthesized by the proteins encoded in the *pqsABCDE* and *phnAB* operons and PqsH and activates its receptor PqsR. Hierarchical regulation of the QS systems exists. The *las* system stimulates the *rhl* system, which in term stimulates PQS synthesis. PQS can feedback and stimulate the *rhl* system.[135] The three systems regulate biofilm, rhamnolipid, alkaline protease, elastase, lipase, pyocyanin, and exotoxin A production and secretion, and motility [136]

QS regulates production of biofilms, a matrix composed primarily of polysaccharides, nucleic acids, proteins, and lipids that surrounds cell aggregates and serves as a physical barrier to protect from environmental stresses. Biofilms are a major virulence determinant and cause of antibiotic resistance. Biofilm production leads to antimicrobial drug resistance by multiple mechanisms. There are three main hypotheses to explain the increase antibiotic resistance of bacteria in biofilms.[137] (1) Penetration of antibiotics through the biofilm is retarded. Antibiotics, such as β-lactams, may be inactivated or positively charged antibiotics, such as aminoglycosides, may be absorbed onto the negatively charged polymers within the matrix. (2) Changes in the microenvironment reduce antibiotic efficacy. Bacteria within deeper layers of the biofilm are anaerobic due to low oxygen levels within the matrix. Aminoglycosides are less effective against anaerobic bacteria than aerobic bacteria. Changes in the osmotic environment may trigger osmotic stress to modulate porin levels and decrease cell permeability. Bacteria may enter a non-replicative state that makes them resistant to antibiotics that target replicating cells, such as penicillin. Although speculative, (3) a small sub-population may develop a unique phenotypic state allowing persistence even with continued antibiotic exposure.

These mechanism along with the genetic flexibility of *P. aeruginosa* provides the ability develop antibiotic resistance and establish both acute and chronic infections. Because of the

importance these mechanisms play in pathogenesis, more intensive efforts to identify novel virulence targets and therapeutics are underway by several groups.

Therapeutic targeting of virulence factors of *P. aeruginosa*

With the problem of growing multi-drug resistant *P. aeruginosa* infections and the lack of novel antibiotics in the drug pipeline, identification of novel targets and therapeutics are critical to treating these problematic infections. Targeting of virulence factors is a novel strategy for developing small molecule antibacterial compounds. Modulation of virulence mechanisms may avoid the pitfalls of current antimicrobials by targeting a system that is not required for viability. There is high selective pressure to develop resistance mechanisms of traditional antibiotics because they target essential processes required for viability. By targeting virulence mechanisms, there is presumably less selective pressure and resistance mechanism presumably less likely to develop or at least develop more slowly. The diversity of virulence mechanisms utilized by bacterial pathogens presents a large array of pharmacological targets with varied functions. Inhibition of virulence factors that are secreted and function outside of bacterium avoids issues with bacterial cell wall penetration and drug efflux. Virulence inhibitors can be used in combination with antibiotic therapies to sensitize bacteria to current therapeutic thereby increasing and prolonging the utility of antibiotics.

Targeting of virulence mechanisms also has its potential obstacles. Previous growth or viability assays used for screening traditional bactericidal compounds are not appropriate for screening inhibitors of virulence, as they will not directly affect viability. New screening methodologies will be required and may need to be highly specific to a particular mechanism or pathogen. For example, the assay conditions must mimic the appropriate *in vivo* environment and conditions necessary for expression and activity of the factor in an *in vitro* assay system and this

may limit the ability to develop screening assays. Inhibitors for some mechanisms, such as quorum sensing, may have broad-spectrum applicability while others may have a very narrow spectrum of activity, like those that target a particular secreted toxin due to variability between species. On the other hand, increased specificity may also lead to reduced cross-resistance mechanism. The temporal variability of virulence factor expression could also limit efficacy of some inhibitors *in vivo*.

Small-molecule inhibitors targeting quorum sensing and the T3SS have been developed as novel therapeutics candidates for *P. aeruginosa* infections. Vaccine therapies have also been developed for targeting flagella[138], type 4 pili[139], and alkaline protease, elastase, and exotoxin A chimeric proteins[140] but are not the focus here.

QS inhibitors

Because QS is a major regulator of many virulence determinants, including biofilm production, efforts by several groups have focused on developing inhibitors of QS. Small-molecule inhibitors of autoinducer synthesis, degradation of autoinducers, or inhibition of the autoinducer-receptor interaction have all been developed to modulate QS.[141] The most commonly employed strategy is disruption of autoinducer binding to its receptor. Several published accounts of synthesized AHL and quinolone analogs demonstrate antagonism QS systems and the downstream processes that they regulate, like biofilm formation. *N*-phenoxyacetyl and *N*-phenylpropionyl HSL potent analogs have been synthesized and shown to inhibit *P. aeruginosa* biofilm production.[142, 143] *N*-acyl cyclopentylamides also inhibit the *las* and *rhl* QS systems and also biofilm, pyocyanin, and rhamnolipid production.[144] Furanone derivatives have successfully inhibited QS in the lungs of infected mice, decreased bacterial burden, improved lung pathology, and prolonged survival time of mice infected with *P.*

aeruginosa.[145] Halogenated furanones increased sensitivity of biofilms to tobramycin treatment and decreased bacterial load in a mouse pulmonary model of infection, however bioavailability may be a limitation to their use.[146]

Screening of natural products has also identified QS inhibitors in grapefruit juice, garlic, and plant extracts. Bergamottin and dihydroxybergamottin, two furocoumarins found in grapefruit juice, inhibited *P. aeruginosa, E. coli*, and *S. typhimurium* biofilm formation.[147] Garlic extracts downregulated *las, rhl*, and PQS QS genes, as well as the genes regulated by QS.[148] Although treatment with garlic extracts alone did not inhibit biofilm production, garlic extracts increased sensitivity of *P. aeruginosa* to tobramycin treatment. Biofilm architecture was altered with garlic treatment alone with a flatter, more undifferentiated biofilm than the classical mushroom architecture of control biofilms. In a *Caenorhabditis elegans* model of infection, garlic extracts decreased lethality of *P. aeruginosa*. Using a mouse model of pathogenesis, garlic extract was found to increase the host inflammatory response and improve bacterial clearance.[149]

Although potent small-molecule inhibitors of virulence factors have been identified and shown efficacious *in vitro* and in some animal models, more preclinical and clinical data is still needed to validate the effectiveness of targeting QS to alleviate *P. aeruginosa* infections. More analysis of the bioavailability and pharmacokinetic parameters of these compounds are needed to fully evaluate potential of these compounds as therapeutics. Commensal bacteria also use similar QS systems. Data on effects of QS inhibitors on commensal flora are also lacking.

T3SS inhibitors

The T3SS plays an essential role in *P. aeruginosa* pathogenesis. Although small-molecule inhibitors of the T3SS of other pathogenic bacteria, including *Yersinia, Salmonella,* and *Chlamydia* species, and *E. coli*,[150] few studies have been performed to identify inhibitors of the

T3SS cytotoxicity by *P. aeruginosa*. In the first screen published, pseudolipasin A, an inhibitor of the T3SS effector ExoU was discovery, but directly inhibited ExoU and not T3SS function.[151] A phenoxyacetamide, MBX 1641, was found to inhibit translocation of T3SS effectors into host cells and have broad spectrum active against *Yersinia* and *Chlamydia*.[152] PscF, the needle protein of the T3SS, is the proposed target of MBX 1641 and its analogs.[153] Hopeaphenol, a resveratrol tetramer, was identified as a *Yesinia pseudotuberculosis* T3SS inhibitor in an irreversible manner.[154] Hopeaphenol also inhibited secretion of the T3SS effector, ExoS, by *P. aeruginosa* and attenuated cytotoxic in HeLa cells. Cross species inhibition suggest a conserved target. Lack of broad-spectrum activity for inhibitors of virulence factors is a drawback to inhibitor utility. The T3SS of many pathogens have highly conserved elements, making broad-spectrum therapeutics possible.

Although quorum sensing and T3SS inhibitors are the primary virulence mechanisms targeted so far for *P. aeruginosa* infections, proof of efficacy has been demonstrated for other virulence determinants of other Gram-negative bacteria. Inhibitors of toxins and bacterial adhesion have demonstrated efficacy *in vitro* and using animal models of infection. Virstatin was identified as a small-molecule inhibitor of cholera toxin production by inhibiting ToxT, the transcription factor responsible for cholera toxin expression, in *V. cholerae*. When administered prophylactically, virstatin treatment resulted in protection of infant mice from intestinal colonization.[155] When administered late after onset of diarrhea systems, virstatin could still reduce bacterial intestinal burden. Inhibitors of both primary toxins secreted by the T3SS, ExoU and ExoS, of *P. aeruginosa* have also been identified. The molecule exosin directly inhibited ADPRT of ExoS to protect CHO cells from ExoS-induced toxicity upon *P. aeruginosa* infection.[156] Pseudolipasin A directly inhibited the PLA_2 activity of ExoU *in vitro* and protected

CHO cells from ExoU-mediated cell toxicity upon *P. aeruginosa* infection. Importantly, pseudolipasin A is an Exo specific inhibitor as it did not inhibit a panel of eukaryotic PLA_2.

Adhesion is a critical initial step for host colonization by many bacteria and as such a bacterial adhesins are potential therapeutic targets. Gram-negative adhesion is primarily mediated by pili. Pilicides are compounds that inhibit pili biogenesis by disrupting chaperone-pilin complexes required for trafficking of pilin monomers to the outer membrane for oligomerization and pili formation. Bicyclic 2-pyridones competitively inhibit chaperone to pilin binding and greatly reduce bacterial adhesion of *E. coli* to bladder carcinoma cells.[157, 158] The chaperone-usher pathway for pili synthesis is highly conserved across bacteria, suggesting potential broad-spectrum efficacy.

In this introduction, I have presented a brief overview of PLD enzymes, specifically focusing on bacterial PLD that have been identified as virulence factors and briefly mentioning points of interest with respect to eukaryotic PLDs that are of relevance in subsequent chapters. As the focus of this project is centered around the enzyme, PldA, which is a secreted effector of the T6SS of *P. aeruginosa*, a summarized description of the T6SS and many of the effectors identified to date is presented here as the T6SS is integral to PldA function and relevance. Efforts to understand microbial pathogenesis have resulted in discovery of several mechanisms of virulence highlighted here for which the T6SS and PldA are included. Issue of drug resistance and unproductive antibiotic development has caused a shift in attention and focus towards targeting virulence mechanism rather than bacterial cell viability. Several studies aimed at virulence inhibitor for *P. aeruginosa* were discussed above.

Of importance to note was the lack attention and understanding of the enzymatic properties of previously described bacterial PLD. There is a significant gap in information about the

catalytic activity and molecular mechanisms by which bacterial PLD induce processes such as cell invasion and survival. In this body of work, I characterize various enzymatic properties of the P. *aeruginosa* PLD, PldA *in vitro* (Chapter II) and upon interaction with human epithelial cells (Chapter III) with the purpose of recognizing potential molecular mechanism involved during PldA interaction with eukaryotic cells. As PldA is important for *P. aeruginosa* infectivity, a second goal of this project discussed in Chapter IV was to develop small-molecule inhibitors of PldA as potential therapeutics.

CHAPTER II

IN VITRO CHARACTERIZATION OF PLDA ACTIVITY AND REGULATION*

Introduction

Phospholipase D, or PLD, is a ubiquitous eukaryotic protein that produces the signaling lipid phosphatidic acid (PtdOH). Roles for human PLD (hPLD) in a variety of signaling processes include cytoskeleton rearrangement, vesicular trafficking, endocytosis, and cell survival[14, 15, 159]. The involvement of hPLD in bacterial infections has long been recognized, but details on its function are lacking. PLD expressed in human leukocytes has been proposed to function in antimicrobial mechanism such as phagocytosis, degranulation, respiratory burst, and chemotaxis[32-35]. Several intracellular pathogens are known to secrete one or more PLD enzymes to promote cell internalization, intracellular survival, or *in vivo* infectivity[38-45, 56]. Cell invasion and intracellular survival for these bacteria often require endocytosis, cytoskeletal rearrangements and membrane trafficking and reorganization, processes hPLD is known to regulate.[159] PLD and phosphatidic acid regulate elements important for bacterial colonization, however host PLD activation often induces microbial killing, bacteria may secrete their own PLD to mimic specific or limited host PLD processes to their advantage.

Bacterial PLDs produced by human pathogens generally have poor sequence homology to the host PLD, but one exception is PldA produced by *Pseudomonas aeruginosa*. Higher order eukaryotic PLDs are generally larger than prokaryotic PLDs and contain one or more regulatory domains, such as phox and pleckstrin homology lipid binding domains or a C2 calcium binding

* Reproduced with permission from Spencer, C. T. and Brown, H. A. (2014) Biochemical characterization of a *Pseudomonas aeruginosa* phospholipase D, *Biochemistry*, in press. Copyright 2015 American Chemical Society.

domain. PldA is comparatively large for a bacterial PLD at 122kDa, but based on primary sequence analysis does not appear to contain these regulatory domains.

PldA is a secreted effector of the H2 Type VI secretion system (H2-T6SS) of *P. aeruginosa*[57]. The enzyme has been associated with promoting chronic infection[56], mediating intra- and inter-species bacterial competition [57], and targeting eukaryotic cells to promote cell invasion by *P. aeruginosa*[52]. Given the sequence similarity of PldA to hPLD isoforms and that PldA targets eukaryotic cells, I hypothesize that PldA will share some critical properties or regulation with human PLD. Those differences may provide insights into the mechanism by which PldA mediates virulence. Although enzymatic activity of PldA has been directly[56], an extensive analysis of catalytic activity and *in vitro* regulation has not previously been performed. In fact, very few PLD virulence factors have been enzymatically characterized. The *in vitro* and *in vivo* substrates are generally not defined. Regulators of protein activity are not evaluated and rarely are the molecular mechanisms by which host cells modulation occurs determined.

Here in Chapter II, I detail my *in vitro* characterization of PldA, identifying its substrate specificities, transphosphatidylation capabilities, divalent cation sensitivity, and lipid and protein activator and interactions. Understandings of the enzymology and regulation of PldA provide a framework from which to investigate its mechanistic roles during infection. Identification of interactions or regulatory mechanisms conserved between PldA and the host PLD provides insights into possible pathways involved or mimicked by PldA during infection.

Experimental Methods

Recombinant expression and purification of PldA

The PldA gene, provided by Dr. Michael Vasil (University of Colorado), was amplified by PCR using a forward primer containing a BamHI site and the reverse primer containing a HindIII

site. The amplified PCR product and pET32b vector were digested with BamHI and HindIII, then ligated using T4 DNA ligase to generate plasmid pET32b.PldA. The plda gene is in frame with the N-terminal thioredoxin fusion protein and His6 tag to generate a Trx-His6-PldA fusion protein. The PldA K169E mutant construct was generated using QuikChange II mutagenesis kit following the manufacturer's protocol. The PldA wild type and K169E mutant sequences were confirmed by sequencing.

E. coli BL21 CodonPlus (*DE3*) RIPL cells were transformed with pET32b.PldA. Bacteria were grown at 37 °C to OD600 of 0.6 in the presence of 100µg/ml ampicillin and 50µg/ml chloramphenicol. Protein expression was induced at 16°C with 0.1 mM IPTG for 12-16h. Cells were lysed by treating with 0.5 mg/ ml lysozyme for 20 min and sonication in wash buffer (8 mM Na_2PO_4, 2 mM KH_2PO_4 (pH 7.5), and 500 mM NaCl) in the presence of Roche protease inhibitor cocktail on ice. PldA was purified using a HiTrap chelating column. Protein was eluted using an imidazole gradient. Elution fractions were concentrated and further purified by size exclusion chromatography using a HiLoad 16/600 Superdex 200 column. Fractions were concentrated and stored at -20°C. PldA K169E mutant was expressed and purified the same as wild type PldA. Protein concentrations were determined using Bradford reagent. Protein purity was analyzed by SDS-PAGE.

To generate tagless wild type and K169 PldA, 10 mU thrombin/µg PldA was incubated with purified PldA fusion protein overnight at 4°C. The reaction was quenched with 1 mM PMSF. Tag removal was complete based on coomassie staining. Thrombin was separated from PldA using a HiTrap chelating column. Untagged PldA was tightly bound to the column even in the presence of 40 mM imidazole despite cleavage of the polyhistidine tag. Cleaved PldA and the 6his-thioredoxin tag were co-eluted with an imidazole gradient. Protein separation was

performed by ultrafiltration using a 50,000 kDa Amicon centrifugal concentrator to remove the thioredoxin tag and imidazole.

Mass spectrometry assay

1.1. General protocol

Purified PldA (50 ng) was incubated with lipids as indicated for each experiment at 37 °C for 10-30 min in reaction buffer (50 mM HEPES (pH 7.5) 100 mM KCl). Lipids in chloroform were dried under an N_2 stream in a glass test tube and resuspended in reaction buffer using bath sonication. PldA, lipid, and cations were combined on ice and reactions initiated by incubating at 37 °C. Reactions were quenched with 2:1 acidified methanol (0.1 N HCl:CH_3OH, 1:1)/chloroform. Lipids were extracted and mass spectrometric analysis of the organic layer was performed as previously described[160]. At least three independent experiments were performed in triplicate.

1.2. Substrate profile analysis

PldA was incubated with 25 μM of each lipid and 3 mM $MgCl_2$. For DOPE and 18:0p/18:1 PE conditions, liposomes were composed of PE and 18:1 SM in a 10:1 ratio.

1.3. Substrate fatty acyl chain saturation analysis

Lipids of various acyl chain compositions (16:0/16:0, 16:0/18:1, 18:1/18:1, 18:0/18:0, and 18:0/20:4) were combined to generate liposomes composed of a single lipid class. 18:0/20:4 PS and PG were not assayed. PldA was incubated with 6 μM of each lipid (30μM total PC or PG and 24 μM total PG) and 3 mM $MgCl_2$.

1.4. Divalent cation regulation

PldA was incubated with 50 μM 10:1 DOPE/SM, DOPG, or DOPC liposomes in the presence of 0.1-10 mM $MgCl_2$ or $CaCl_2$.

1.5. Transphosphatidylation analysis

PldA was incubated with 50μM 10:1 DOPE/SM, DOPG, or DOPC liposomes and 0.3% methanol, ethanol, n-butanol, ethanolamine, or glycerol, or 50 mM choline chloride and 3 mM $MgCl_2$.

1.6. Phosphatidylinositol-4,5-bisphosphate (PI(4,5)P_2) regulation

PldA was incubated with 50 μM 10:1 DOPE/SM or DOPG liposomes in the absence and presence of 5 mol% porcine brain PI(4,5)P_2 and 3 mM $MgCl_2$.

Exogenous PLD assay

Phospholipase D activity was measured using the modified exogenous PLD assay[161]. In brief, 50ng purified PldA was incubated with liposomes containing 50 μM DOPC, 2.5 μCi ^{3}H-DPPC, and any other indicated lipids added for 10-30 min at 37 °C. To assay the effect of human PLD protein activators on PldA activity, a range of concentrations (1-100 nM) of Protein Kinase Cβ II, Arf1, or Cdc42 were added. Protein Kinase Cβ II, Arf1, or Cdc42 were purified as described previously.[162] Final reaction conditions contained 50 mM HEPES (pH 7.5), 100 mM KCl, and 3 mM $MgCl_2$. The reaction was quenched with 10% trichloroacetic acid and bovine serum albumin on ice. Protein and lipid were removed by centrifugation. Supernatant was removed and free tritiated choline was measured by scintillation counting. At least three independent experiments were performed in triplicate.

Modified Amplex Red assay

Steady state kinetics was determined using a modified version of the Phospholipase D Amplex Red kit. In a 96-black well plate, the following components were combined on ice to yield final concentrations of: 50 ng PldA, 50 mM Tris (pH 7.5), 80 mM KCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 50 μM Amplex Red reagent, 0.1U/ml choline oxidase, and 1U/ml horseradish

peroxidase. Substrate, 12:0 PC or 14:0 PC, in chloroform was dried down under a N_2 stream and resuspended in 50 mM Tris (pH 7.5) and 80 mM KCl buffer. Lipid was added to the reaction mixture and fluorescence emission at 590nm (excitation = 530nm) was read continuously at 90s intervals for 60 min at 37 °C using a fluorescence plate reader. Background signal was removed by subtracting the signal at each lipid concentration without PldA. All reactions were performed in triplicate. Initial velocity was determined from the linear component of the fluorescence signal. Fluorescence units were converted to nmol choline by comparison to a choline chloride standard curve.

All kinetic analyses were performed using GraphPad 6.0. Initial velocity versus substrate concentration plots were analyzed using the Michaelis-Menten model (Equation 1) or substrate inhibition model (Equation 2)

$$v = \frac{V_{max}*[S]}{K_M+[S]} \qquad (1)$$

$$v = \frac{V_{max}*[S]}{K_M+[S]*\left(1+\frac{[S]}{K_i}\right)} \qquad (2)$$

PldA thermal stability was measured using the Amplex Red assay described above with some modifications. Two different concentrations of PldA, a dilute solution of 0.5 μg/ml and a concentrated solution of 0.5 mg/ml were incubated at various temperatures for 30 min. All reaction components and concentrations are the same as above, except a single concentration of 0.5 mM 14:0 PC was used. Activity was normalized to samples incubated at 0 °C.

Liposome binding assay

Lipids in chloroform and 3.2 μCi [^{3}H]-DPPC were dried under a stream of nitrogen. Lipid was suspended in 50 mM HEPES (pH 7.5), and 176 mM sucrose buffer by vortexing vigorously. Lipids were allowed to hydrate for 1 hour at room temperature. Lipids were subjected to 5 cycles

of freezing in liquid N_2 and thawing in a warm water bath. Vesicles were diluted 1:5 in an isotonic buffer (50 mM HEPES (pH 7.5), 100 mM KCl) and pelleted by centrifugation at 15,000 x g for 20 min at room temperature. Lipids were resuspended in the isotonic buffer. Final lipid concentrations were calculated based on [^{3}H]DPPC radioactivity.

For the binding assay, PldA was added to vesicles in a 1 ml reaction and incubated at room temperature for 5 minutes. Samples containing PldA without lipids were used as controls to determine background. The final reaction conditions were 2μg PldA 50 mM HEPES (pH 7.5), 100 mM KCl, 3 mM $MgCl_2$, and 100 μM lipid. Lipid-bound protein was isolated by centrifugation at 15,000 x g for 20 min at 4°C. The supernatant was removed and the lipid and protein resuspended in 1X SDS loading buffer.

For Western analysis, protein was transferred to a nitrocellulose membrane. The membrane was blocked with TBST containing 5% non-fat milk and incubated with anti-PldA rabbit polyclonal antibody. After washing, membranes were probed with HRP conjugated rabbit secondary antibody and developed using ECL reagent.

Akt kinase assay

The Akt phosphorylation of PldA was measured using a non-radioactive *in vitro* kinase assay. Akt1 was overexpressed with a polyhistidine tag in Sf21 insect cells, as described previously.[163] Briefly, 0.5 μg recombinant Akt was used with 0.5 μg PldA or 0.5 μg GSK3α/β fusion protein (Cell Signaling #9237) as substrates in the presence of 200 μM ATP in a kinase assay buffer [20 mM Tris (pH 7.5), 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM Na_3VO_4, and 10 mM $MgCl_2$]. The reaction was incubated at 30°C for 30 minutes. Reactions were terminated with SDS-PAGE sample buffer. Phosphorylation of PldA and GSK3α/β fusion

protein was detected by immunoblotting with a phospho-Akt substrate antibody (Cell Signaling #9614). Akt1 was detected using a pan-Akt antibody (Cells Signaling #9272)

Results and Discussion

Expression and purification of PldA

In order to perform *in* vitro studies, PldA was overexpressed in *E. coli* and purified. The PldA gene was cloned into the pET32b vector to produce a PldA fusion protein with N-terminal thioredoxin and polyhistidine tags (Figure 4A). Optimal protein expression required incubations at temperature below 18 °C. Lowering the induction temperature enhanced the stability of the enzyme by reducing accumulation of protein fragments. The Trx-His6-PldA fusion protein was isolated using immobilized metal affinity chromatography. Protein was concentrated and further purified using size exclusion chromatography. Protein yields after IMAC and SEC were 8.1 mg/L and 6.4 mg/L, respectively. For select experiments, a catalytically dead mutant was needed as a negative control. Site directed mutagenesis was used to generate mutant PldA K169E to render PldA catalytically inactive. For some experiments, tagless PldA was required. The thrombin cleavage site of the PldA fusion protein was utilized to remove the thioredoxin and polyhistidine tags and generate tagless wild type PldA and tagless PldA K169E constructs. SDS-PAGE analysis after purification for wild type PldA, PldA K169E, tagless PldA and tagless PldA K169E is shown in Figure 4B. All experiments were formed with the wild type Trx-His6-PldA fusion protein, hereafter referred to as PldA, unless otherwise stated. Efficient expression and purification of PldA was somewhat surprising, as active, recombinant hPLD has never been accomplished using *E. coli* expression systems. Heterologous expression of bacterial PLDs is also difficult to achieve in *E. coli*.[164]

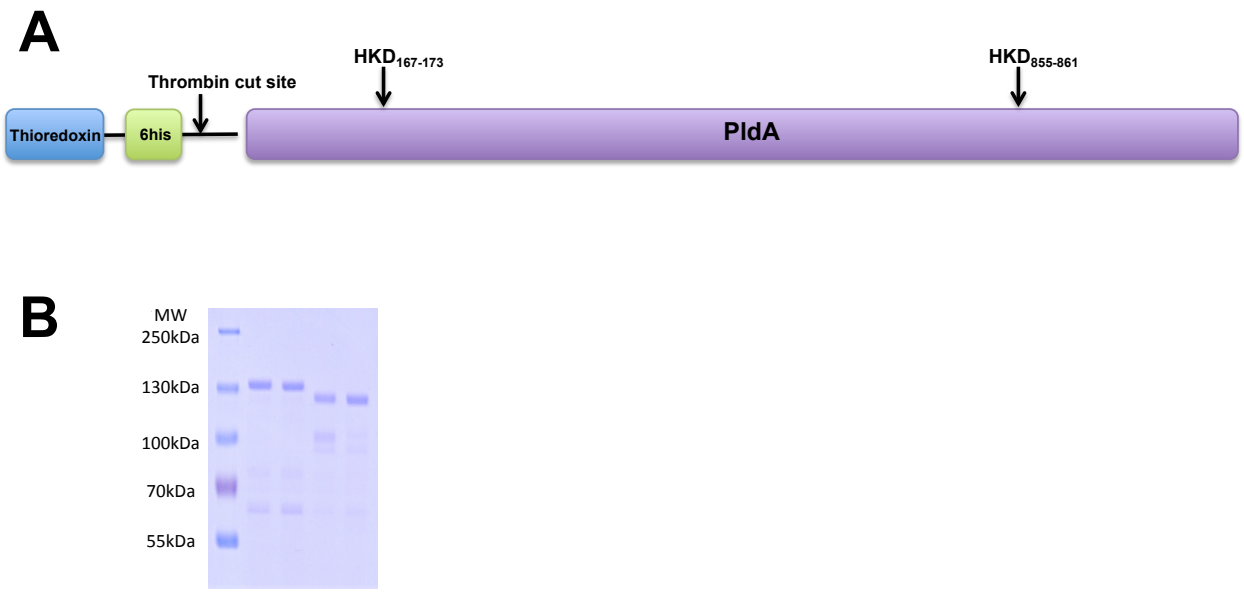

Figure 4. Purified recombinant PldA. (A) Scheme of PldA fusion protein construct. (B) SDS-PAGE analysis of recombinant PldA. Lane 1, molecular weight marker; lane 2, wild type PldA; lane 3, K169E PldA; lane 4, tagless wild type PldA; and lane 5, tagless K169E PldA.

pH and thermal stability of PldA

As little characterization of PldA has previously been performed, optimal reaction pH was determined. pH stability of PldA was assessed by measuring PC hydrolysis by PldA in reaction mixtures of various pH values (Figure 5A). Five difference buffer systems were used to obtain the desired pH of each reaction. Analysis of the pH stability of PldA reveals PldA activity over a range of pH values (5 – 9) with maximal activity at pH 7, however a precipitous decline in activity was observed at pH of 10. PldA activity was subsequently assayed at pH 7.4 for all *in vitro* experiments. PldA is active over a wide pH range, suggesting it can have activity in a variety of neutral and acidic suborganelles.

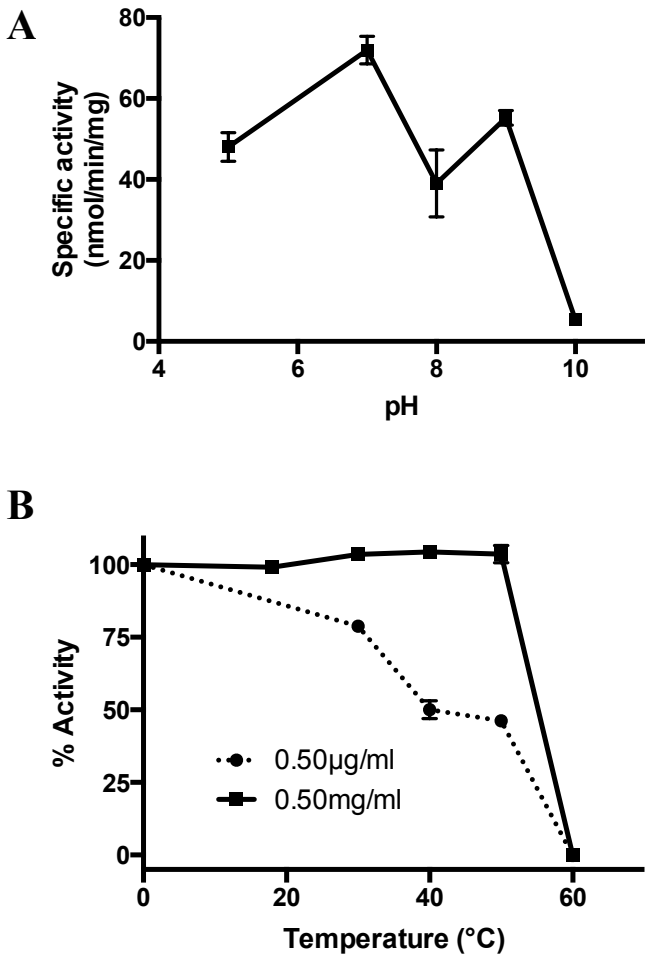

Figure 5. pH and thermal stability of PldA. (A) PLD activity was measured using exogenous assay. pH values were obtained using following buffer systems: pH 5, sodium acetate; pH 7, HEPES; pH 8, Tris; pH 9, imidazole; pH 10, sodium bicarbonate. (B) Dilute (0.5 μg/ml) or concentrated (0.5 mg/ml) PldA solutions were pre-incubated at indicated temperatures for 30 min prior to assaying PLD activity. Activity is normalized to samples incubated at 0 °C.

Initial characterization of PldA thermal stability was performed by pre-incubating PldA for 30 min at various temperatures prior to measuring PC hydrolysis at 37 °C using the exogenous assay. When dilute samples of PldA at 0.5 μg/ml were pre-incubated at elevated temperatures a

gradual decline in PldA activity occurred over a broad range of temperatures (Figure 5B). Upon further analysis, 1,000-fold more concentrated protein samples (0.5 mg/ml) showed a very sharp loss of activity only between 50 °C and 60 °C. For either dilute or concentrated PldA solutions complete loss of activity was observed at 60 °C. Protein concentration clearly has a significant impact on PldA thermal stability.

In vitro substrate analysis

Next, a fairly exhaustive analysis of potential PLD substrates was performed. The ability of diverse glycerophospholipids to serve as PldA substrates was assessed based on various chemical lipid features, including headgroup composition, fatty acyl chain unsaturation, and acyl or ether linkage to the glycerol backbone. Shown in Figure 6 are the structures of lipids assayed for hydrolysis by PldA. The ability of PldA to hydrolyze the major glycerophospholipids, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), and phosphatidylserine (PS) at a single concentration was determined. Due to the inherent instability of PE in aqueous solutions, DOPE (dioleoyl PE) and 18:1 sphingomyelin (SM) in a 10:1 ratio was used to generate stable lipid solutions. All further experiments with DOPE shown also include 18:1 SM. PldA utilizes all of these lipids as substrates, except PI, with variable hydrolysis rates (Table 2). The order of substrate preference was: DOPE>>DOPG>DOPS>DOPC. Lysolipids were also observed to be substrates for PldA, but the order of preference does not mirror that of the diacyl lipids (LPE>>LPC>LPG>LPS). Most lysolipids were also hydrolyzed to a lesser extent than their diacyl counterpart, except LPC. The hydrolysis rate of 18:1 LPC was significantly higher (8.6-fold) than hydrolysis of DOPC. PldA was not found to have any detectable sphingomyelinase activity. No hydrolysis of 18:1 SM was observed under any conditions including 10:1 PE/SM liposomes.

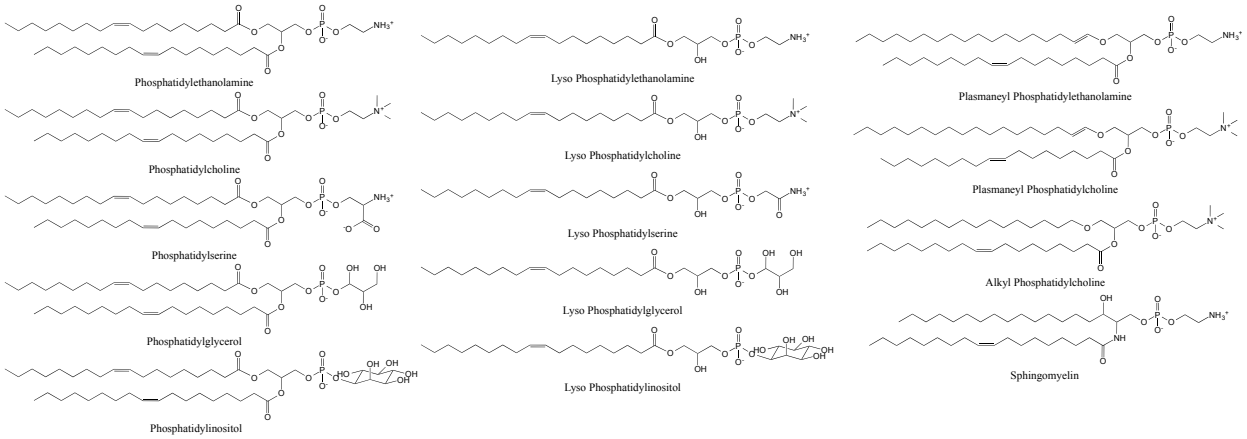

Figure 6. Structures of lipids analyzed for hydrolysis by PldA.

Table 2. Specific activity of recombinant PldA with various lipids[a]

Substrate	Specific activity (nmol/min/mg)
DOPC	15.3±3.6
DOPG	344.1±22.0
DOPE	2871.9±218.1
DOPS	229.3 ±25.8
DOPI	ND
18:1 LPC	130.4±17.9
18:1 LPG	57.3±2.6
18:1 LPE	1345.6±94.9
18:1 LPS	9.0±5.9
18:1 LPI	ND
18:0p/18:1 PC	46.3±5.6
16:0e/18:1 PC	12.3±2.8
18:0p/18:1 PE	1714.6±79.2
18:1 SM	ND

ND– not detected, [a] Values are given as mean ± SEM.

Despite the protein sequence similarity between PldA and eukaryotic PLD family members[52, 56], the substrate preference of PldA is noticeably different from that of the human isoforms and explains how PldA is able to significantly modulate bacterial and eukaryotic cellular lipids. Human PLD1 and PLD2 utilize PC as the primary substrate *in vitro* and *in vivo*, while PldA hydrolyzes PE and PG more readily than PC in a reconstitution assay. PE is the second most abundant phospholipid of most human cells thereby providing a significant substrate pool.[165] If PC were the primary substrate of PldA like human PLD, PldA would not be able to induce cell toxicity of bacteria, which generally contain little to no PC.

In order to further evaluate substrate specificity, alkenyl, or plasmalogen, and alkyl ether phospholipids were assessed as potential substrates. The hydrolysis rate of 18:0p/18:1 plasmenylethanolamine and 18:0p/18:1 plasmenylcholine was 50% and 300% that of the corresponding diacyl-linked lipids, DOPE and DOPC, respectively. To improve solubility of 18:0p/18:1 plasmenylethanolamine in solution, 18:1 SM was added in a 10:1 ratio of PE to SM. The alkyl-linked lipid 16:0e/18:1 PC also displayed a similar hydrolysis rate relative to DOPC. *In vitro* PldA can hydrolyze acyl-linked phospholipids as well as ether-linked lipids commonly found in eukaryotic cells.

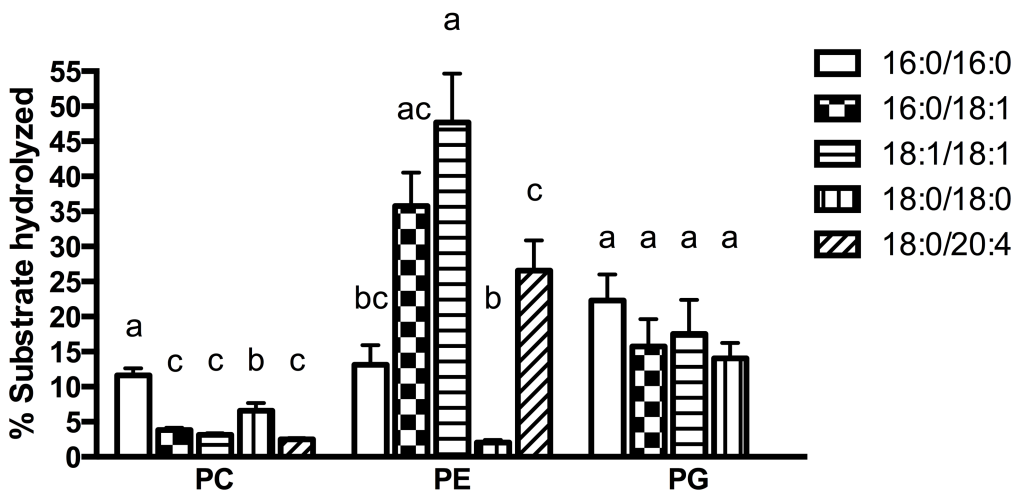

Figure 7. PldA displays variable substrate hydrolysis based on phospholipid fatty acyl chain composition. 18:0/20:4 PG was not assayed. Mean values are shown ± SEM. Statistical analysis performed using One-way ANOVA with Tukey post-hoc test. Groups not differing ($p>0.05$) share common letters.

We next examined whether PldA has preference for substrates of particular fatty acyl compositions. The effects of fatty acid unsaturation on PldA lipid hydrolysis were evaluated for three lipid substrate classes (Figure 7). PldA was incubated with mixed liposomes containing a single lipid class, such as PC, but a variety of fatty acyl moiety combination, such as 18:1/18:1, 16:0/18:1, 16:0/16:0, etc in equal proportions. In terms of PC hydrolysis, lipids with two saturated acyl chains were preferentially hydrolyzed over species containing one or more unsaturated acyl chains. This trend is opposite that of PE substrates, in which lipids containing unsaturated acyl chains were preferentially hydrolyzed over di-saturated PE lipids. As for PG substrates, little preference in hydrolysis was observed based on fatty acid unsaturation.

Whether PldA recognizes specific structural features of phospholipids or whether biophysical properties of these lipids, such as membrane fluidity and headgroup accessibility, directly modulate activity is unclear. *P. aeruginosa* lung infections over time significantly impact lung function in part by decreasing surfactant function, which is composed of roughly 90% lipid of which DPPC, dipalmitoyl PC, constitutes approximately 40%[166-168]. The major lipid components of surfactant, PC and PG, are all PldA substrates. Over time, PldA could contribute to destruction of surfactant lipids further reducing lung function.

Transphosphatidylation

PLD hydrolyzes phospholipids to generate phosphatidic acid; however many enzymes are capable of using primary alcohols as a nucleophile to produce a transphosphatidylation product. The ability of PldA to perform transphosphatidylation using a series of primary alcohols was evaluated (Figure 8A). PldA was capable of using methanol (MeOH), ethanol (EtOH), and *n*-butanol (BuOH) to produce transphosphatidylation products. The order of transphosphatidylation rates correlated with the order of hydrolysis rates in the absence of primary alcohols for the lipid classes (DOPE>DOPG>DOPC) (Table 2). Interestingly, the trends for transphosphatidylation rates for the alcohols varied for each substrate. When using DOPC as a substrate, transphosphatidylation rates with BuOH were slightly lower than with MeOH or EtOH. But when DOPG served as the substrate, transphosphatidylation rates with BuOH were slightly higher than with MeOH or EtOH. And finally when DOPE served as the substrate, transphosphatidylation rates were significantly higher with MeOH than other two alcohols.

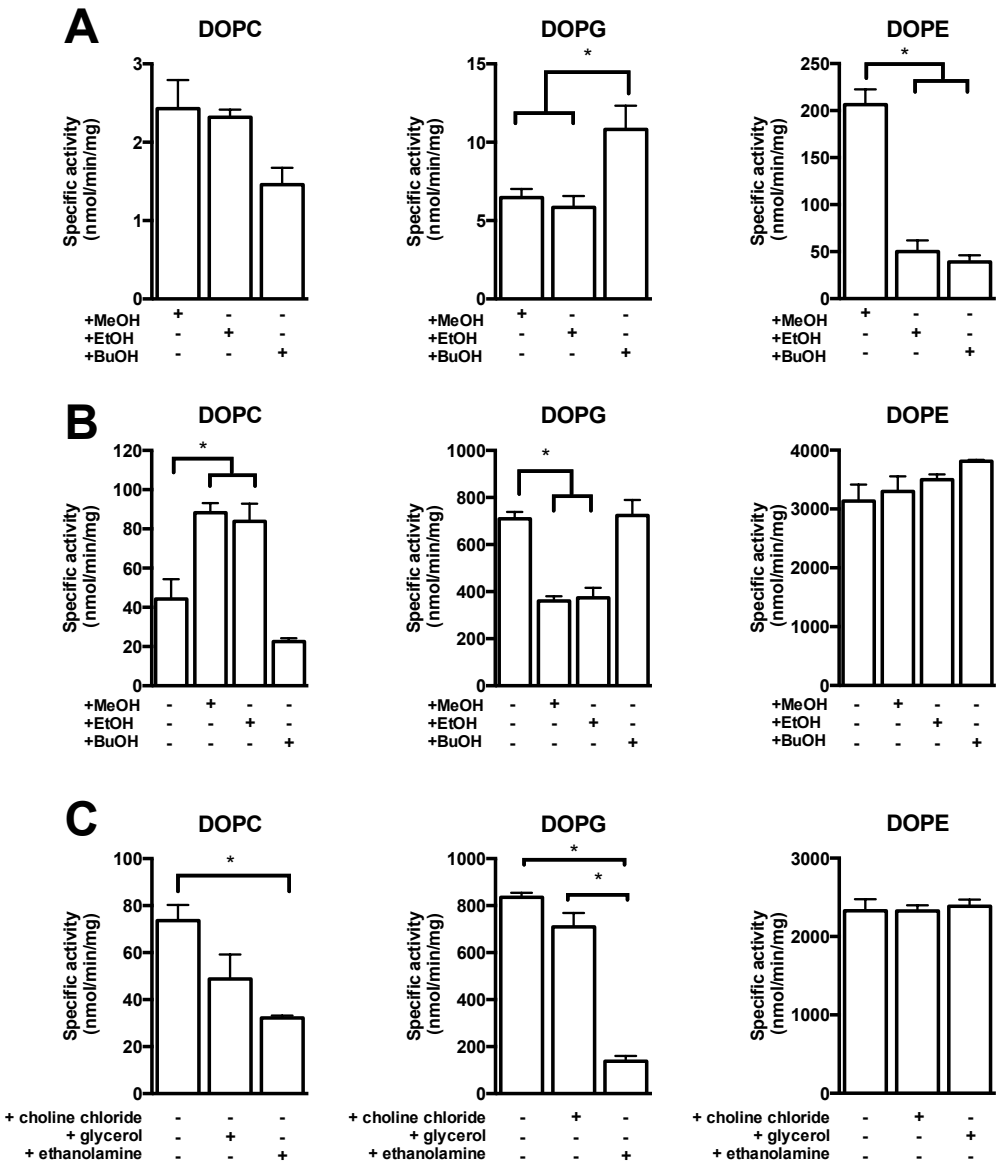

Figure 8. PldA transphosphatidylation and hydrolysis activity in the presence of primary alcohols. (A) PldA transphosphatidylation and (B) hydrolysis activity in the presence of methanol, ethanol or *n*-butanol. (C) Effects of free phospholipid headgroups on PldA hydrolysis activity. Substrates are shown above each panel. Mean values are shown ± SEM. Statistical analysis performed using One-way ANOVA with Tukey post-hoc test. *$p<0.05$

The effects of MeOH, EtOH, and BuOH on PldA substrate hydrolysis are summarized in Figure 8B. Changes in hydrolysis rates of DOPC and DOPG in the presence of MeOH, EtOH, and BuOH trended with phosphatidylalcohol production. A major difference was the stimulation of DOPC hydrolysis by MeOH and EtOH, but an inhibition of DOPG hydrolysis by MeOH and EtOH. In the case of DOPE hydrolysis, the addition of any of the alcohols resulted in a slight increase in hydrolysis rates that was not significant. Most interestingly, in many cases, these primary alcohols were ineffective at inhibiting PtdOH production, especially PE, which is the major substrate of PldA. Observed transphosphatidylation rates were low relative to hydrolysis rates. Transphosphatidylation rates for PG were only 1.5-1.8% of the hydrolysis rates in the presence of the alcohols, and transphosphatidylation rates using PC and PE were only 2-6% of the hydrolysis rates. Butanol between 0.1%-1% is commonly used to inhibit cellular hPLD mediated PtdOH production to probe the function of PLD in various cellular processes, but 0.3% alcohol was ineffective inhibiting *in vitro* PldA activity.

Because of the transphosphatidylation efficiency of some bacterial PLDs, many have been studied for use as biocatalysts for industrial production of phospholipids, such as PG, PE, and PS, from PC.[169] Transphosphatidylation activity of PldA with physiologically relevant headgroups, like choline, ethanolamine, and glycerol was also investigated (Figure 8C). The order of inhibition of PtdOH formation correlated with the order of substrate preference (ethanolamine>glycerol>choline). Essentially, the headgroup of a preferred substrate, such as ethanolamine of PE, inhibits the hydrolysis of the poorer lipid substrates. In the presence of 0.3% ethanolamine, hydrolysis of both DOPG and DOPC was significantly inhibited. The addition of 0.3% glycerol produced a modest decrease in DOPC hydrolysis, while DOPE hydrolysis was not altered. The addition of 50 mM choline chloride had no impact on DOPG or DOPE hydrolysis.

Glycerol was the only headgroup used by PldA under these conditions to generate a transphosphatidylation product and only from DOPE substrate with a transphosphatidylation rate of 114.2±10.5 nmol/min/mg, which is only 5% of the hydrolysis reaction (Table 2). So even with physiologically relevant alcohols PldA transphosphatidylation is low.

The transphosphatidylation reaction is a commonly observed property of HKD-containing PLDs. Although PldA can perform this reaction, the hydrolysis rates are significantly higher than the transphosphatidylation rates. *n*-Butanol is commonly used to inhibit cellular PtdOH production by hPLD isoforms. For some PLDs, primary alcohols like EtOH and BuOH are better nucleophiles than water, resulting in higher transphosphatidylation than hydrolysis activity.[170] In most cases assayed here, MeOH, EtOH, and BuOH were poor inhibitors of PtdOH production suggesting that these primary alcohols would likely be ineffective tools to study and modulate PldA activity in cells. Primary alcohols have been used as tools in the literature to investigate the role of bacterial PLD in virulence without verifying hydrolysis or transphosphatidylation activity.[55] It is important to perform these kinds of analyses, rather than assume all HKD-containing PLDs can perform the transphosphatidylation reaction and that the primary alcohols will decrease PtdOH production, as both these properties can be highly variable across different PLDs.[170-175]

Phosphatidylcholine substrate inhibition

Efforts were made to calculate kinetic parameters for PldA substrates, but proved complex. Interfacial enzymes, such as PLD, that interact with their substrate at an interface, in this case, a lipid membrane, do not follow Michaelis-Menten kinetics. Instead surface dilution kinetics is used to calculate the kinetic parameters associated with the enzyme.[176] This requires a bulk diluent, which can be a lipid or detergent, that does not interact at the substrate binding site but

can be mixed with substrate at an interface. Since most glycerophospholipids are substrates for this enzyme, interfacial kinetic analysis was complicated to perform due to the lack of a suitable bulk phospholipid diluent. Efforts to assay enzyme activity using lipid/detergent micelles resulted in very low activity and large error within replicates. Instead, we initially chose to define apparent kinetic constants ($K_{M,app}$ and $V_{max,app}$) for DPPC. Hydrolysis rates were plotted as a function of substrate concentration for DPPC liposomes (Figure 9A). When analyzed with non-linear regression, the substrate inhibition model (Equation 2) was a better fit to the data than the Michaelis-Menten model (Equation 1), however, this simple substrate inhibition model is still not the best fit for this system, suggesting that a more complex inhibition is occurring. To determine if this observation was specific for DPPC, we analyzed short chain lipids, 14:0 PC and 12:0 PC (Figure 9B and 9C, respectively) using a fluorescence assay. Although the decrease in activity was less pronounced than with DPPC, both of these substrates also produced data more amenable to the substrate inhibition model.

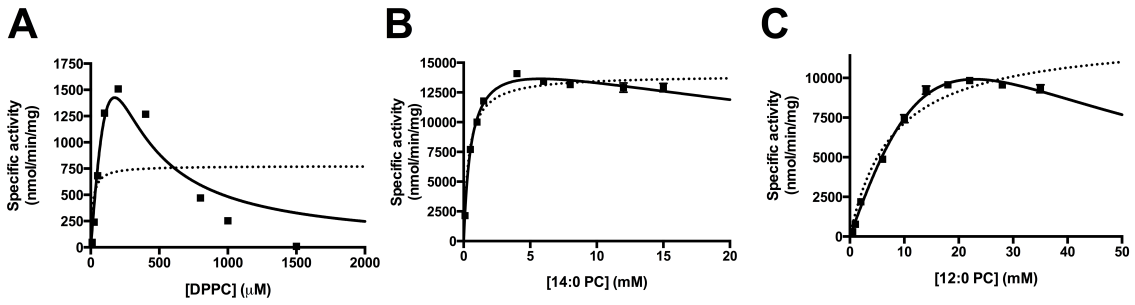

Figure 9. Phosphatidylcholine induces substrate inhibition of PldA. Steady state kinetics analysis was performed using (A) DPPC, (B) 14:0 PC, and (C) 12:0 PC and the Amplex Red assay. Solid line, Substrate inhibition model fitting; dotted line, Michealis-Menten model fitting.

The fit of the substrate inhibition model was not sufficient for generating kinetic parameters for DPPC. The confidence intervals were very large for the 12:0 PC data set, despite the high R^2 value bringing into question the accuracy of the values. For 14:0 PC, the fit was sufficient to calculate a V_{max} of 16.4 μmol/min/mg, a K_m of 0.58 mM, and a K_i was 57.2 mM.

Efforts to characterize the kinetic parameters for DOPG and DOPE were also problematic. As the fluorescence assay used for PC is specific to PC hydrolysis, mass spectrometry (MS) is required for measuring PG and PE turnover. Because of the micromolar to millimolar range of lipids needed, there were technical issues in quantitation of product formation by MS. The large excess of substrate relative to product leads to ion suppression and inaccurate quantitation. With large amounts of lipids, it is possible to saturate the LC column. To deal with this the samples can be diluted, but there is still the issue of low product to substrate ratio. For these reasons, we did not determine the kinetic parameters for DOPG and DOPE.

Overall attempts to generate kinetic rate constants for PldA proved to be technically challenging. Because most phospholipids were substrates of PldA, a suitable bulk diluent was not identified. Lipid-detergent micelles were unreliable and MS analysis of PtdOH in the presence of high concentrations of substrate was impractical. PC hydrolysis was instead characterized using a fluorescence assay, but appeared to induce complex substrate inhibition independent of lipid presentation. The underlying mechanism or physiological significance of substrate inhibition by PC is unknown.

Divalent cation stimulation

For some PLD enzymes, activity is stimulated by or dependent on divalent cations, such as magnesium and calcium[177-179]. PldA activity as a function of Ca^{2+} and Mg^{2+} concentrations was subsequently measured. Robust hydrolysis of DOPE did not require either of these ions and only

a modest 1.6-fold increase in activity at 10 mM $MgCl_2$ was observed (Figure 10A). Increasing concentrations of $CaCl_2$ produced a modest, but significant decrease in DOPE hydrolysis at 5 mM and 10 mM. In contrast, a large concentration dependent stimulation of DOPC and DOPG hydrolysis was observed with maximal activity seen above 2.5 mM $MgCl_2$ and $CaCl_2$ (Figure 10B and 10C). DOPC activity did require Mg^{2+} and Ca^{2+}, however PG hydrolysis was barely detectable without the ions present. Interestingly, for DOPG, Mg^{2+} and Ca^{2+} produced similar changes in the absolute specific activity at comparable concentrations, however, for DOPC, Mg^{2+} and Ca^{2+} produced similar patterns of stimulation, but Mg^{2+} was more effective at stimulating higher specific activity at comparable cation concentrations.

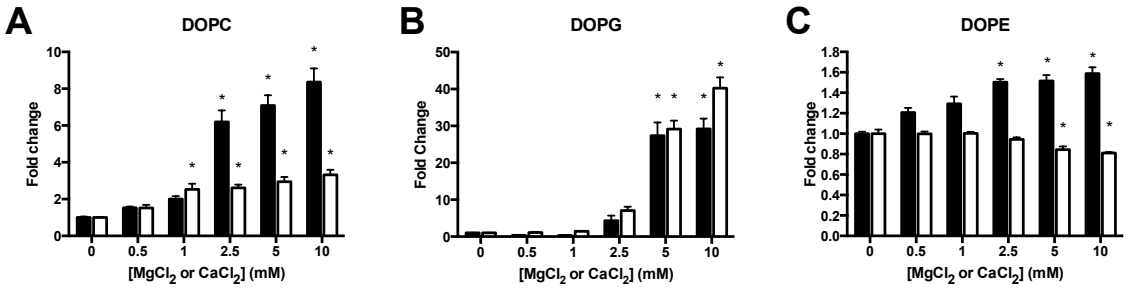

Figure 10. Divalent cations stimulate PldA activity *in vitro* in a concentration dependent manner. $MgCl_2$ (black bar) and $CaCl_2$ (white bar). Substrates are shown above each panel. Mean values are shown ± SEM. Statistical analysis performed using One-way ANOVA with Tukey post-hoc test. *$p<0.05$

Divalent cations often stimulate catalytic activity of PLDs[171, 177, 178]. PldA does not display an absolute requirement of divalent cations for catalytic activity, however robust stimulation does occur in a concentration dependent manner. Although the concentrations of free intracellular

Mg^{2+} (0.5-1 mM) and Ca^{2+}(0.3-0.5 mM)[180] in human cells are below the levels observed for PldA stimulation in this study, the effects of the cations may be primarily due to increased PldA-lipid interactions rather than direct modulation of PldA. If divalent cations were required for catalysis, all substrates would be expected to be equally effected, but divalent cations were required only for the hydrolysis of the anionic substrate PG.

Stimulation by host PLD regulators

Eukaryotic PLDs are regulated through a variety of protein-protein and protein-lipid interactions.[16] Since PldA is a secreted protein and due to the significant sequence homology to the hPLD isoforms, some of these interactions for hPLD were investigated as regulators of PldA. hPLD activity is stimulated *in vitro* and *in vivo* by PKC and small GTPases, such as Arf and Cdc42[162, 181-184]. Activators were used in a range of 1:6 to 16:1 activator/PldA. Stimulation of PldA activity *in vitro* was not observed for these small GTPases or PK C, even with 16-fold excess at 100 nM (Figure 11). At most, a 50% increase in activity was seen with Arf1 at the two highest concentrations, in contrast to the potent >70-fold stimulation of hPLD by Arf1[162].

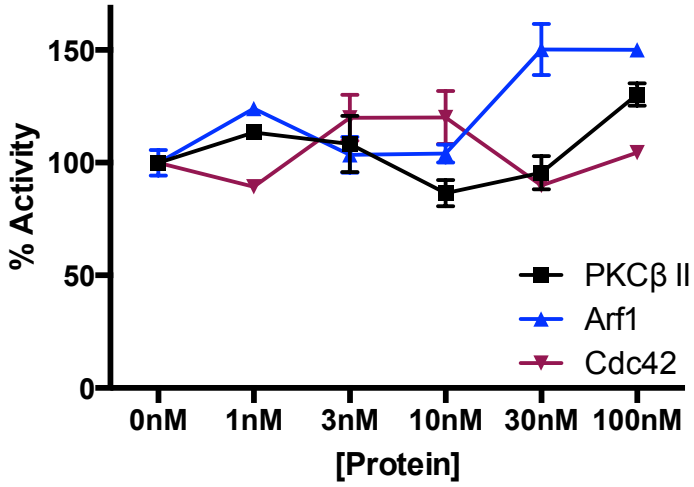

Figure 11. Human PLD activators do not stimulate PldA activity *in vitro*. PLD activity was measured using exogenous assay in the presence of Protein kinase Cβ II, Arf1, or Cdc42 at indicated activator concentrations. Mean values are shown ± SEM.

In many cell types, hPLD1 and hPLD2 basal activity is very low or not detectable without activation by a signaling event, which activates PKC or small GTPases, such as RhoA, Cdc42, and Arf. No stimulation of PldA was observed *in vitro* by these protein activators over a range of concentrations; however, this isn't too unexpected as most of the mapped interactions between the hPLD isoforms and these regulators are localized to regions not conserved in PldA, such as the PX and PH domains or C-terminus of human PLD.[184-186] I speculate that by secreting a PLD that is not as tightly regulated as the host PLD, *P. aeruginosa* can directly induce PLD signaling networks while circumventing unnecessary or potentially disadvantageous signaling networks. Perhaps only specific protein-protein interactions or regulatory mechanisms are conserved between the bacterial PLD and the host PLD affording only a limited subset of signaling pathways to be activated by PldA. This theory draws on the observation with *C. trachomatis*, in

which activation of the host PLD in epithelial cells decreased intracellular bacterial viability however whereas *C. trachomatis* PZ-PLD increased survival.[40, 55]

Phosphoinositide stimulation

Phosphatidylinositol-4,5-bisphosphate, $PI(4,5)P_2$, is a potent lipid stimulator of hPLD activity both *in vitro* and *in vivo*.[187-189] hPLD contain N-terminal PH and PX homology domains that are important for binding phosphoinositides[190] however, based on sequence analysis PldA does not appear to contain either of these domains. Here $PI(4,5)P_2$ stimulation of PldA was also examined. Based on sequence alignment using ClustalW[191], PldA does possess a partially conserved $PI(4,5)P_2$ binding domain between its two HKD motifs that was identified in the catalytic domain of eukaryotic PLDs[192], but there are number of clusters of basic amino acid throughout PldA that may facilitate phosphoinositide binding and stimulation (Figure 12A). One potential mechanism by which $PI(4,5)P_2$ can increase PLD activity is to increase lipid binding affinity. To examine this possibility, protein binding of PldA to liposomes comprised of 100 mol% DOPC or 95 mol% DOPC: 5 mol% $PI(4,5)P_2$ was assessed. Based on the Western blotting results in Figure 12B, $PI(4,5)P_2$ caused a significant increase in binding to DOPC liposomes.

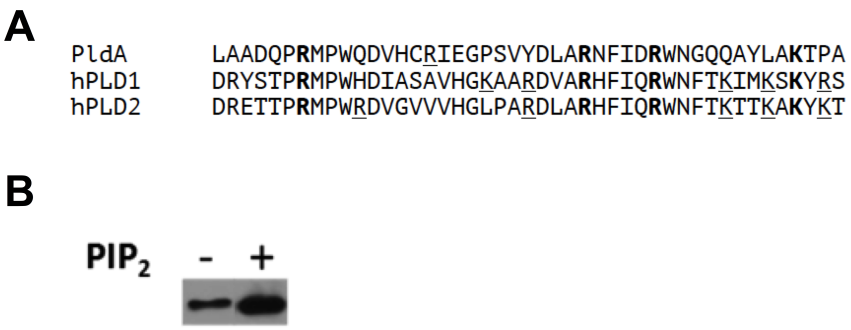

Figure 12. PldA contains partially conserved PI(4,5)P_2 binding site. (A) Sequence alignment of phosphatidylinositol-4,5-bisphosphate binding site of human PLD1 (hPLD1) and human PLD2 (hPLD2) with corresponding homologous region in PldA. (B) PIP_2 increases PldA binding to DOPC liposomes

As shown in Figure 13, the addition of 5 mol% PI(4,5)P_2 to DOPC liposomes resulted in an 8-fold stimulation of PC hydrolysis. To determine if this stimulation is specific for DOPC, hydrolysis of DOPG and DOPE liposomes in the absence and presence of 5 mol% PI(4,5)P_2 was compared. (Figure 13) The addition of PI(4,5)P_2 to DOPG liposomes, resulted in a modest 1.2-fold increase in PldA activity, while PI(4,5)P_2 caused a 20% reduction in DOPE hydrolysis. PI(4,5)P_2 stimulation of PC hydrolysis by PldA was also concentration dependent as seen in Figure 14.

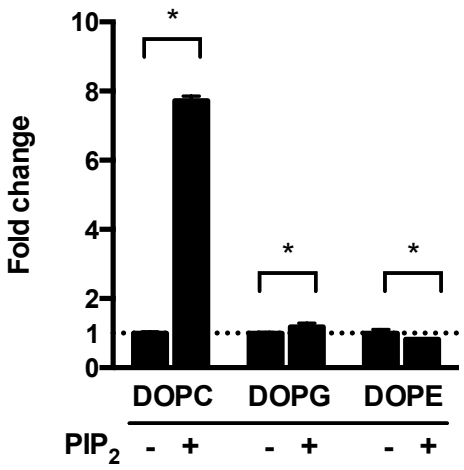

Figure 13. PIP_2 stimulates PldA hydrolysis of PC and PG *in vitro*. PLD activity was measured using LC-MS. Mean values are shown ± SEM. Statistical analysis performed using student's t-test. *$p<0.05$

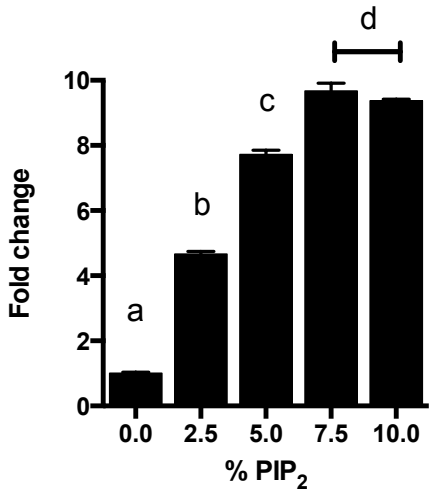

Figure 14. $PI(4,5)P_2$ stimulation of DOPC hydrolysis is concentration dependent. PLD activity measured using exogenous assay. Statistical analysis performed using One-way ANOVA with Tukey post-hoc test. Groups not differing ($p>0.05$) share common letters.

We also investigated the specificity of the $PI(4,5)P_2$ stimulation by assaying the effects of other phosphoinositides on PldA activity. PldA hydrolysis of DOPC liposomes containing 5 mol% PI, phosphoinositides, or diacylglycerol pyrophosphate (DGPP) was measured. DGPP was included as another anionic lipid control. PI did not stimulate PC hydrolysis, however all phosphoinositides stimulated hydrolysis to varying extents (Figure 15). The order of potency for phosphoinositides was PIP_3>PIP_2>PIP. The difference in stimulation was not dependent on the location of phosphate on the inositol head group, but rather the overall number of charges. DGPP also stimulated PldA activity to a similar extent as the mono-phosphorylated phosphoinositides.

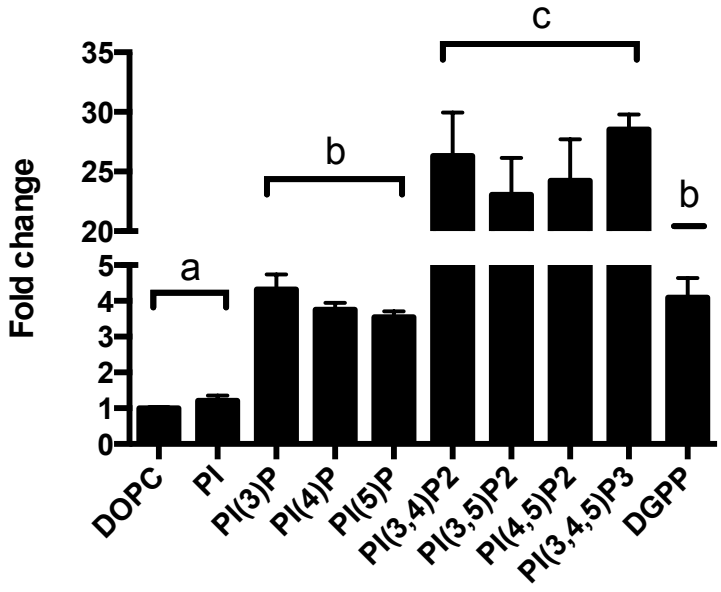

Figure 15. Phosphoinositides stimulate DOPC hydrolysis. All phosphoinositides were 36:2 species, except for brain PI(4)P. PC and phosphoinositides were in 20:1 ratio. PLD activity was measured using exogenous assay. Statistical analysis performed using One-way ANOVA with Tukey post-hoc test. Groups not differing ($p>0.05$) share common letters.

As a strong divalent cation requirement was seen with hydrolysis of the anionic lipid PG (Figure 10), leading me to speculate that divalent cations may be needed to facilitate interactions with anionic lipids, this hypothesis was extended to PI4,5)P_2 stimulation of PldA. Is PI(4,5)P_2 stimulation of PldA Mg^{2+} or Ca^{2+} dependent? In fact, no stimulation of PC or PG hydrolysis by PI(4,5)P_2 occurs in the absence of a divalent cation supporting the notion of divalent cations mediating protein-lipid binding. (Figure 16)

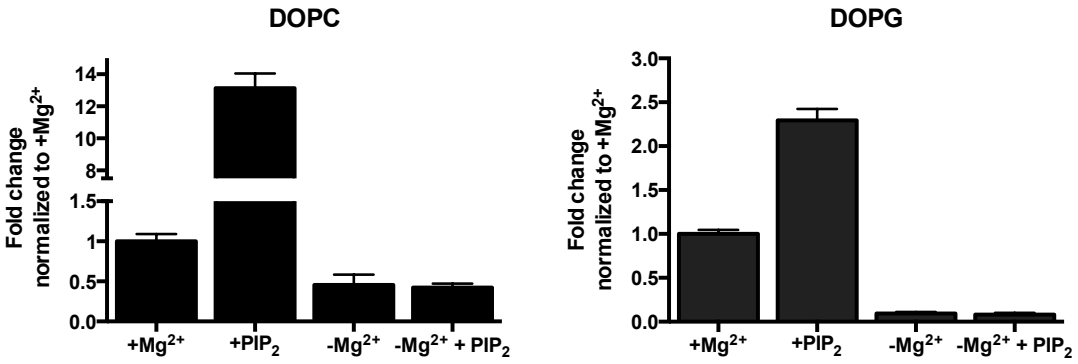

Figure 16. Divalent cations are required for PIP_2 stimulation of PC and PG hydrolysis. Substrates are designated at top of graph. PLD activity was measured with LC-MS. 5 mol% PIP_2 and 3 mM $MgCl_2$ were used in $+PIP_2$ and $+Mg^{2+}$ treated samples.

In eukaryotic cells, phosphoinositides commonly serve as unique docking sites for proteins and help to delineate various intracellular membrane compartments, primarily those involved in vesicular trafficking[193]. hPLD1 in a variety of cell lines has a basal localization at perinuclear vesicles that is dependent on phosphoinositide binding[194, 195] and upon stimulation hPLD1 mediates vesicular trafficking that is dependent on catalytic activity[194]. Both PldA lipid binding and activity are increased in the presence of phosphoinositides, as they are for hPLD. Phosphoinositides may modulate PldA activity and localization once the enzyme is translocated

into a host cell. Many pathogens hijack host vesicular trafficking during infection to promote bacterial survival.[47] Based on these findings, one can hypothesize that PldA is able to mimic host PLD mediated vesicular trafficking as well. This effect is studied later in Chapter III. Phosphoinositide stimulation is also very interesting in the context of PC hydrolysis. It is unclear if PC substrate inhibition (Figure 9) is physiologically relevant however if significant suppression of activity occurs in the presence of PC, phosphoinositides may serve as a potential mechanism to increase enzyme activity. This is particularly relevant in infected cells. PC is the most abundant cellular lipid of most human cells[165] and as such PldA binding and hydrolysis at PC-rich membranes may be very low. I speculate that phosphoinositides serve as means of localizing PldA and modulating PldA activity within PC-rich eukaryotic cells.

Phosphorylation and activation by Akt

Akt is serine/threonine kinase that regulates a wide array of cellular processes including endocytosis, vesicular trafficking, and cell proliferation.[196] *P. aeruginosa* cell invasion was previously demonstrated to be PI3K- and Akt-dependent.[51, 61] Interestingly, hPLD2 directly binds Akt and stimulates Akt activity through PtdOH production.[163] It was previously published that Akt can bind PldA, and Akt activation upon *P. aeruginosa* infection of epithelial cells was PldA-dependent.[52] A Scansite search[197] was used to identify putative protein binding and phosphorylation motifs. Based on this search, PldA contains the Akt phosphorylation consensus sequence RXRXXS/T and is predicted to be phosphorylated at threonine 55. To determine if Akt can phosphorylate PldA, PldA was assayed as a substrate in an *in vitro* Akt kinase assay with recombinant, purified human Akt1. GSK3α/β fusion protein was also included as a positive control. Akt phosphorylation was probed using a phospho Akt substrate antibody. Using recombinant Akt1 and PldA, Akt phosphorylates PldA in an ATP-dependent manner. (Figure 17)

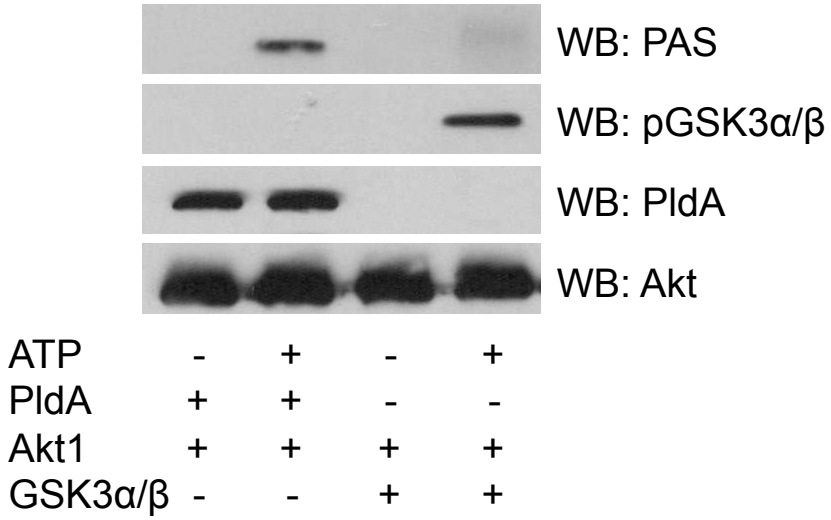

Figure 17. Akt1 phosphorylates PldA *in vitro*. Western blot analysis of Akt kinase assay with PldA and GSK3α/β fusion protein. PAS, phospho Akt substrate.

Recombinant purified Akt1 phosphorylates PldA *in vitro*, however the significance is unclear and whether this occurs in cells is unknown. To determine if Akt phosphorylation alters PldA activity, PldA was treated with Akt in a 1:1 molar ratio with PldA in the presence and absence of ATP to identify phosphorylation and non-phosphorylation dependent effects on PldA activity. Addition of Akt increased PldA activity 3-fold even in the absence of ATP (Figure 18A). The addition of ATP and hence phosphorylation of PldA did not further increase PldA activity. Because of the nature of the fluorescence assay used DTT present in the Akt purification buffer and kinase reaction buffer caused significant increase in background noise negatively affecting the signal-to-noise. Because of this interference it cannot be confirmed that PldA activity was still linear and the standard error was large across conditions, so there is uncertainty as to the accuracy of the activity measured.

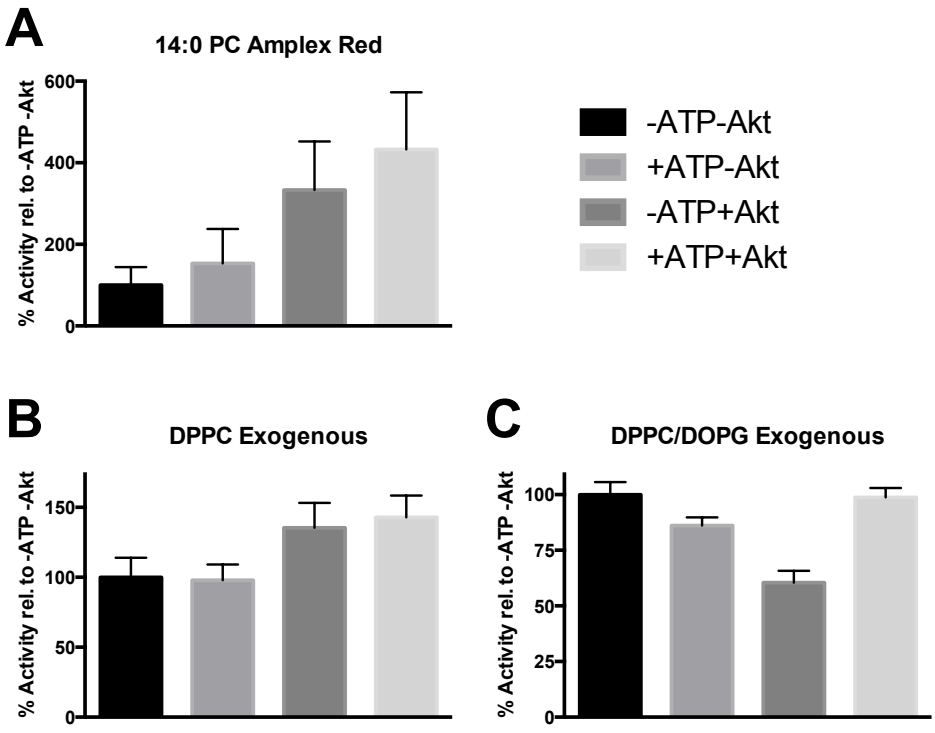

Figure 18. Effect of Akt on PldA activity *in vitro*. PldA was used as substrate in Akt kinase assay under the conditions indicated. PLD activity was subsequently measured using the Amplex Red with (A) 14:0 PC or exogenous assay with (B) DPPC liposomes or (C) 1:1 DOPG/DPPC liposomes.

This experiment was repeated using the exogenous assay, another PLD assay for which DTT interference is not an issue. The results were not consistent across two different vesicle compositions used. For DPPC vesicles, the results modestly recapitulated the Amplex Red data with Akt stimulating PldA activity in an ATP-independent manner (Figure 18B) although very modestly. For PG/PC mixed liposomes, however, Akt did not stimulate PC hydrolysis above the control condition without ATP and Akt (Figure 18C). Addition of Akt without ATP actually

decreased PldA activity, however the addition of ATP increased PldA activity back to control conditions. Anionic lipids such as PIP_3, PtdOH[163], and PS[198] increase Akt lipid binding to PC liposomes. With PC/PG liposomes, perhaps Akt has some low affinity for the anionic PG lipid and Akt binding of liposomes interfered with PldA binding under these conditions. The effects of Akt on PldA activity in these experiments are inconsistent and significantly more work is needed to determine what effect if any Akt binding has on PldA activity. Through binding, Akt could also affect localization of PldA, and vice versa, within cells. This possibility also needs to be explored.

PldA is a secreted effector of the H2-T6SS of *Pseudomonas aeruginosa* that targets bacterial and eukaryotic cells, however little characterization of its catalytic activity has been performed. Our findings demonstrate that PldA hydrolyzes a wide variety of phospholipids *in vitro*, and despite homology to eukaryotic PLD, such as hPLD, the substrate preference is very different. The preference for PE, instead of PC, perfectly positions PldA to hydrolyze cellular lipids over prokaryotic and eukaryotic organisms. PC, a relatively poor substrate, is capable of inducing substrate inhibition *in vitro*, however the significance remains to be determined. PldA activity is stimulated *in vitro* by divalent cations and phosphoinositides; both of which may be factors in regulating PldA localization and/or activity when targeting eukaryotic cells. Unlike many HKD-containing PLDs, transphosphatidylation by PldA is highly inefficient and highlights the importance of confirmation of specific catalytic properties of PLDs. Understanding both the differences and similarities between the host PLD and the bacterial homologue provide rational vantage points from which the role of PldA in virulence can be assessed. Some hypotheses and future directions this *in vitro* characterization has inspired have been briefly alluded to throughout the text and are discussed in more detail later (Chapter V).

CHAPTER III

EFFECTS OF PLDA ON HUMAN LUNG EPITHELIAL CELLS AND *E. COLI*[*]

Introduction

PldA has been shown to target both bacterial[57] and eukaryotic[52] cells, factors that may both play a role in promoting *P. aeruginosa* infections. Given the sequence similarity of PldA to hPLD isoforms and that PldA targets eukaryotic cells, it is of interest to determine similarities and differences between the enzymes. In Chapter II, an extensive analysis of catalytic activity and *in vitro* regulation was detailed. Here in Chapter III, I continue analysis of PldA activity when expressed in bacterial cells, in particular *E. coli*, and after uptake into human lung epithelial cells, specifically the A549 cell line. No previous identification of cellular substrates of a PLD virulence factor has been characterized for eukaryotic hosts. Changes in *P. aeruginosa* lipids due to PldA have been previously published.[57] Because of the sequence similarities between PldA and hPLD and partial conservation of regulatory mechanisms, such as phosphoinositide stimulation and Akt interactions my next objective was to identify hPLD-associated functions recapitulated by PldA. Cell viability, modulation of cellular lipids, and effects on cellular signaling were assessed for *E. coli* and A549 cells. This work details the first characterization of the eukaryotic cellular substrates of a bacterial PLD. Lipid product species generated by PldA in A549 cells closely resembled those of hPLD, despite the difference in substrates compositions within cells and substrate hydrolyzed. Despite producing significant increases in PtdOH with *E.* coli when overexpressed, PldA does not induce cell toxicity further supporting the observed requirement of periplasmic localization for toxicity. Based on imaging

[*]Reproduced with permission from Spencer, C. T. and Brown, H. A. (2014) Biochemical characterization of a *Pseudomonas aeruginosa* phospholipase D, *Biochemistry*, in press. Copyright 2015 American Chemical Society.

studies internalized PldA was capable of mediating activity-dependent translocation of intracellular vesicles that eventually resulted in vesicle secretion. Here I also expand on *in vitro* studies, which demonstrated interactions between human Akt and PldA to look at the effects of PldA uptake on Akt activation. Data here identify the changes to cellular lipids induced by PldA, which may directly affect vesicular trafficking and Akt signaling processes also associated with hPLD activity.

Experimental Methods

Plasmid generation

The PldA sequence was amplified by PCR using a forward primer containing a EcoRI site and the reverse primer containing a BglII site. The amplified PCR product and pCMV-HA mammalian expression vector were digested with EcoRI and BglII, then ligated using T4 DNA ligase to generate plasmid pCMV.HA.PldA. The plda gene is in frame with the N-terminal HA tag. The PldA H167N mutant plasmid (pCMV.HA.PldA.H167N) was generated using QuikChange II mutagenesis kit following the manufacturer's protocol. The PldA wild type and H167N mutant sequences were confirmed by sequencing.

Cell culture

A549 cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C in 5% CO_2.

E. coli cell viability assay

BL21 Codon Plus (*DE3*) RIPL *E. coli* transformed with plasmids expressing wild type PldA (pET32b.PldA) or catalytically dead PldA (pET32b.PldA.H167N) were grown from frozen glycerol stocks in LB media with 100 μg/ml ampicillin and 50 μg/ml chloramphenicol at 37 °C.

Cells were adjusted to an OD600 of 0.5 at time 0 h. OD600 was measured 1, 2, and 3 h after induction with 0.1 mM IPTG at 25 °C.

E. coli mass spectrometric analysis

BL21 Codon Plus RIPL *E. coli* transformed with plasmids expressing wild type PldA (pET32b.PldA) or catalytically dead PldA (pET32b.PldA.H167N) were grown from frozen glycerol stocks in LB media with 100 μg/ml ampicillin and 50 μg/ml chloramphenicol at 37 °C. Cells were adjusted to an OD600 of 0.5 at time 0 h. Cells uninduced at time 0 h and cells induced with 0.1 mM IPTG 1, 2, and 3 h post-induction were collected. Cells were pelleted at 6,000 x g and then washed with 1X PBS and pelleted again. Cells were resuspended in 1X PBS and lipids extracted in 800 μl 1:1 0.1 N HCl/MeOH and 400 μl $CHCl_3$. Samples were analyzed by mass spectrometry as described earlier (Chapter II).

Trypan blue cell viability assay

A549 cells were seeded into a 12-well plate at 1.5 x 10^5 cells/well in media containing DMEM, 10% FBS, and 1% penicillin/streptomycin 18 h before transfection. The medium was changed to DMEM and 10% FBS before transfection. Cells were transfected in triplicate witb Lipofectamine 2000 in Opti-MEM according to manufacturer's protocol. Each sample was transfected with 0.5 μg empty vector control (pCMV.HA) or plasmids expressing HA tagged wild type PldA (pCMV.HA.PldA) or catalytically dead PldA (pCMV.HA.PldA.H167N). Cells were trypsinized and cell viability was measured 48 h and 72 h post-transfection by trypan blue staining. Cells were lysed in 10 mM Tris, 150 mM NaCl, and 1% SDS buffer. Protein expression was monitored by western blotting using anti-HA primary antibody and anti-mouse secondary antibody. β-actin was probed as a loading control using an anti-β-actin primary antibody and anti-mouse secondary antibody.

WST-1 cell viability assay

A549 cells were seeded into a 96-well plate at 1.5 x 10^4 cells/well in media containing DMEM, 10% FBS, and 1% penicillin/streptomycin 18 h before transfection. The medium was changed to DMEM and 10% FBS before transfection. Cells were transfected in triplicate witb Lipofectamine 2000 in Opti-MEM according to manufacturer's protocol. Each sample was transfected with 0.2 μg empty vector control (pCMV.HA) or plasmids expressing HA tagged wild type PldA (pCMV.HA.PldA) or catalytically dead PldA (pCMV.HA.PldA.H167N). Cells viability was measured 24 h, 48 h, and 72 h post-transfection using the WST-1 assay following the manufacturer's protocol.

PldA internalization

In a 12-well plate, A549 cells were seeded at 1.5x10^5 cells/well in DMEM, 10% FBS, and 1% penicillin/streptomycin 18h prior to treatment. 2 μM PldA, PldA K169E, tagless PldA, or tagless PldA K169E was incubated with A549 cells in DMEM, 10% FBS medium for 3.5 h at 37 °C in 5% CO_2. Control cells were vehicle treated. Cells were washed three times with PBS and lysed on the plate with 100 μg/ml digitonin in PBS with protease inhibitor cocktail at room temperature for 10 min. Cytosol was collected, and cell membranes scraped in PBS with protease inhibitor to collect membrane fraction. For time course, PldA was detected by western blotting as described earlier using a rabbit anti-PldA polyclonal antibody and HRP conjugated anti-rabbit secondary antibody. β-actin was used as a loading control.

Mass spectrometry analysis of A549 cells

For mass spectrometric analysis, in a 12-well plate, A549 cells were seeded at 1.5x10^5 cells/well in DMEM, 10% FBS, and 1% penicillin/streptomycin 18h prior to treatment. 2 μM PldA or PldA K169E was incubated with A549 cells for 3.5 h at 37 °C in 5% CO_2. Cells were

washed three times with PBS and lysed in 800 µl 1:1 0.1 N HCl/methanol. Lipids were extracted with 400µl chloroform. LC-MS analysis was performed as previously published.[160]

Confocal microscopy

A549 cells were seeded at $1.5x10^4$ cells/well in a 35 mm glass bottom plate (MatTek) in DMEM, 10% FBS, and 1% penicillin/streptomycin 18 h prior to treatment. PldA and PldA K169E were labeled using the Alexa Fluor 488 Protein labeling kit according to the manufacturer's protocol to generate WT-488 or K169E-488. 1 µM WT-488 or K169E-488 was incubated with A549 cells for 4 h at 37°C in 5% CO_2. Extracellular PldA was removed by washing three times with PBS followed by addition of 10% FBS in DMEM. Cells were imaged using Zeiss LSM 510 inverted microscope at 5 h and 7 h post-incubation using a 63x 1.4 N.A. (Plan-Apochromat) oil immersion objective. Acquisition settings were held constant unless noted otherwise.

Liposome binding assay

The liposome binding assay was adapted from the procedure of Buser and McLaughlin.[199] Lipids in chloroform and 3.2 µCi ^{3}H-DPPC were dried under a stream of nitrogen. Lipids were suspended in 50 mM HEPES (pH 7.5), and 176 mM sucrose buffer by vortexing vigorously and allowed to hydrate for 1 hour at room temperature. Vesicles were subjected to 5 cycles of freezing in liquid N_2 and thawing in a warm water bath after which, vesicles were extruded through two 0. 1 µm polycarbonate filters. Vesicles were diluted 1:5 in an isotonic buffer (50 mM HEPES (pH 7.5), 100 mM KCl), pelleted by centrifugation at 100,000 x g for 1h at room temperature and resuspended in isotonic buffer. Final lipid concentrations were calculated based on [^{3}H]DPPC radioactivity.

PldA or PldA K169E was added to vesicles in a 1ml reaction and incubated at room temperature for 5 minutes. Samples containing PldA without lipids were used as controls to determine background. The final reaction conditions were 2 μg PldA, 50 mM HEPES (pH 7.5), 100 mM KCl, 3 mM $MgCl_2$, and 100 μM lipid. Lipid-bound protein was isolated by centrifugation at 15,000 x g for 20 min at 4 °C. The supernatant was removed and the lipid and protein resuspended in 1X SDS loading buffer.

For western blot analysis, protein was transferred to a nitrocellulose membrane. The membrane was blocked with TBST containing 5% non-fat milk and incubated with anti-PldA rabbit polyclonal antibody. After washing, blots were probed with HRP conjugated rabbit secondary antibody and developed using ECL reagent.

Results and Discussion

Effects of PldA on E. coli cell viability and lipid composition

PldA has been demonstrated to induce membrane lysis and cellular death of *P. aeruginosa*[57], *Pseudomonas putida*[57], and *E. coli*[52]. PldA is proposed to be translocated into the periplasmic space of targeted cells. Significant hydrolysis of PE and accumulation of PtdOH was observed in *P. aeruginosa* cells in which PldA was expressed, but without an "immunity protein" that was genetically deleted[57]. Many T6SS effectors are localized within the genome adjacent to an immunity protein that is co-expressed with the effector that serves to neutralize the effector within the cell to prevent deleterious results before the effector is secreted.[200] Without the PldA immunity protein, *P. aeruginosa* itself is susceptible to lysis induced by PldA.

Despite the observed membrane sensitization and cell death associated with PldA in bacterial cells, PldA can be overexpressed in *E. coli* to produce significant yields of recombinant protein

with no obvious cell toxicity issues. (Figure 3) To directly address whether overexpression of PldA in *E. coli* promotes cell toxicity, wild type (WT) and catalytically dead (H167N) PldA were overexpressed in *E. coli* BL21 CodonPlus (DE3) RIPL cells at 25 °C over several hours (Figure 19). Cell density prior to IPTG induction was the same for both constructs. No difference in cell growth post-induction was observed for several repeated trials. (Figure 19) This discrepancy in observed toxicity by PldA may be explained by the localization of PldA during expression. Here, the expression construct allows for only cytoplasmic localization of PldA. Most T6SS effectors, including PldA, are thought to be translocated into the periplasmic space of other bacterial cells. In earlier studies, it also note that PldA overexpression in *E. coli* cytoplasms did not induce cell death, however if PldA was expressed with a localization signal that directs it into the periplasmic space, cell viability was impaired.[52] This implies that modulation of cellular lipids on the cytoplasmic leaflet of *E. coli* does not induce membrane disruption; instead specific hydrolysis of phospholipids on the periplasmic leaflets impairs viability.

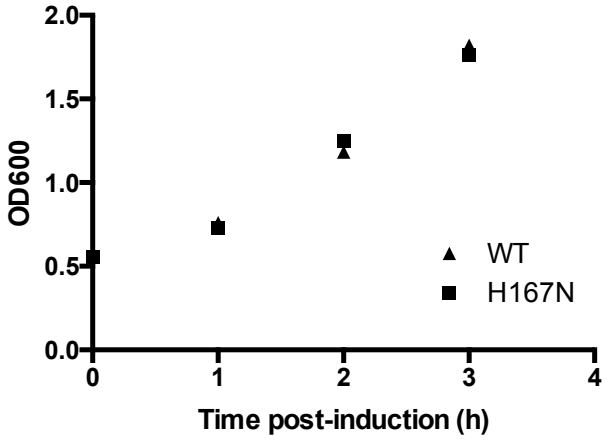

Figure 19. Wild type cytosolic PldA is not toxic to *E. coli*. PldA wild type (WT) or catalytic mutant (K169E) expression was induced with IPTG for 1 h to 3 h. Cell viability was measured by OD600.

Another alternative explanation is that PldA is not catalytically active within the bacterial cytoplasm. To evaluate this possibility, the lipid composition of *E. coli* overexpressing either wild type (WT) or mutant (H167N) PldA before and after protein induction were compared (Figure 20). Although there was leaky expression of both WT and H167N PldA prior to induction (data not shown), the proportions of the lipid classes were comparable. After 3 h of induction, PtdOH comprised 12% of the total cellular lipids of WT expressing cells, while PtdOH of H167N expressing cells remained low at 1%. *In vitro* analysis of PldA substrates shows that PE is the preferred PldA substrate, but PG is also very efficient hydrolyzed. In *E. coli*, PE appears to be the only substrate used for PtdOH production. In fact, absolute picomoles of PG were higher in WT cells than H167N cells. These data clearly demonstrate that PldA overexpressed in the *E. coli* cytoplasm is catalytically active and causes significant changes in

PE and PtdOH levels in cells, however elevated PtdOH on the cytoplasmic leaflet is not sufficient to perturb cell viability.

Perturbation of cell viability seems heavily dependent on the localization of PldA and either the degradation of the phospholipid bilayer of the periplasmic face or the accumulation of PtdOH in this region. Perhaps degradation of PE or accumulation of PtdOH causes significant changes in the membrane properties of the phospholipid bilayer that induces a more porous or leaky membrane.

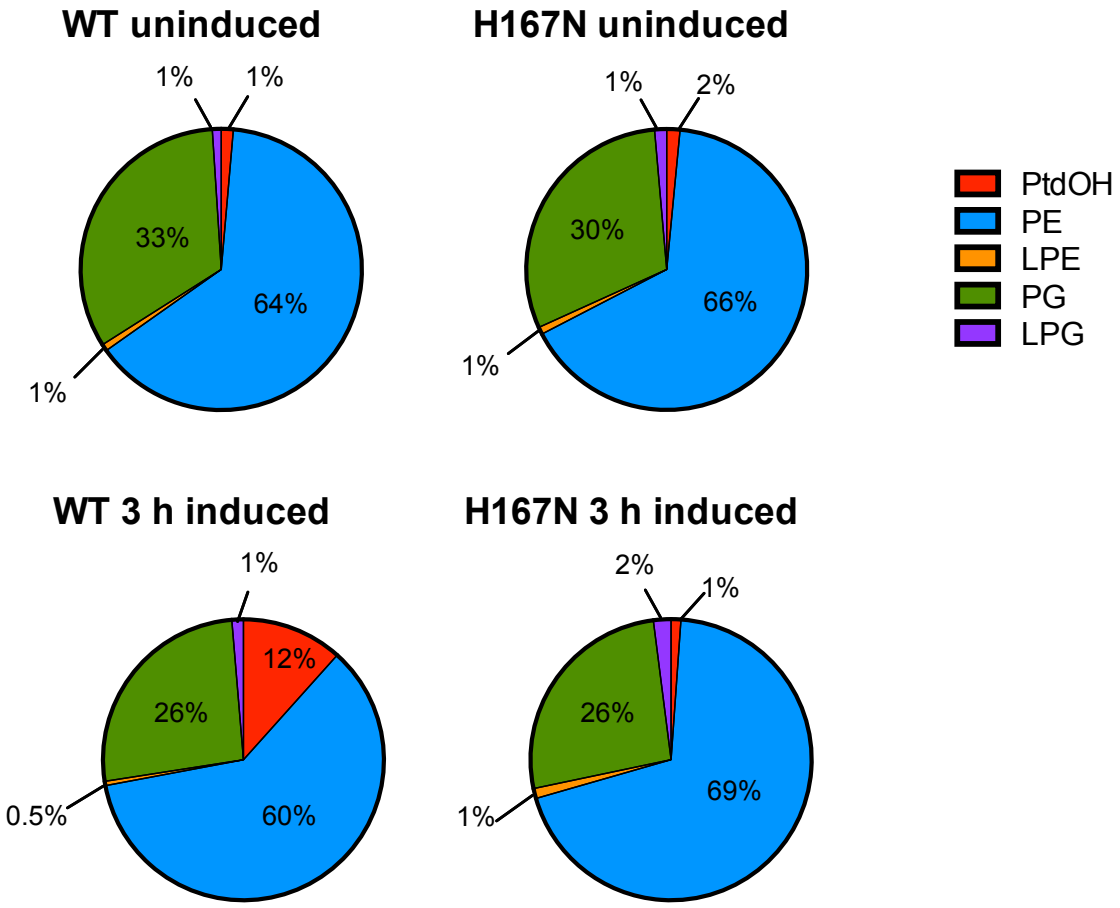

Figure 20. Overexpression of PldA in *E. coli* causes a significant changes in cellular lipids. PldA wild type (WT) or catalytic mutant (H167N) expressing cells were uninduced or induced with

IPTG for 3 h. *E. coli* lipids were extracted and analyzed by LC-MS. The distribution of each lipid class is presented as a percentage of the total.

Effects of PldA on human lung epithelial cell viability

As PldA also targets eukaryotic cells, I chose to assess whether PldA has an effect on human cell viability using A549 cells, a human lung adenocarcinoma epithelial cell line. For these experiments, A549 cells were transfected with a CMV-promoter based mammalian expression vector. Cell viability was measured using WST-1 reagent (Figure 21) and trypan blue staining (Figure 22). Initially viability was measured using the WST-1 assay, which is a colorimetric assay that gauges cell metabolism, however due to the nature of the effects of PldA on bacterial membrane stability, trypan blue staining was also performed to analyze membrane integrity of A549 cells. Cells were transfected with an empty vector control to account for effects on cell viability due to the transfection process and vectors containing wild type (WT) or catalytically dead (H167N) PldA. As seen with *E. coli,* no difference in A549 cell viability was observed with expression of PldA based on both cell viability assays.

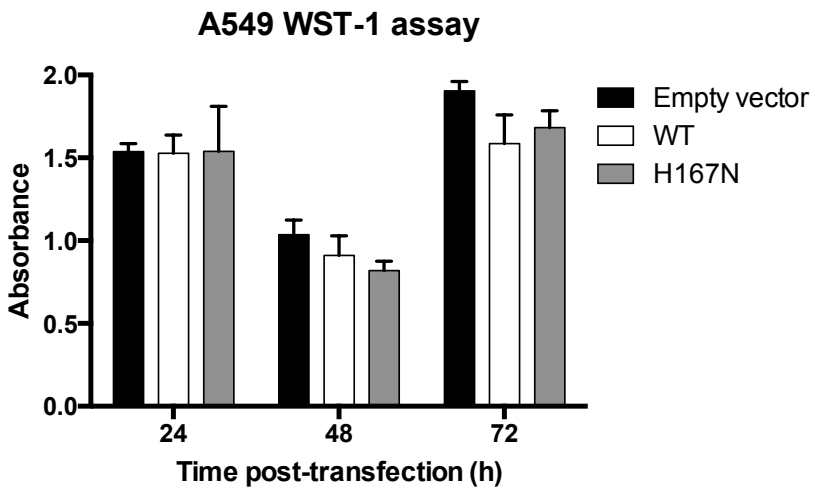

Figure 21. Overexpression of PldA in A549 cells does not induce cell death. A549 cells were transfected with an empty vector control or vector expressing wild type PldA (WT) or catalytic mutant (H167N) PldA. Cell viability was measured using WST-1 assay 24 – 72 h post-transfection. Mean values are shown ± SEM.

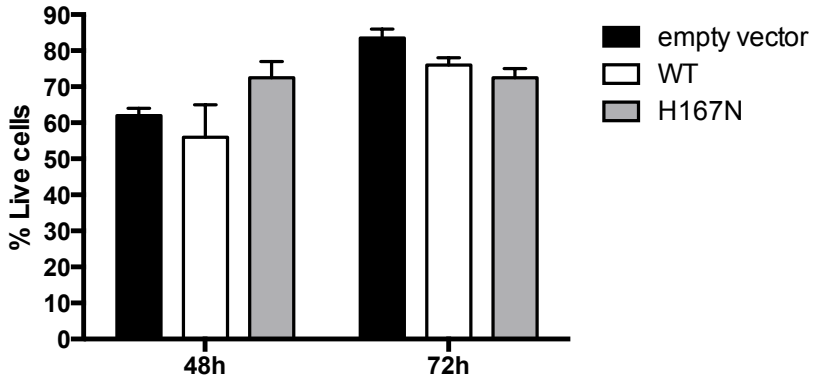

Figure 22. Overexpression of PldA in A549 cells does not induce membrane rupture. A549 cells were transfected with an empty vector control or vector expressing wild type PldA (WT) or catalytic mutant (H167N) PldA. Cell viability was measured using trypan blue staining 48 – 72 h post-transfection. Mean values are shown ± SEM.

Once again, I looked to confirm PldA activity was present after transfection. Unlike *E. coli*, no detectable increase in PtdOH was observed 24 to 72 h post-transfection (data not shown). Earlier time points (6 h and 12 h) were also screened for PldA activity, however expression of PldA was very low and again no change in PtdOH was observed. The lack of detectable activity may be due to several factors. Transfection efficiency is very poor with A549 cells. PldA may be active under these conditions however low signal to noise may have prevented detection of activity. Over several days, cells may have been able to compensate for any increased PLD activity in the cells, either by neutralizing PldA itself or increasing turnover of PtdOH.

Intracellular uptake and localization of PldA in A549 cells

The ability of PldA to promote epithelial cell internalization of *P. aeruginosa*[52] is presumably due to catalytic activity as most functions of PLDs are thought to be mediated by PtdOH. As transfection was not an efficient means of measuring the effects of PldA on human cells, instead, I explored the effects of introducing exogenous PldA and determining if it was capable of internalizing into human cells and eliciting changes in lipid composition. PldA used throughout the *in vitro* studies was generated as a fusion protein with thioredoxin, a protein that has been shown to translocate across the plasma membrane of human cells[201, 202]. To determine whether the thioredoxin tag promotes PldA internalization, the thioredoxin tags of PldA and PldA K169E were removed utilizing thrombin, and internalization of tagless PldA and thioredoxin-PldA fusion proteins were analyzed by western blotting (Figure 23). Based on Western analysis of cell cytosolic and membrane fractions, PldA levels are significantly higher than PldA K169E levels after several hours of incubation. Tagless PldA and tagless PldA K169E were detected in the cytosolic and membrane fractions at levels lower than the PldA wild type fusion protein, suggesting that the thioredoxin tag promotes a significantly higher level of internalization of

PldA. Low digitonin concentrations were used to selectively permeabilize the plasma membrane without rupturing intracellular organelle membranes. Cell fraction purity was assessed using cytosolic, plasma membrane, and lysosomal markers, CAD, or carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase, Na^+/K^+ ATPase, and LAMP1, respectively. No or low contamination of the cytosolic fraction with Na^+/K^+ ATPase or LAMP1 suggests that the PldA observed in the cytosolic fraction was not leached from intracellular organelles or due to membrane contamination.

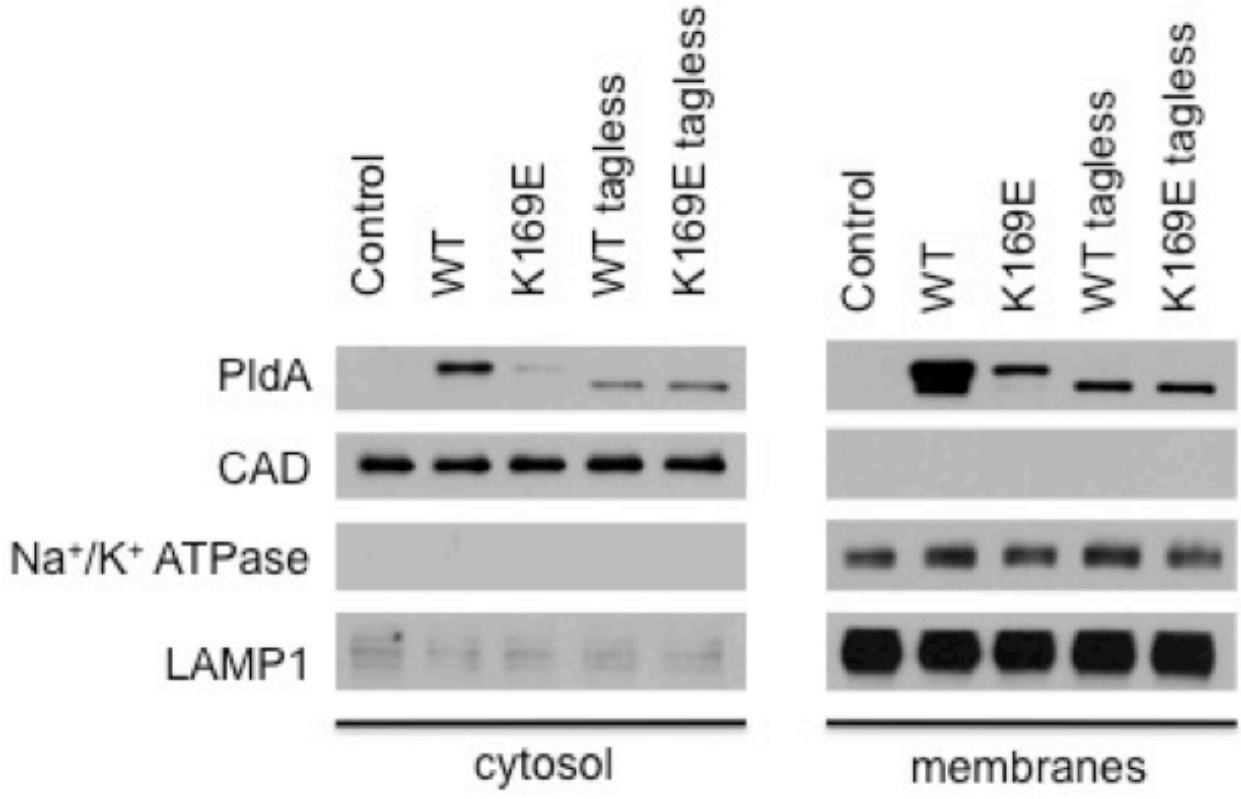

Figure 23. The thiodoxin tag mediates efficient uptake of recombinant, exogenous PldA by A549 cells. Cells were treated with PldA construct for 3.5 h. PldA in cytosolic and membrane fractions was analyzed using western blotting. CAD, Na^+/K^+ ATPase, and LAMP1 served as cytosolic, plasma membrane, and lysosomal markers.

The large discrepancy in protein levels of PldA and PldA K169E fusion proteins led us to investigate whether the loss of catalytic activity was affecting internalization or stability of PldA K169E. To answer this question, internalization of PldA and PldA K169E over time was determined by Western blotting (Figure 24). At early time points, (30 min to 2 h post-incubation), wild type and K169E PldA proteins are present at comparable levels within A549 cells, however by 4 h post-incubation, protein levels for PldA are still increasing while those for PldA K169E have significantly declined, suggesting that catalytic activity is important for stability after internalization, but not for internalization itself.

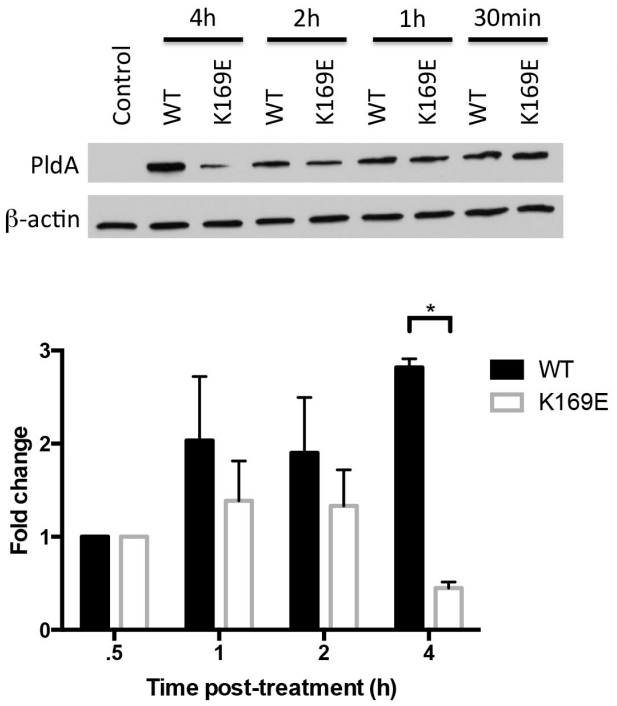

Figure 24. Intracellular stability of internalized PldA is activity dependent. Cells were treated with wild type (WT) or mutant (K169E) PldA for 30 min to 4 h. Top panel, representative western blot of experiment. Bottom panel, quantitation using densitometry of three independent

experiments. WT and K169E data normalized to actin loading control and respective signals at 30 min. Mean values are shown ± SEM. Statistical analysis performed using student's t-test. *$p<0.05$

To visually confirm internalization of exogenous recombinant PldA, A549 cells were treated with Alexa Fluor 488 labeled PldA and PldA K169E and imaged using confocal microscopy (Figure 25). Based on confocal imaging, both catalytically competent fluorophore-labeled (WT-488) and incompetent (K169E-488) PldA were localized to the perinuclear space within A549 cells 5 h post-treatment, however over time differences were observed. At 5 h and 7 h post-incubation WT-488 fluorescence intensity are comparably robust. Using the same acquisition settings, K169E-488 fluorescence intensity was much lower at 7 h compared to 5 h post-K169E-488 treatment or 7 h post-WT-488 treatment. This is consistent with results shown in Figure 24. Both wild type and mutant PldA initially localize to perinuclear vesicles, but over time wild type PldA vesicles disperse towards the plasma membrane and extracellular vesicles were detected that co-localize with PldA (data not shown). Localization of mutant PldA however remained constant over time and no vesicle secretion was observed.

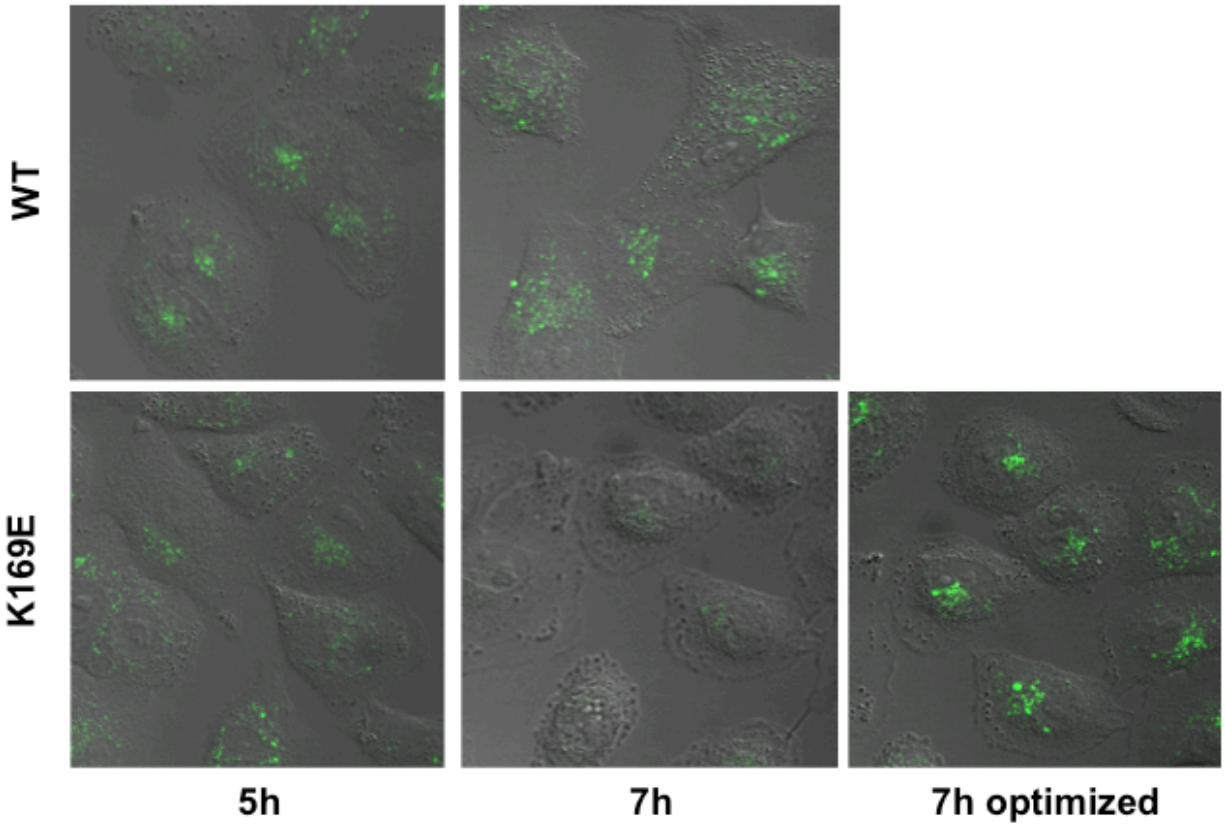

Figure 25. Intracellular localization and stability of PldA are activity dependent. Fluorescence and DIC overlay images of A549 cells treated for 4 h with wild type and mutant PldA labeled with Alexa Fluor 488. Cells were imaged 5 h and 7 h post-treatment.

PldA induced vesicle secretion by A549 cells

If PldA is added in the absence of serum, vesicle secretion into the media was further accelerated (Figure 26). The physiological significance of this vesicle secretion is unclear. There are no references in the literature of *P. aeruginosa* induced vesicle secretion of infected cells. There is a published report of *P. aeruginosa* inducing membrane blebbing and shedding resembling apoptotic blebbing after cell internalization[203], however visually that phenomenon appears different from this observed membrane release.

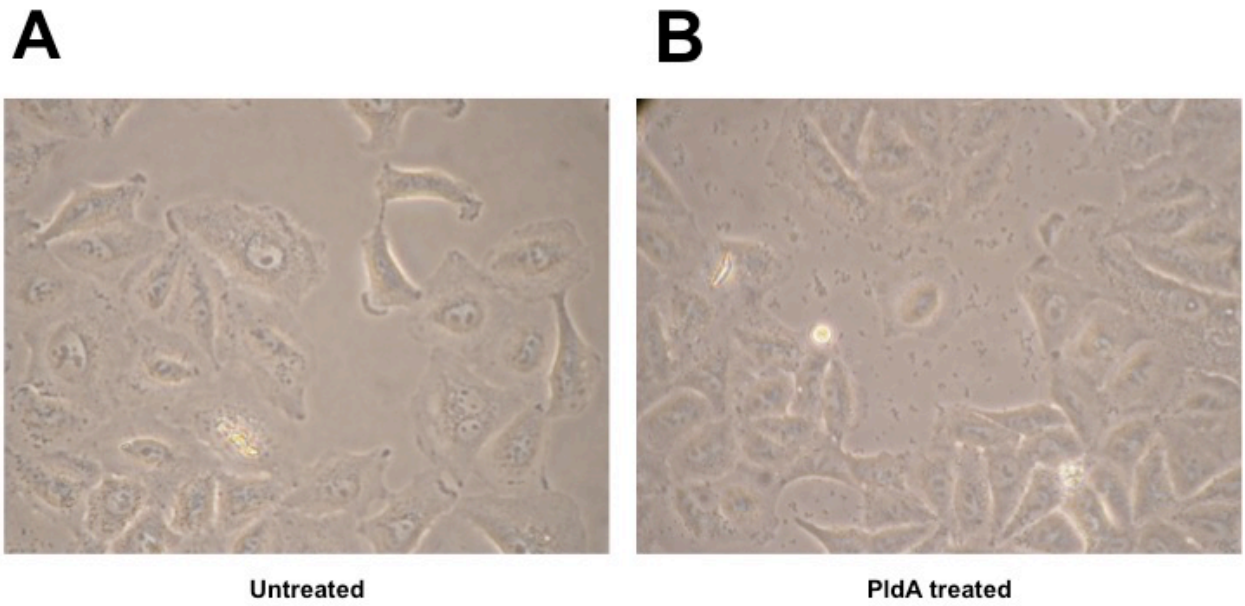

Figure 26. PldA induces vesicle secretion of A549 cells. A549 cells were left A) untreated or B) treated with wild type PldA in the absence of serum for 2 h.

In eukaryotic cells, phosphoinositides commonly serve as unique docking sites for proteins and help to delineate various intracellular membrane compartments, primarily those involved in vesicular trafficking.[193] hPLD1 in a variety of cell lines has a basal localization at perinuclear vesicles that is dependent on phosphoinositide binding[194, 195] and upon stimulation hPLD1 mediates vesicular trafficking that is dependent on catalytic activity.[45] Both PldA lipid binding and activity are increased in the presence of phosphoinositides (Figures 12 and 13), as they are for human PLD. I speculate that phosphoinositides may modulate PldA activity and localization once the enzyme is translocated into the host cell cytosol. In this study, exogenous PldA was taken up into A549 cells and associated with both the membrane and cytosolic fractions. As little diffuse cytosolic localization was seen in A549 cells after exogenous uptake (Figure 25), or after overexpression with transfection in HeLa cells[52], cytosolic PldA appear to concentrate at

punctate vesicular structures and based on *in vitro* characterization (Chapter II) this localization may be regulated by phosphoinositides.

Internalized active, but not mutant, PldA induced changes in vesicular trafficking in A549 cells. Many pathogens hijack host vesicular trafficking during infection to promote bacterial survival.[204] Human PLD also promotes vesicle release and secretion of a variety of secretory cells.[28] Although it is unknown whether vesicle release by A549 cells is physiologically relevant in this case, PldA was capable inducing vesicle secretion of A549 cells. Based on these findings, one can hypothesize that PldA is able to mimic host PLD mediated vesicular trafficking during infection after translocation into host cells as well. The nature of these intracellular vesicles co-localized with PldA and the effects of PldA on various components of vesicular trafficking networks requires further description.

Effects of PldA on human lipid composition

Once internalization was confirmed visually, the effects of PldA on A549 cellular lipids was evaluated. The lipid composition of A549 cells treated with PldA or PldA K169E was determined by LC-MS analysis (Figure 27). Unlike the transfection experiments, exogenous treatment of A549 cells with PldA caused significant changes to host lipids. The primary PtdOH species generated (32:1, 34:2, 34:1, and 36:2 PtdOH) correlated nicely with significant reductions in the corresponding species of PE (32:1, 34:2, 34:1, and 36:2) and PG (34:1 and 36:2). *In vitro*, PldA was also observed to hydrolyze plasmalogen PE. In A549 cells, the 36:1p PE species was the only plasmalogen whose levels were reduced with wild type PldA treatment, although the change was not significant. The only PC species whose level decreased with PldA treatment relative to PldA K169E treatment was DPPC, although the change was also not

statistically significant. It is interesting to note here that DPPC was observed to be the preferred PC substrate *in vitro* as well (Figure 7).

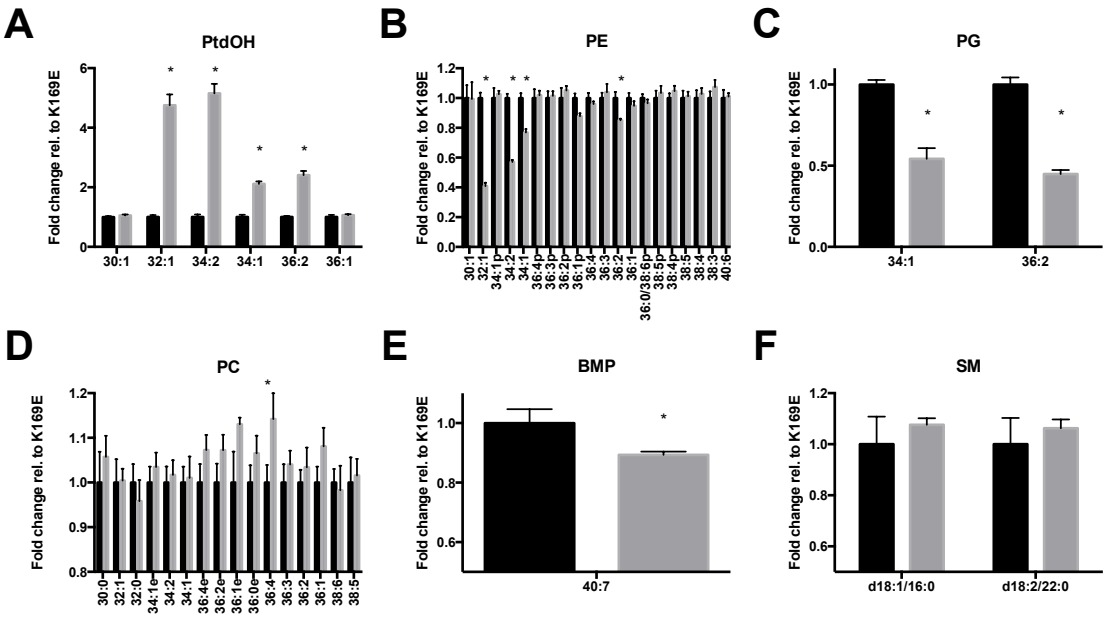

Figure 27. Exogenous treatment of A549 cells causes significant changes in cellular lipids. A549 cells were treated with wild type or mutant (K169E) PldA for 3.5 h. A549 cellular lipids were extracted and analyzed by LC-MS. Lipids levels are normalized to cells treated with PldA K169E. Mean values are shown ± SEM. Statistical analysis performed using student's t-test.

Bis(monoacylglycero)phosphate, or BMP, was the only lipid class that significantly decreased with PldA treatment that was not a substrate. BMP is specifically and exclusively localized to the late endosome or lysosome within cells and is important for intraluminal vesicle formation of late endosomes, sphingolipid metabolism, and cholesterol trafficking.[205, 206] BMP is a highly stable lipid and PldA likely does not directly hydrolyze BMP, instead the significant loss of PG, the starting material for BMP *de novo* synthesis[205], most likely limited biosynthesis

of BMP. The decline in BMP with wild type PldA treatment suggests that PldA can alter the lipid membranes of endosomal vesicles. Changes to BMP would be expected to have an impact on sphingolipid metabolism, as BMP is an activator of many sphingolipid-metabolizing enzymes.[206] Although we could not analyze most sphingolipids using this specific LC-MS method, quantification of sphingomyelin was possible. Treatment of A549 cells with wild type PldA caused an increase in SM levels, although not significant, suggesting a reduction in lipid turnover or an increase in SM synthesis. The decrease in BMP, which normally stimulates acid sphingomyelinase hydrolysis of sphingomyelin, suggests that the increase is due to a decline in SM turnover. As noted earlier, with confocal microscopy, PldA localized puncta appears to transition from the perinuclear region closer to the cell periphery, eventually resulting in vesicle secretion. This effect is dependent on catalytic activity. The synthesis of PtdOH, a lipid that promotes vesicular trafficking in human cells, and/or the modulation of endosomal lipids such as BMP may mediate these trafficking changes and promote stability of PldA. Based on confocal imaging and mass spectrometry, internalized PldA is capable of altering vesicle trafficking and lipid signaling within A549 cells.

In A549 cells treated with PldA, increased production of only four species of PtdOH was detected. Interestingly, these PtdOH species are the same characteristic signaling species of PtdOH produced by the human isoforms in cells.[207, 208] This selectivity is not based on substrate species abundance. The most abundant PE species contain polyunsaturated fatty acids (PUFAs). The PE species that were PldA substrates constituted only 17% of the total cellular PE, while the five most abundant PE species, all of which contain PUFAs, constituted 55% of total PE (Figure 28). By mimicking host PLD signaling lipids, PldA may be able to induce PLD-associated signaling pathways, such as vesicle trafficking (Figure 25) or Akt signaling (Figure29).[52, 163]

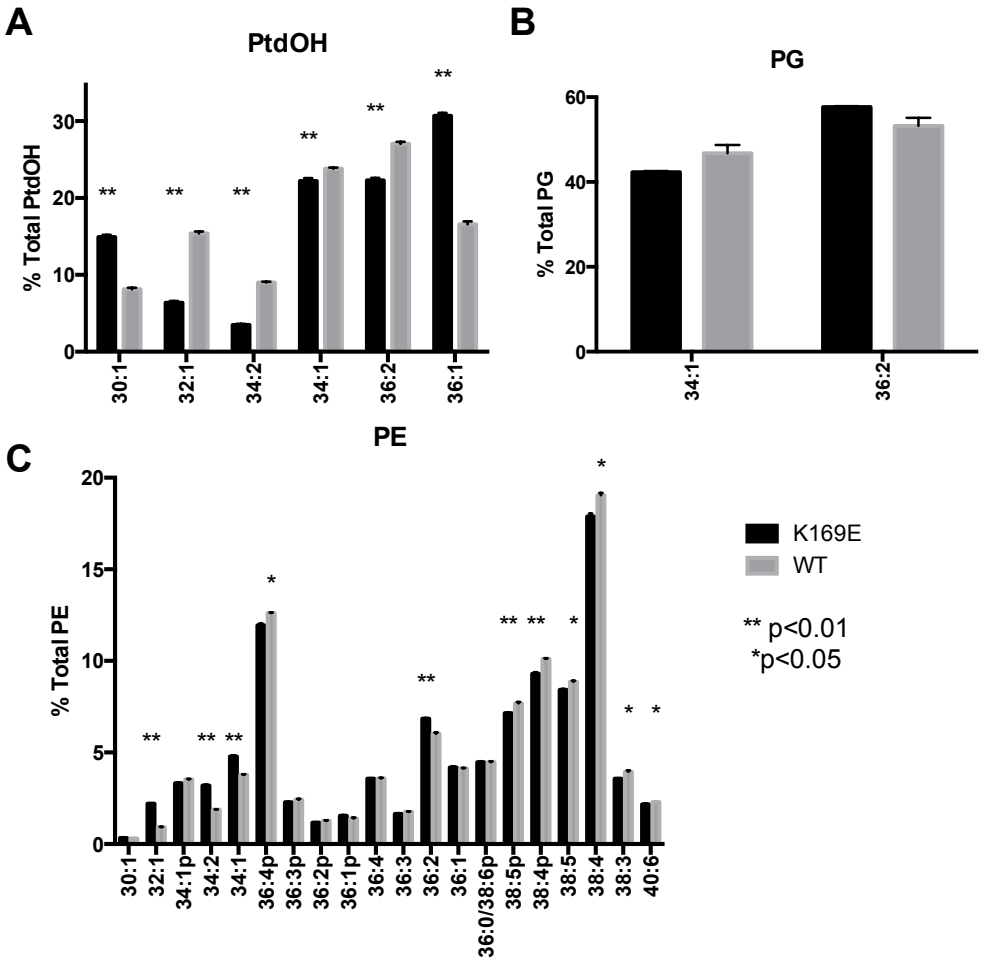

Figure 28. PldA treatment of A549 cells induces redistribution of lipids species for PtdOH and PE. Species distribution of A) PtdOH B) PG and C) PE in A549 cells after treatment with wild type (WT) or mutant (K169E) PldA. Mean values are shown ± SEM. Statistical analysis performed using student's t-test.

PldA induced Akt activation requires PldA activity

Previous accounts demonstrated a link between PldA and human Akt that served to mediate cell internalization of *P. aeruginosa*.[51, 52, 61] Herein, we assessed whether PldA internalized from

the medium also induces Akt activation in A549 cells. Initial efforts focused at the 3.5 h time point, as that was where the most profound difference in wild type and mutant PldA levels were observed (Figure 29A). No significant difference was in Akt phosphorylation between WT and K169E treated cells. However at earlier time points (30 min to 1 h) post-treatment a reproducible and quantified increase in Akt phosphorylation was observed with WT treated cells, while K169E treated cells resembled control, or vehicle, treated cells. (Figure 29B) Independent of the mechanism for internalization, either the T6SS[52] or thioredoxin induced exogenous uptake, PldA stimulates Akt activation in epithelial cells.

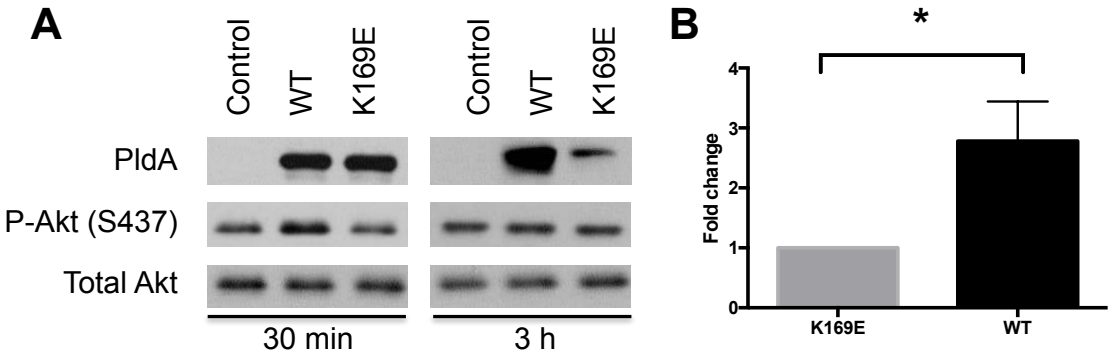

Figure 29. PldA induces a transient stimulation of Akt in A549 cells upon exogenous treatment. A) Wild type PldA stimulates Akt phosphorylation 30 min after exogenous addition and uptake, but not after 3 h. Representative experiment shown. B) Quantification of difference in Akt phosphorylation between WT and K169E treated cells after 30 min using densitometry. Fold change was normalized to total Akt and PldA K169E treated samples. Mean values are shown ± SEM. Statistical analysis performed using one sample t-test. * $p < 0.05$

The *Neisseria gonorrhoeae* PLD, NgPLD, was observed to directly bind and stimulate host Akt signaling.[43, 44] Previous findings confirm that PldA also interacts with Akt and stimulates its phosphorylation and activation upon *in vitro P. aeruginosa* epithelial cell infection. Here, we reproduce these earlier findings of PldA inducing Akt phosphorylation using exogenous PldA that is capable of internalizing A549 cells. The ability to reproduce these earlier findings of Akt activation support the notion that other observed effects, vesicular trafficking and lipid changes, in A549 cells are relevant despite the difference in the method of delivery of PldA into cells.

Understanding of the parallels between the human isoforms of PLD and PldA contribute to predictions of its mechanism of activity as a virulence factor. In this work, we have identified for the first time, the substrate specificity of PldA in eukaryotic cells, as well as the products produced, which closely match those of the host PLD. Several similarities between hPLD isoforms and PldA suggest that PldA may mimic some of the functions of the host PLD, such as vesicular trafficking or Akt activation. Of particular interest for future studies is the putative role of phosphoinositides in regulating PldA localization and activity in eukaryotic cells. Further investigation is needed to confirm the physiological relevance of these *in vitro* regulators of PldA activity to better understand the function of PldA during *P. aeruginosa* infection. Given the central importance of this bacterial virulence factor, such studies are likely to be illuminating.

CHAPTER IV

SCREENING OF SMALL MOLECULE INHIBITOR OF PLDA*

Introduction

Based on phylogenetic analysis, PldA is closely related to eukaryotic PLD.[52, 56] In fact, PldA has regions of high homology with the mammalian enzyme including possessing two catalytic HKD motifs, but does not share much homology with those enzymes from prokaryotes. Decreasing success in development of novel antibacterial compounds inhibiting traditional antibiotic targets along with increased drug resistance of many bacteria have caused a shift in antibacterial drug discovery towards modulation of virulence factors, factors produced by pathogens that contribute to disease progression. PldA is as a secreted effector of the T6SS of *P. aeruginosa* that targets human epithelial cells to promote bacterial internalization[52] and bacterial cells to promote intra- and inter-bacterial species competition[57], both of which may be important components for establishing and maintaining infection. Efforts to target virulence mechanism such as quorum sensing and T3SS have confirmed that small-molecule inhibitors of virulence mechanisms can abrogate bacterial infections *in vitro* and *in vivo*. Genetic ablation of PldA is associated with decreased persistence of *P. aeruginosa* in lung infections, but can PldA be targeted pharmacologically to decrease *P. aeruginosa* infectivity.

Over the past decade isoform selective, direct small-molecule inhibitors of mammalian PLD1 and PLD2 have been generated.[209-212] Compounds **1-3** (Figure 30) have been used to elucidate new roles for human PLD in cancer and neurodegeneration[16, 213]; however, these potent and

* Reproduced in part with permission from Scott, S. A., Spencer, C. T., O'Reilly, M. C., Brown, K. A., Lavieri, R. R., Cho, C.-H., Jung, D.-I., Larock, R. C., Brown, H. A., and Lindsley, C. W. (2014) Discovery of Desketoraloxifene Analogues as Inhibitors of Mammalian, *Pseudomonas aeruginosa*, and NAPE Phospholipase D Enzymes, *ACS Chem. Biol*. in press. Copyright 2014 American Chemical Society

highly selective inhibitors of human PLD have never been tested against PldA or other non-HKD containing PLDs.

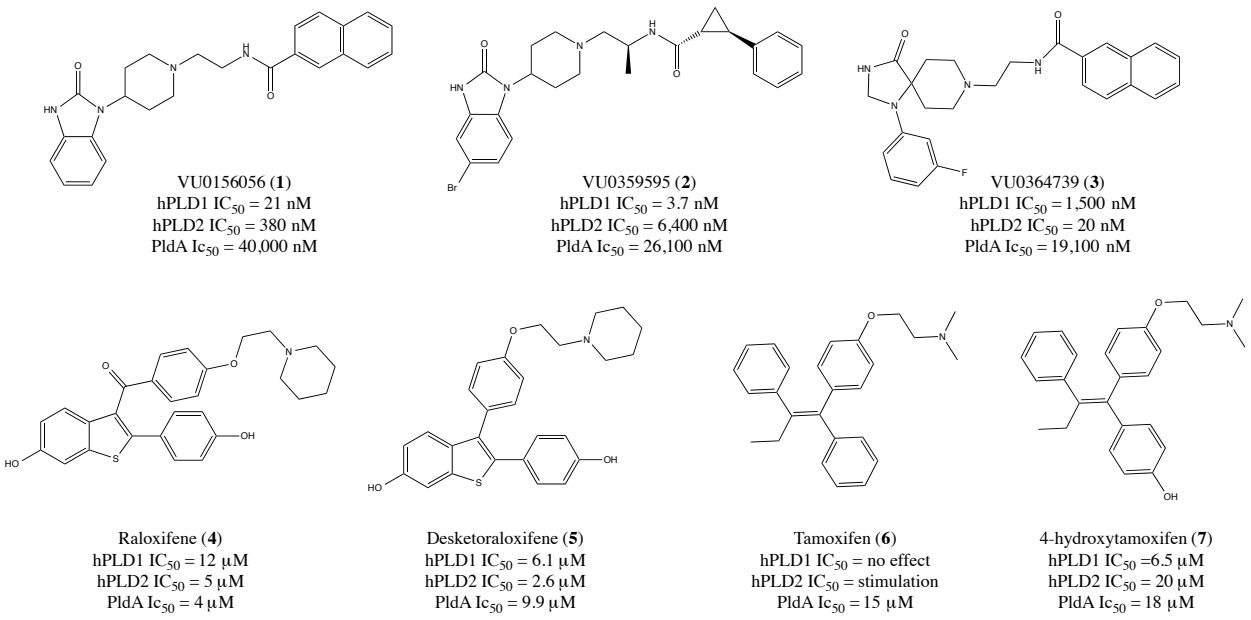

Figure 30. Efficacy of potent human PLD inhibitors on PldA. IC_{50} values of previously identified human PLD inhibitors for hPLD1, hPLD2, and PldA. hPLD values were previously published for **1-3**.[209-212]

NAPE-PLD, or N-acyl phosphatidylethanolamine phospholipase D, which hydrolyzes N-acyl phosphatidylethanolamine to generate N-acylethanolamines (NAE), such as anandamide, and phosphatidic acid, is a representative non-HKD PLD of great interest.[2] Anandamide is a major endocannabinoid shown to have antinociceptive and analgesic properties, play a role in several neurodegenerative disorders, and to have anti-proliferative, anti-metastatic and pro-apoptotic effects of cancer cells in culture and *in vivo*.[214]

These four enzymes are divergent, yet the identification of a ligand that could bind and inhibit, with broad-spectrum activity would be highly desirable to dissect their physiological roles and assess therapeutic potential. Very few published accounts of bacterial PLD inhibition currently exist. Early work on the non-HKD containing *Streptomyces chromofuscus* PLD utilized a non-hydrolyzable phosphoramidate substrate mimic to modestly inhibit enzymatic activity,[215] and the literature does not contain any subsequent reports on small molecule inhibitor development for bacterial HKD or non-HKD PLDs. In the case of NAPE-PLD, most insights have been garnered through studies in NAPE-PLD (-/-) mice.[216]

In Chapter IV, my goal was to identify potent small-molecule inhibitors of PldA. Although previous identified human PLD inhibitors containing benzimidazole and triazaspirone scaffolds were comparably poor inhibitors of PldA, we identified lead compounds based on selective estrogen receptor modulators with modest micromolar potency against recombinant PldA. Several analogs of desketoraloxifene were synthesized and parallel screening against PldA, hPLD1, hPLD2, and human NAPE-PLD was undertaken. Although PldA-selective compounds were not identified, this work is the first step towards developing potent inhibitors of PldA for assessing PldA as a viable therapeutic target of *P. aeruginosa*. Further steps needed to demonstrate proof of efficacy are discussed in more detailed in Chapter V.

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Experimental Methods

Modified Amplex Red assay.

Amplex Red assay kit was purchased from Invitrogen (Cat#A12219) and used with modifications detailed below. In a 96-black well plate, in a final reaction volume of 200 µL the following components were combined on ice to yield final concentrations of: 50 mM Tris (pH 7.5), 80 mM KCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 50 µM Amplex Red reagent, 0.1 U/mL choline oxidase, 1 U/mL horseradish peroxidase, 1.9 nM PldA, 20 µM (or 5 µg/mL) test compound and 0.5 mM 14:0 PC. Recombinant PldA was expressed in *E. coli* and purified as described earlier. (Chapter II) Lipid in chloroform was dried under a N2 stream in a glass test tube and resuspended in 50 mM Tris (pH 7.5) and 80 mM KCl buffer. All components were combined on ice and the reaction was initiated by incubating at 37° C and reading fluorescence emission at 590 nm (excitation = 530 nm) continuously at 90s intervals for 60 min using a fluorescence plate reader. The signal was normalized by subtracting the background signal produced in the presence of DMSO and absence of PldA. Initial velocities were determined from the linear component of the fluorescence signal. Data are presented as percent total activity normalized to vehicle control. All experiments were performed once in triplicate.

To detect false positive hits, each compound was tested again under identical conditions described above, except PldA and PC substrate were removed and substituted with 50 µM choline chloride. This experimental setting provides a way of assessing the ability of the compounds to inhibit either the choline oxidase of horseradish peroxidase enzymes independent of their effect on PldA. Compounds that were potential false positives were screened at 20 µM in the exogenous PLD assay to confirm PldA inhibition.

To generate concentration response curves for each hit, the assay as described above was used except a range of concentrations (0.1 μM-50 μM) were assayed for each compound.

Exogenous measurement of human PLD activity.

In vitro PLD activity was measured with an exogenous substrate assay as previously described.[161, 162] For a detailed procedure, see supporting information. Briefly, PLD activity was measured as the release of free [^{3}H]-choline from [choline-methyl-^{3}H] dipalmitoyl-phosphatidylcholine ([^{3}H]-DPPC). 3–50 nM PLD1 or PLD2 was reconstituted with phospholipid vesicle substrates composed of 10 μM dipalmitoyl-PC, 100 μM PE (bovine liver), 6.2 μM PIP2 (porcine brain), 1.4 μM cholesterol and 2.5 μCi [^{3}H]-DPPC. Reactions were stopped with the addition of trichloroacetic acid and bovine serum albumin. Free [^{3}H]-choline was separated from precipitated lipids and proteins by centrifugation, and was analyzed by liquid scintillation counting. Experiments were performed once in triplicate. The data were analyzed by nonlinear curve fitting and statistical analysis using GraphPad Prism 6.0. Data are plotted as mean ± SEM unless otherwise stated.

Exogenous measurement of PldA activity. Phospholipase D activity was measured using a modified exogenous assay.[161, 162] In brief, 6 nM purified PldA was incubated with liposomes containing 90 μM 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol), 10 μM 1,2-dipalmitoyl-sn-glycero-3-phosphocholine, and 2.5 μCi [^{3}H]-DPPC. The reaction was quenched with 10% trichloroacetic acid and bovine serum albumin on ice. Free [^{3}H]-choline was measured by scintillation counting. Experiments were performed once in triplicate. Data are presented as percent total activity normalized to vehicle control. Data were analyzed by non-linear regression and IC50 values generated using GraphPad Prism 6.0. Data are plotted as mean ±SEM unless otherwise stated.

Endogenous PLD assay of cell lines.

Endogenous PLD activity was determined using a modified *in vivo* deuterated 1-butanol (1-butanol-d10) PLD assay.[161, 212] Cells were seeded into 12-well tissue culture plates to reach 90% confluence at time of assay. Cells were pretreated in the presence of test compound (5 nM to 50 μM) or DMSO (vehicle control) in DMEM for 5 minutes at room temperature. After pretreatment media was removed, cells were treated with DMEM + 1 μM PMA + 0.3% 1-butanol-d10 and either test compound or DMSO vehicle control, or DMEM alone for 30 minutes at 37 ºC. After treatment, samples were extracted, internal standard added, and samples analyzed by mass spectrometry. Experiments were performed once in triplicate. IC50 values were determined using a concentration response curve and nonlinear curve fitting and statistical analysis performed using GraphPad Prism 6.0. Data are plotted as mean ± SEM unless otherwise stated. Data were collected and analyzed by Dr. Sarah Scott.

Steady state kinetics of PldA in the presence of raloxifene.

Amplex Red assay kit was purchased from Invitrogen and used with modifications detailed below. In a 96-black well plate, in a final reaction volume of 200 μL the following components were combined on ice to yield final concentrations of: 50 mM Tris (pH 7.5), 80 mM KCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 50 μM Amplex Red reagent, 0.1 U/mL choline oxidase, 1 U/mL horseradish peroxidase, 1.9 nM PldA. 14:0 PC in chloroform was dried under a N2 stream in a glass test tube and resuspended in 50 mM Tris (pH 7.5) and 80 mM KCl buffer. PldA activity was measured over a range of 14:0 PC concentrations (0.1–4 mM) in the presence of a single concentration of raloxifene at 0μM, 1μM, or 10μM). All components were combined on ice and the reaction was initiated by incubating at 37° C and reading fluorescence emission at 590 nm (excitation = 530 nm) continuously at 90s intervals for 60 min using a fluorescence plate reader.

The signal was normalized by subtracting the background signal produced in the presence absence of PldA at each different substrate concentration. Initial velocities were determined from the linear component of the fluorescence signal. Data are presented as the change in fluorescence intensity over time. All experiments were performed once in triplicate. Non-linear regression and statistical analyses were performed using GraphPad Prism 6.0.

Recombinant NAPE-PLD expression and purification.

E. coli expressing the plasmid pQE-80-PLDHis encoding C terminus 6× His tagged human NAPE-PLD was provided by Dr. Sean Davies (Vanderbilt University). Protein purification was performed as described previously.[217] Protein was concentrated and buffer exchanged into 10 mM Na_2HPO_4 500 mM NaCl.

NAPE-PLD activity assay.

In a final reaction volume of 100 μL, the following components were combined: 50 mM Tris, pH 7.5, 10 μM 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-nonadecanoyl (diC18:1, C19 NAPE), 1% N-octylglucoside or 0.1% Triton X-100, 0.5 μg partially purified NAPE-PLD and incubated at 37 °C for 30 min – 1 hr. At the end of the reaction the lipid products were extracted with 2:1 chloroform/methanol solution. The lipids were analyzed using previously described glycerophospholipid mass spectrometric methodology.[160, 161]

E. coli mass spectrometric analysis

BL21 Codon Plus (*DE3*) RIPL *E. coli* transformed with plasmids expressing wild type PldA (pET32b.PldA) or catalytically dead PldA (pET32b.PldA.H167N) were grown from frozen glycerol stocks in LB media with 100 μg/ml ampicillin and 50 μg/ml chloramphenicol at 37 °C. Cells were adjusted to an OD600 of 0.5 at time 0 h. Cells uninduced at time 0 and cells induced with 0.1 mM IPTG 1, 2, and 3 h post-induction were collected. Cells were pelleted at 6,000 x g

and then washed with 1X PBS and pelleted again. Cells were resuspended in 1X PBS and lipids extracted in 800 µl 1:1 1 N HCl/MeOH and 400 µl CHCl3. Samples were analyzed by mass spectrometry as described earlier.

E. coli mass spectrometric analysis after raloxifene treatment

BL21 Codon Plus RIPL *E. coli* transformed with plasmids expressing wild type PldA (pET32b.PldA) were grown from frozen glycerol stocks in LB media with 100 µg/ml ampicillin and 50 µg/ml chloramphenicol at 37 °C. Cells were adjusted to an OD600 of 0.5 at time 0 h. Cells were treated with 20 µM raloxifene or DMSO at time 0 h. Cells uninduced at time 0 and cells induced with 0.1 mM IPTG 1, 2, and 3 h post-induction were collected. Cells were pelleted at 6,000 x g and then washed with 1X PBS and pelleted again. Cells were resuspended in 1X PBS and lipids extracted in 800 µl 1:1 0.1 N HCl/MeOH and 400 µl $CHCl_3$. Samples were analyzed by mass spectrometry as described earlier (Chapter II).

Results and Discussion

PLD Fluorescence assay development

In order to screen compounds for inhibition of recombinant PldA, a modified version of the commercially available Amplex Red kit was used due to its convenience and high throughput potential. This assay allows for detection of direct modulators of PldA activity in a simple reconstitution assay. The Amplex Red assay utilizes a three-step enzymatic process to produce the fluorescent compound, resorufin. (Figure 31) Free choline liberated from PC by a PLD is then used by choline oxidase (CO) to generate H_2O_2. Horseradish peroxidase (HRP) in turn uses H_2O_2 to oxidize the Amplex Red compound to generate resorufin. The production of resorufin over time serves as a proxy for choline liberation and hence PLD activity. The most reproducible activity using the Amplex Red assay was with the synthetic short chain 1,2-diheptanoyl-sn-

glycero-3-phosphocholine (14:0 PC) substrate used below its critical micelle concentration, as opposed to a long chain PC in liposomes.

The strategy for addressing and identifying compounds with off-target effects during screening was to test each compound in the presence of choline chloride without PldA present to ensure that changes in the observed fluorescence were not attributable to inhibition of CO or HRP. For this reason it is important to further validate leads using the previously published and well established exogenous PLD assay as an alternative assay system.[161] The Amplex Red assay was developed to assess phospholipase activity and could be useful to screen large libraries of compounds for possible PLD inhibition; however it is critical to fully investigate the possibility of false positives through inhibition of CO or HRP.

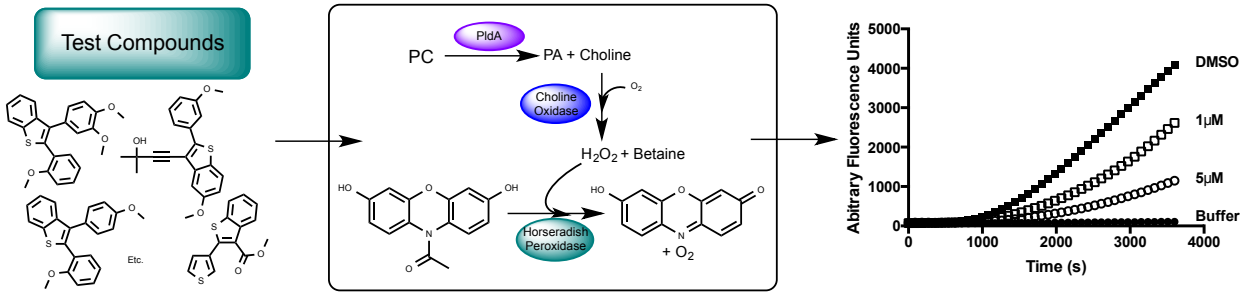


Figure 31. Amplex Red PldA inhibitor screening assay reaction scheme.

The exogenous PLD assay measures hydrolysis of PC in liposomes by recombinant, purified PLD enzymes in an *in vitro* system. Each assay system has its own merits and limitations. The Amplex Red assay uses monomeric substrate, while substrate is presented as a liposome in the exogenous assay. Hydrophobic compounds can intercalate into membranes causing inhibition due to disruption of the interaction with the lipid interface as opposed to directly altering

catalytic activity, leading to false positives. The use of a monomeric, soluble substrate allows examination of the effects of the small molecules on PldA activity independent of protein binding to lipid membrane, and enables selection of molecules that directly impact PldA activity. These compounds are highly hydrophobic with limited water solubility. Decrease in potency in the exogenous assay may be attributable to inhibitor sequestration in liposomal membranes or within vesicles that alter the concentration of inhibitor accessible to PldA.

Inhibitor screening of human PLD inhibitors against PldA

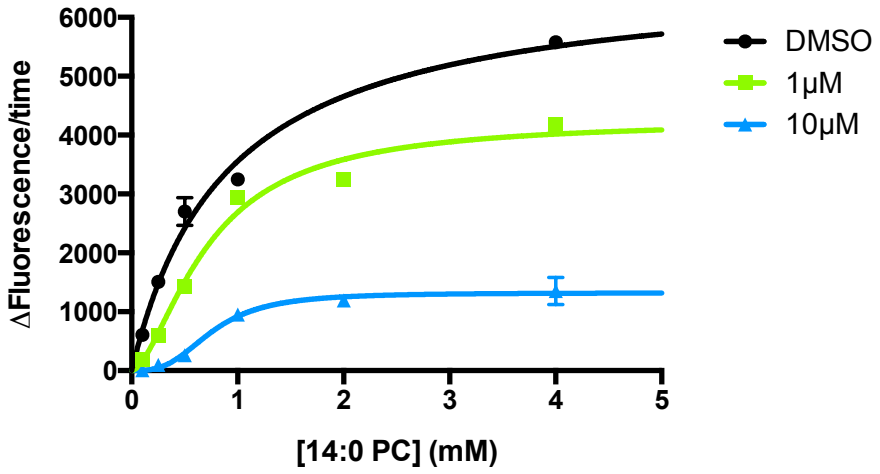
Potent isoform selective and dual inhibitors of hPLD1 and hPLD2 containing benzimidazole and triazaspirone scaffolds 1-**3** have been previously reported (Figure 30).[209-212] Because of the homology between the human isoforms and PldA, these compounds were screened for inhibition of PldA. Concentration response curves (CRCs) of representative hPLD1-, hPLD2-, or dual hPLD1/2-selective PLD inhibitors **1-3** were generated to evaluate their potency against PldA. A 1,000 to 10,000-fold discrepancy exists between potency against PldA and hPLD isoforms. These compounds, which inhibit hPLD1 and hPLD2 in the low nanomolar range, are comparably poor inhibitors of PldA, suggesting that these compounds bind to a region of the hPLD that is lacking or not well conserved in PldA.

Although the benzimidazole and triazaspirone inhibitor scaffolds are ineffective against PldA, there are other reported inhibitors of human PLD, including resveratrol, honokiol, polyisoprenyl phosphate, halopemide, and diethylstilbestrol[218-225], one of which is the selective estrogen receptor modulator (SERM), raloxifene (**4**). Selective estrogen receptor modulators (SERMs) (**4**-**7**) have long been used for the successful treatment of breast cancer. Raloxifene is often used in the treatment of osteoporosis in post-menopausal women and is also used as a preventative measure for women at a high risk for breast cancer development. Raloxifene was

developed as an anti-estrogen, but SERMs have since been shown to have differential effects based on findings of anti-proliferative effects in ER negative breast cancer, suggesting an ancillary mechanism of action for these compounds.[226] A previous report from our lab suggests off-target inhibition of human PLD as a potential ER-independent mechanism for the efficacy of these SERMs.[218]

Raloxifene was also tested for inhibition of PldA, and interestingly the raloxifene IC_{50} (3.9 μM) was 5- to 10-fold more potent than the hPLD1-selective, hPLD2-selective, and dual hPLD1/2 inhibitors, **2** (26.1 μM), **3** (19.1 μM), and **1** (40 μM), respectively. In contrast to the benzimidazole and triazaspirone scaffolds, the IC_{50} of raloxifene against hPLD1 and hPLD2, (12 μM and 5 μM, respectively) was very similar to the potency against PldA. In fact, all SERMs tested (**4- 7**) inhibited PldA (IC50' s 4 − 18 μM) (Figure 30). This data further suggest that although PldA is evolutionarily similar to the human PLD isoforms, there is differential conservation of inhibitor binding sites for these varied scaffolds. The identification of two variable lead scaffolds with differential binding sites supports the possibility of developing specific inhibitors of PldA.

To further characterize the effects of raloxifene on PldA, I performed steady state kinetics analysis in the presence of various concentrations of raloxifene to determine its mechanism of inhibition. (Figure 32) Substrate concentration was varied in the presence of a single concentration of raloxifene to generate dose response curves. PldA activity was measured using, 14:0 PC below its CMC, in the Amplex Red assay in order to perform Michaelis-Menten kinetics. With increasing concentrations of raloxifene, the V_{max} decreased, however the K_M remained constant for the substrate suggesting non-competitive inhibition.



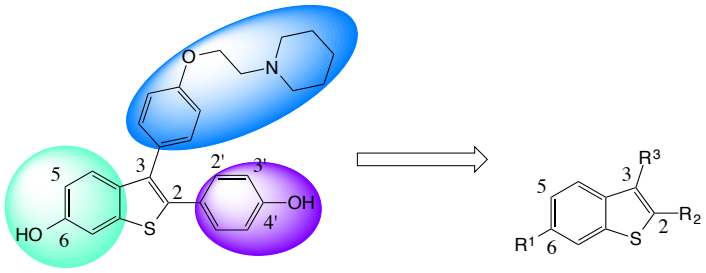
[Raloxifene]	K_M	V_{Max}
DMSO	0.8955±0.10	6741±284
1μM	0.7209 ±0.07	4260±203
10μM	0.7576±0.08	1322±82

Figure 32. Raloxifene acts as a non-competitive inhibitor of PldA. Steady state kinetics analysis was performed in the presence of DMSO or raloxifene at 1 μM or 10 μM using Amplex Red assay. Mean values are shown ± SEM.

These finding led us consider that other SERM analogues may also exhibit broader spectrum PLD inhibitory activity and prompted a more thorough investigation of structure-activity relationships within scaffold **5**. The use of the well-characterized SERMs **4**-**7** as starting points for inhibitor development aimed at human PLD and PldA proves attractive due to the vast amount of preclinical, clinical, and pharmacokinetics information already known about these compounds. Here, we report a novel desketoraloxifene-based scaffold that inhibited not only two human PLDs but also PldA and NAPE-PLD by a multifaceted chemical biology approach.

Novel scaffold PLD inhibitor screening and hit validation

To follow-up on the unique PLD inhibitory profile of **5**, we began to design analogue libraries; however, we took note of a library of analogues of **8** reported (Figure 33) by Larock and co-workers in conjunction with the Kansas University Center for Methodology and Library Development that surveyed three regions of the core. [227] Desketoraloxifene was developed by Eli Lily in the late 90s as a raloxifene analog that lacked the important carbonyl hinge in the parent molecule.[228] We not only screened PldA using the Amplex Red and exogenous assays, but also hPLD1 and hPLD2 using a cellular assay along with the exogenous assay.



Desketoraloxifene (**5**) 77 analogs (**8**)

Figure 33. Three regions of **5** surveyed in a diversity library of desketoraloxifene analogues **8**.

Cellular hPLD activity was assessed using a PLD transphosphatidylation reaction unique to this enzyme.[212] Instead of the traditional biological nucleophile water, in the presence of a primary alcohol (*n*-butanol), PLD will alternatively use the alcohol to produce a metabolically stable transphosphatidylation product, phosphatidylbutanol (PtdBuOH), instead of producing PA. PtdBuOHs were then isolated and detected using mass spectrometry (Figure 34).

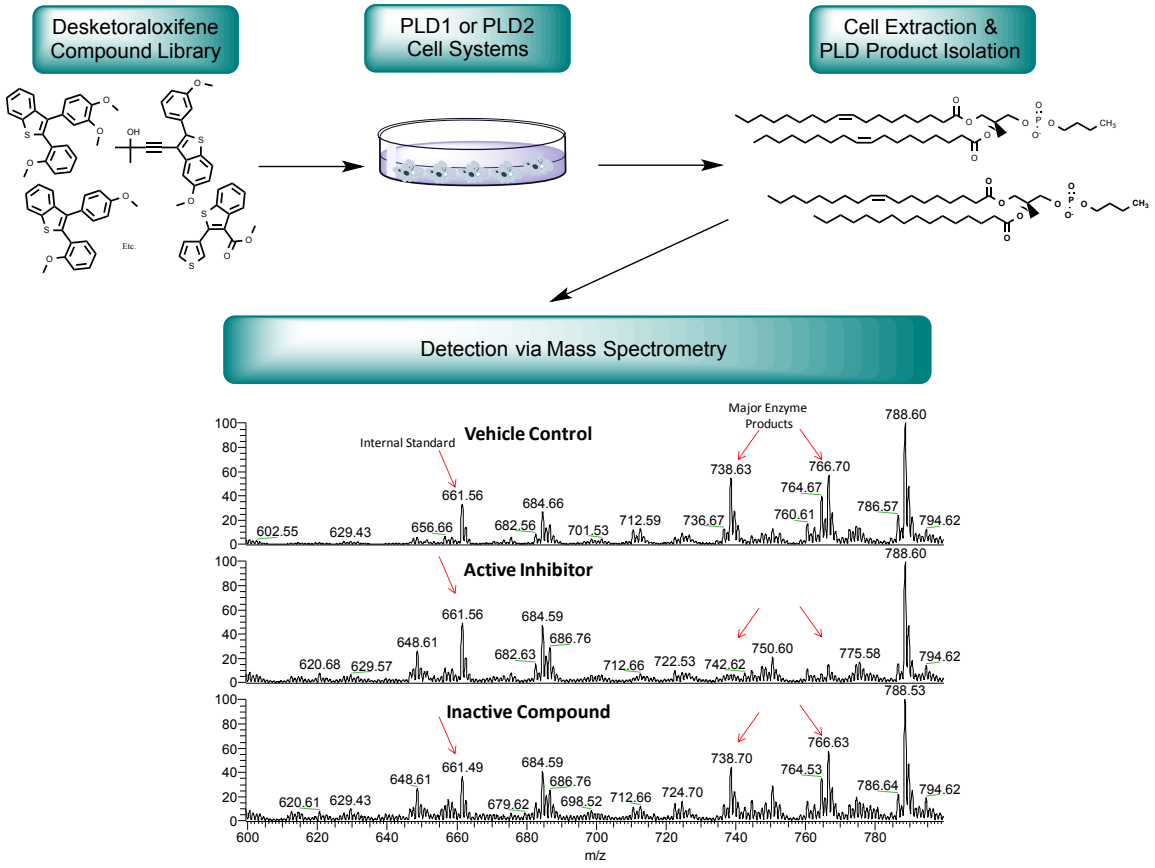


Figure 34. Mammalian PLD inhibitor screening assay scheme.

The cellular model systems for human PLD1 and PLD2 require that the molecules be cell penetrant, which is both a positive and a negative requirement, and the system is more complex allowing for indirect effects on PLD activity. The exogenous PLD assay utilizes recombinant purified human PLD enzyme and reconstituted liposomes in an *in vitro* system. Cell penetrance is no longer an issue and effects are likely more direct. The exogenous assay with human PLD isoforms also has the same caveats as discussed with PldA inhibitor screening.

We were graciously provided 77 analogues (**8**), and we first screened the library for inhibition of PldA using the Amplex Red assay tested at 5 μg/mL and inhibition of cellular hPLD1 and hPLD2 at tested at 10 μg/mL. From the single-point data, a number of analogues **8**

displayed inhibition of PldA. A number of analogues **8** possessed human PLD inhibitory activity, with a general preference toward inhibition of hPLD1 over hPLD2 (Figure 35A and 35B). Excitingly, as shown in Figure 35C, a number of analogues **8** displayed inhibition of PldA also. Data from the three assays enabled the assessment of favorable structure activity relationship (SAR) trends for inhibition of PLD1/2, PldA, or both (Figure 36).

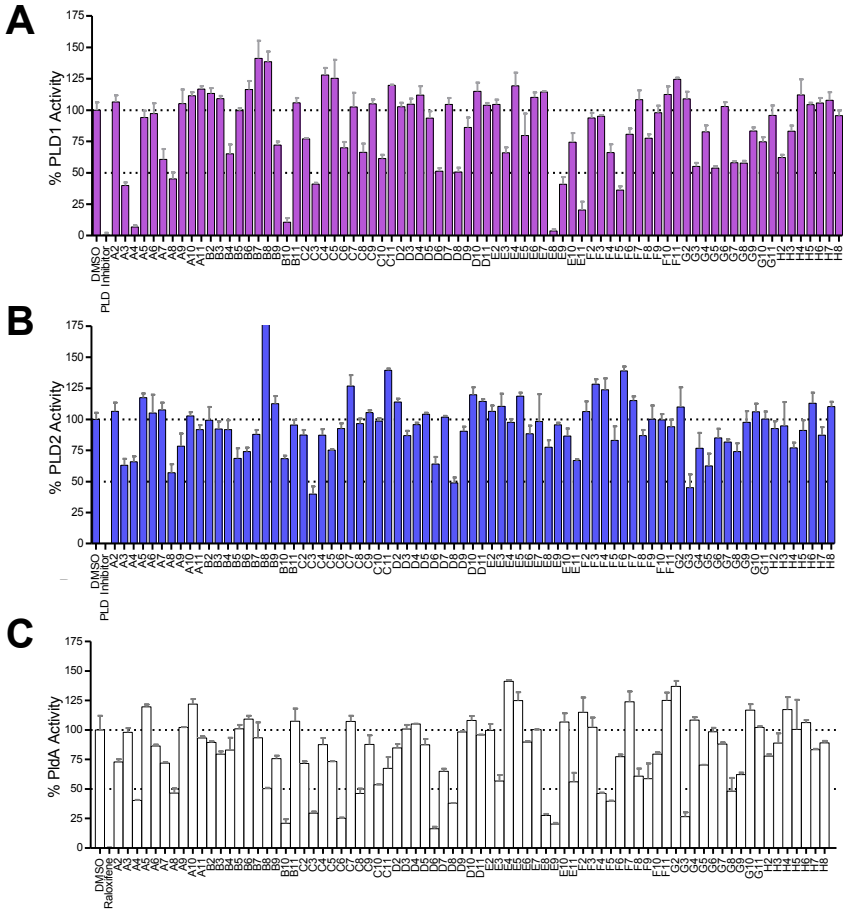


Figure 35. Single-point screen of analogs **8**. (A) Desketoraloxifene analog **8** inhibition of cellular PLD1 cells (B) or cellular PLD2 when tested at 10 μg/mL. (C) Single point desketoraloxifene analogs **8** inhibition of PldA when tested at 5 μg/mL.

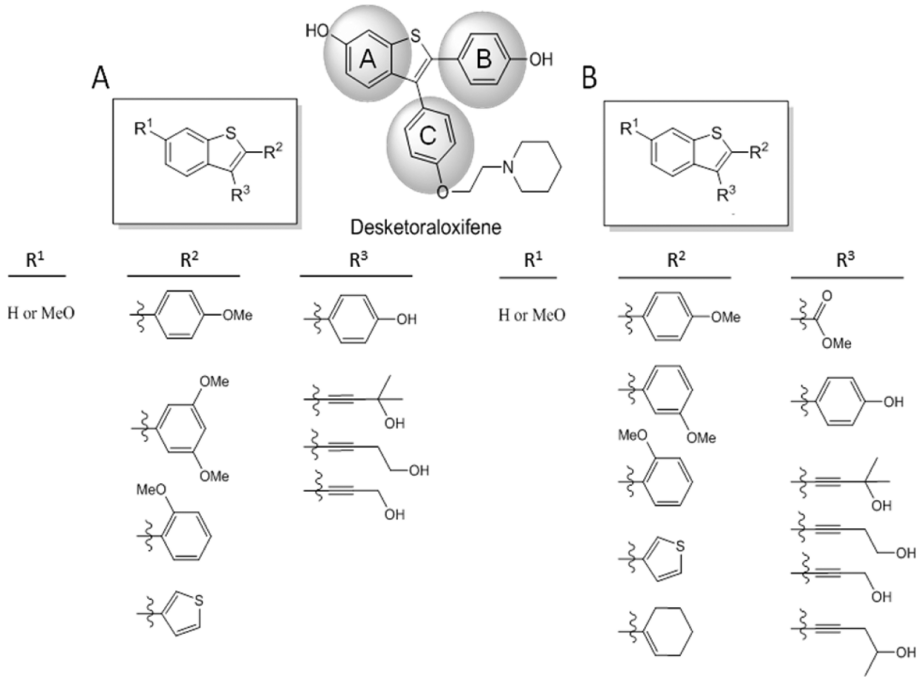


Figure 36. Structure activity relationship of hits of analogues **8** for (A) hPLD and (B) PldA.

Using the SAR trends observed from the initial desketoraloxifene analogue library, additional targeted compounds were synthesized for the further development of small molecule mammalian and bacterial PLD inhibitors, but due to unfavorable DMPK (drug metabolism and pharmacokinetics) properties and potential safety concerns, the acetylenic analogues were not pursued further; rather, synthesis was focused on aryl substitution patterns not represented in the provided library of analogues **8**.

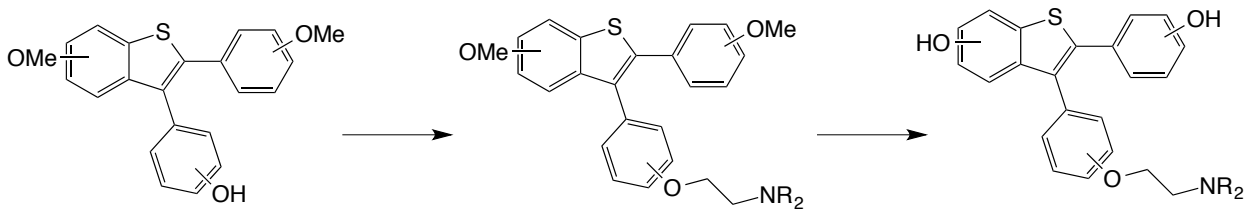


Figure 37. Desketoraloxifene analogues **9-11** synthesized based on analogues **8** SAR trends.

Novel analogues **9-11** were synthesized based on the SAR trends observed in the initial published library and were assessed for their ability to inhibit PldA, hPLD1, and hPLD2. (Figure 37) Analogues **9-11** were synthesized in the Dr. Craig Lindsley lab by Matthew O'Reilly and Kyle Brown as published.[229] Compounds were also screened against PldA at 20 μM using the modified Amplex Red assay. They were further validated as hits using the exogenous PldA assay and found to inhibit PldA activity >50% at 20 μM. Human isoform inhibition was again assessed in the PLD1 and PLD2 model cellular systems at a single set 20 μM concentration. Inhibitory activity was determined by comparison with DMSO vehicle control and hits were identified as compounds that inhibited >50% activity or showed an isoform selective profile between PLD1 and PLD2 (Figure 38). Many compounds that significantly inhibited PldA activity were similar to those that inhibited the human enzymes (Figure 38). Thus, we were excited to find novel desketoraloxifene analogs that inhibited both human PLD and PldA from this parallel library effort.

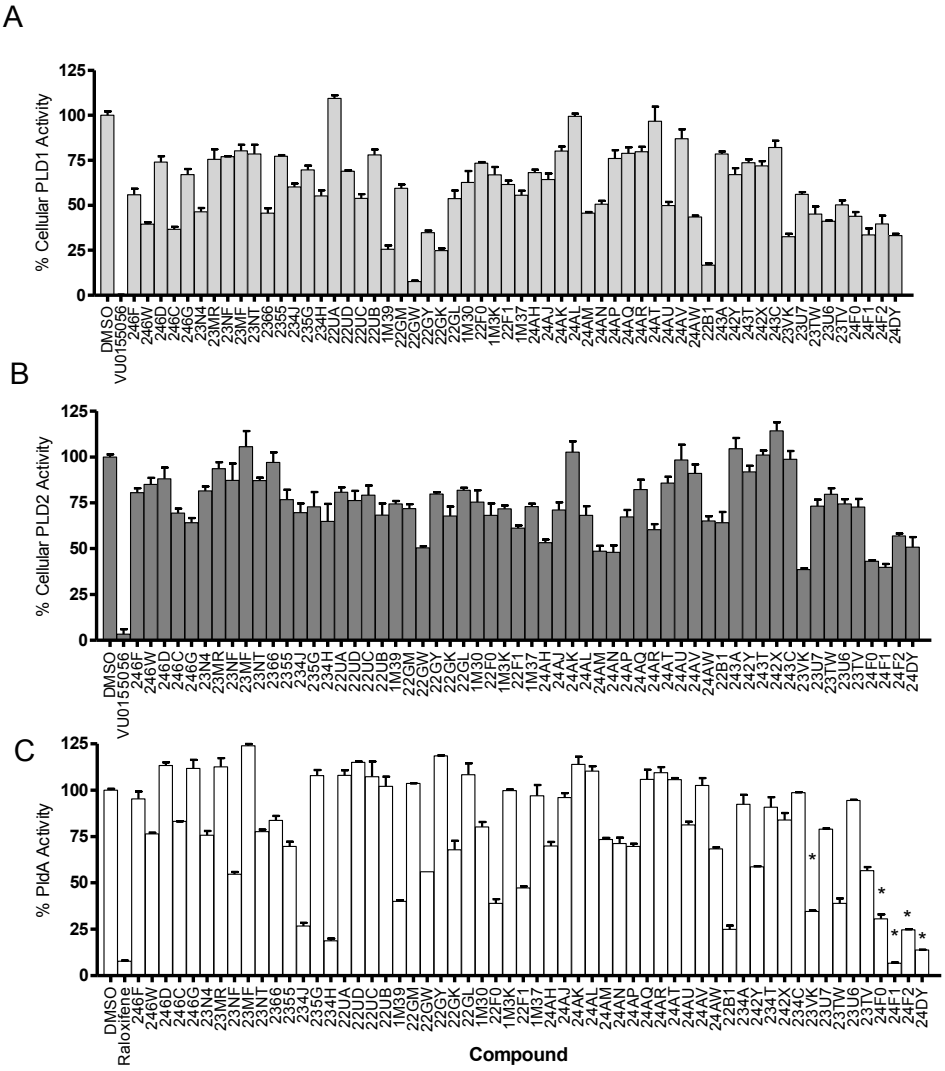


Figure 38. Single point screen of analogues **9-11** against (A) hPLD1, (B) hPLD2, and (C) PldA. Compounds were screened at 20 μM.

As mentioned earlier, because of the enzyme-coupled system used in the Amplex Red assay, off-target inhibition of CO or HRP is possible. To illustrate this, analogues **9-11** were screened in the Amplex Red assay in the presence of choline chloride but absence of PldA and PC (Figure 39). Choline as a positive control induces an increase in resorufin production. Analogs **11e-11h**

inhibited resorufin production independent of PldA suggesting off-target inhibition of CO or HRP. These compounds were later screened using the exogenous assay with PldA and found to inhibit PldA activity (Table 3).

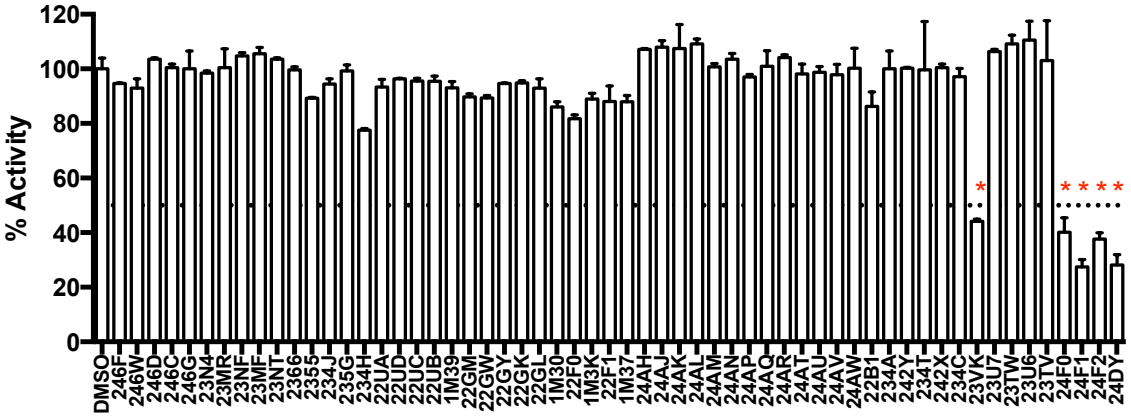
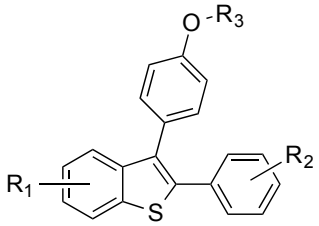


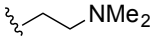
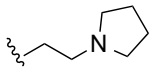
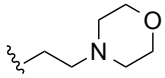
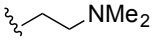
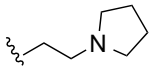
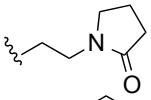
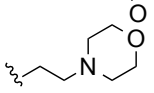
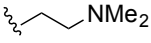
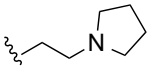
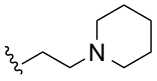
Figure 39. Amplex Red off-target screen of analogues **9-11** using choline chloride. Compounds screened at 20 μM. *Inhibition of fluorescence signal in the absence of PldA.

Concentration response curves of lead compounds were generated using the cellular model systems and exogenous assay for the human isoforms, or using the modified Amplex Red assay and exogenous assay for PldA (Table 3). These compounds are highly hydrophobic with limited water solubility. Decrease in potency in the exogenous assay may be attributable to inhibitor sequestration in liposomal membranes or within vesicles that alter the concentration of inhibitor accessible to PldA. A similar phenomenon seems to occur for the human isoforms. There was a selective inhibition of PLD1 over PLD2 using the cellular screening assay, however analysis with recombinant protein using the exogenous assay demonstrates that the compounds directly affect the activity of both PLD isoforms. Differences in the subcellular localization, and

therefore accessibility, of these compounds to PLD1 and PLD2 within these cells may also explain this discrepancy.



11

Cmpd	R_1	R_2	R_3	Cellular PLD1 IC_{50} (μM)[a]	Cellular PLD2 IC_{50} (μM)[a]	Exogenous PLD1 IC_{50} (μM)[a]	Exogenous PLD2 IC_{50} (μM)[a]	Exogenous PldA IC_{50} (μM)[a]	Amplex PldA IC_{50} (μM)[a]
11a (1M39)	H	3-OMe	H	13.4±0.9	>20	>20	>20	7.3±1.0	6.4±1.1
11b (22GW)	H	3-OMe		2.6±0.3	>20	4.7±2.2	7.1±2.4	16.1±1.7	7.5±0.7
11c (22GK)	H	3-OMe		11.3±1.5	>20	7.6±2.2	1.8±0.6	17.6±2.1	>20
11d (22GY)	H	3-OMe		12.1±0.6	>20	>20	>20	>30	>20
11e (24F1)	5-OH	4-OH		6.9±0.6	15.6±2.2	19.7±5.1	5.8±1.0	10.6±0.9	ND
11f (24DY)	5-OH	4-OH		7.3±2.0	>20	10.8±1.5	4.7±0.7	12.0±1.2	ND
11g (23VK)	5-OH	4-OH		5.8±2.6	>20	2.9±0.6	3.5±0.6	6.8±0.9	ND
11h (24F0)	5-OH	4-OH		10.1±0.9	>20	11.2±01.6	3.3±0.5	12.1±0.5	ND
11i (22B1)	H	3-OH	H	5.6±0.9	>20	11.5±01.5	6.2±0.7	17.8±1.0	2.1±0.2
11j (2336)	H	4-OMe	H	15.1±2.7	>20	10.1±1.1	3.2±1.5	>30	>20
11k (234J)	H	4-OMe		>20	>20	>20	>20	22.4±1.2	5.5±0.2
11l (234H)	H	4-OMe		>20	>20	17.6+5.3	8.2±3.4	22.±1.1	2.95±0.3
5	5-OH	4-OH		10.1±1.7	>20	6.1±0.5	2.6±2.4	9.9±0.8	8.7±0.7

[a]Each IC_{50} is the average of three experiments and expressed with ±SEM; ND = not determined.

Table 3. IC50 values against PldA and human PLD

In general, compounds closely related to the desketoraloxifene parent structure **5** were the most favorable *pan*-PLD inhibitors. These compounds contained modest modifications to the C ring of the scaffold but retained hydroxyl substitutions in the A and B rings. Many compounds that inhibited mammalian PLDs also inhibited the bacterial enzyme (**11a-c**, **11e-i**, **11k**, **11l**) although the overall SAR trends were not completely conserved. Several compounds, e.g., **11a** and **11l**, displayed enhanced inhibition of PldA compared to the mammalian PLD1 and PLD2. Alternatively, **11c** and **11j** preferentially inhibited mammalian PLD1 and PLD2 over PldA (Table 1). However, the SAR was far from robust, and highlights the power of preparing libraries of analogs to capture serendipitous bioactivity.

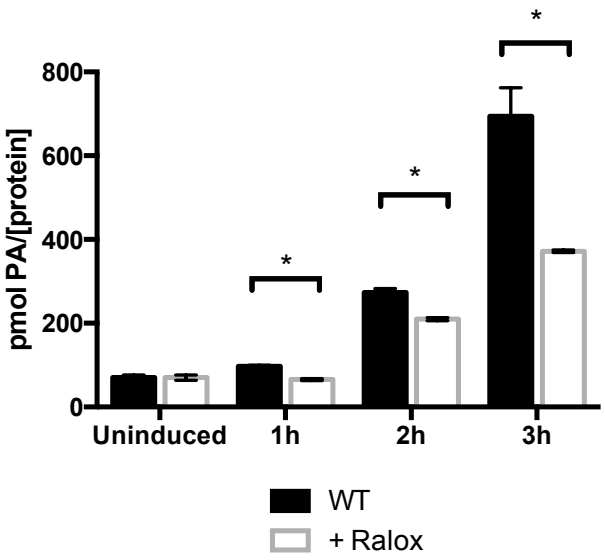


Figure 40. Raloxifene inhibits PldA in *E. coli* overexpressing PldA. Cells were treated with DMSO or 20 μM raloxifene and induced with IPTG for the times indicated. *E. coli* lipids were extracted and analyzed by LC-MS. Statistical analysis performed using student's t-test. *$p < 0.01$

One limitation of these studies is the lack of a physiologically relevant assay for PldA. We could utilize a cellular based assay for evaluating PLD inhibition by SERM analogues, however we did not have a cellular system to look at PldA activity. Instead, I chose to use the *E. coli* overexpression system used for production of recombinant PldA. *E. coli c*ells were pre-treated with 20 μM raloxifene and left uninduced or induced with IPTG to express PldA over 3 h (Figure 40). PtdOH was measured at various time points by LC-MS. PtdOH levels increased in cells expressing wild type PldA over time. The treatment of cells with raloxifene inhibited PldA activity at each time point. The data suggest that raloxifene is *E. coli* cell-penetrant and can inhibit PldA in bacterial cells. The next steps would be to determine these inhibitors can augment PldA activity during infection and augment *P. aeruginosa* infectivity.

Effect of human PLD and desketoraloxifene inhibitors on NAPE-PLD enzymes.

As previously mentioned, other enzymes, such as NAPE-PLD, exhibit phospholipase D-like phosphodiesterase activity; however they are not structurally related to HKD-containing PLD family, which include human PLD1 and PLD2 and PldA. The potency of various structural classes of PLD inhibitors developed against HKD-containing PLD proteins were evaluated on recombinant human NAPE-PLD. Each compound was assayed at a single 20 μM final concentration (Figure 41A). PtdOH production by NAPE-PLD was assessed utilizing mass spectrometry.[160] The PLD1-selective, PLD2-selective, and dual PLD1/2 inhibitors **1**-**3** have poor potency against NAPE-PLD under these conditions, and like the data for PldA, suggest the mammalian allosteric site is absent in this PLD enzyme as well. Raloxifene (**4**) was also observed to not inhibit NAPE-PLD activity (at 20 μM) although it was capable of inhibiting PLD1, PLD2 and PldA. We also evaluated desketoraloxifene as an inhibitor of the highly structurally divergent NAPE-PLD and discovered that it also inhibited NAPE-PLD (IC_{50} = 58

μM), albeit weakly. Moreover, the desketoraloxifene analog **11b** also displayed inhibition of NAPE-PLD, with an IC_{50} of 67 μM (Figure 41B). Inhibition of NAPE-PLD by other documented inhibitors, such as MAFP, or methyl arachidonyl fluorophosphates, was likely due to perturbations of the lipid-detergent micelles disrupting protein-lipid interactions attributable to the high concentrations required for inhibition (IC_{50} = 1mM). Due to the hydrophobicity of these compounds and the high concentrations required for inhibition of NAPE-PLD, the inhibition may be indirect. The compounds may be perturbing the detergent-lipid micelles to inhibit the protein-lipid interaction. No assay with soluble protein is available to test this effect. While weak, **5** and **17b** are rare examples of NAPE-PLD inhibitors, and the first examples of a chemotype that can universally inhibit human PLD1/2, bacterial PldA and human NAPE-PLD, suggesting a potential common binding site amongst structurally and phylogenetically distinct PLD enzymes.

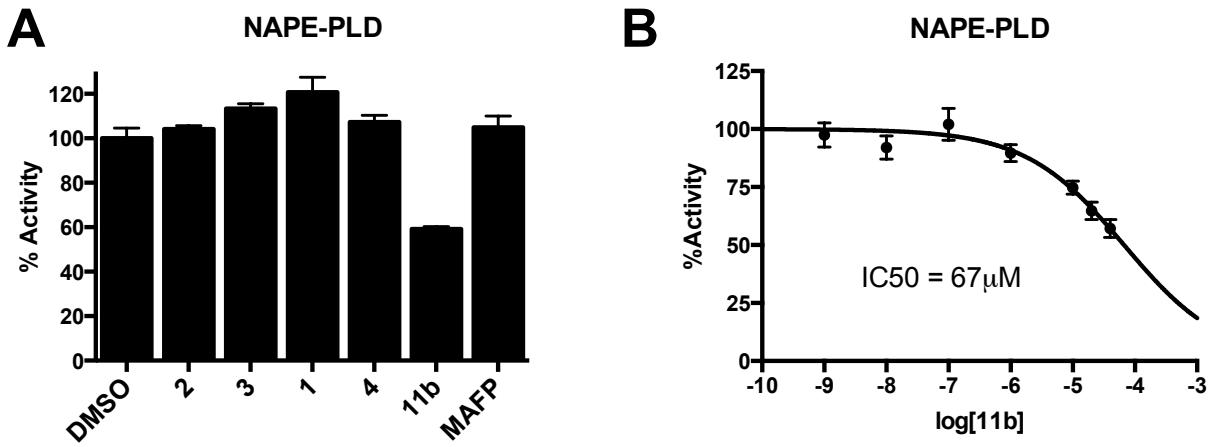


Figure 41. Screening of unique structural PLD inhibitors against human NAPE-PLD. Compounds were screened against NAPE-PLD via a mass spectrometry assay at 20 μM, except for MAFP, which was used at 500 μM. (C) CRC for 11b on human NAPE-PLD (IC_{50} = 67 μM).

A new class of PLD inhibitors has been classified based on the SERM desketoraloxifene **5**. Herein we identify the first published inhibitors of an important bacterial PLD, *Pseudomonas aeruginosa* PldA, as well as weak, yet improved, inhibitors of NAPE-PLD. The compounds are modest dual hPLD1/2 inhibitors, but the first to inhibit a diverse range of structurally and phylogenetically diverse PLDs. PldA, has been implicated in promoting chronic infection of the opportunistic pathogen *Pseudomonas aeruginosa*.[56] Therapeutics targeting this bacterial PLD may serve as viable strategies for treating infections. The identification of a sub-millimolar inhibitor of NAPE-PLD will help to better define the role of this enzyme in cellular processes. SERM compounds are often well tolerated in the patients, have good pharmacokinetics and have been used for decades in the clinic, which makes this class of analogs an ideal starting point for the development of novel, universal inhibitors of mammalian (PLD1/2, NAPE-PLD) and bacterial PLD (PldA). These are significant findings, which have provided a lead series for further, focused optimization efforts towards potent *pan*-PLD inhibitors for *in vivo* studies.

CHAPTER V

SUMMARY AND FUTURE DIRECTIONS

Several PLD have been identified as virulence factors of bacterial pathogens crucial for host colonization, cell adherence, cell invasion, and altering host cell signaling.[38, 44-48, 52, 55-57] PldA is one such example, which is important for establishing persistent infection[56], but also functions to enable intra- and inter-species bacterial competition[52, 57]. Although bacterial PLD contribute to the infectivity of a range of pathogens, little characterization regarding their enzymatic activity, regulation, and direct molecular mechanism by which they contribute to virulence has been thoroughly examined.

The objective of this study was to biochemically characterize the enzymatic properties of PldA *in vitro* and from these studies identify potential protein and lipid regulatory factors in eukaryotic cells. Although preliminary studies of PldA activity have been performed[56], an extensive analysis of PldA substrates *in vitro* and in host cells is lacking. Given the sequence similarity of PldA to mammalian PLD and that PldA targets eukaryotic cells, I hypothesized that during infection PldA serves to mimic host PLD in some capacity during infection. In order to do this, PldA may need to share some critical properties or regulation with hPLD. Therefore in Chapter II, I began by examining the substrate specificity, transphosphatidylation capabilities, divalent cation sensitivity, and potential lipid and protein activators and interactions of PldA. Interesting deviations between hPLD and PldA were observed, specifically, substrate preference (Table 2), transphosphatidylation efficiency (Figure 8), and stimulation by human PLD protein activators (Figure 11), however noteworthy similarities were also identified, including divalent cation simulation (Figure 10), phosphoinositide stimulation (Figure 12-16), and Akt binding (Figure 17). Identification of interactions or regulatory mechanisms conserved between PldA and

the host PLD provides insights into possible pathways involved or mimicked by PldA during infection.

PldA was observed to have differential substrate preference relative to hPLD isoforms, but despite this, the products generated are remarkably similar (Table 2, Figure 27). hPLD primarily hydrolyzes PC in cells to generate mono- and di-unsaturated PtdOH.[207, 208] The predominant PC species in most human cells are the corresponding PtdOH species generated by hPLD[208] making it difficult to assess whether hPLD displays substrate preference based on acyl chain composition in cells. PldA on the other hand displays preference for mono- and di-unsaturated PE in A549 cells (Figure 27) even though these species are significantly less abundant than PUFA containing PE species (Figure 28). PldA produces hPLD-like PtdOH species, in spite of the lower abundance of corresponding PE substrate species. The ability to mimic hPLD generated PtdOH may be crucial as the acyl composition may be important for downstream signaling cascades and proteins that directly bind PtdOH.

Transphosphatidylation is a trademark reaction performed by HKD-containing PLD. Most PLD are capable for utilizing primary alcohols, such as ethanol and butanol, to generate phosphatidylalcohols, however this is not completely generalizable.[170] Historically these primary alcohols have been used to investigate the cellular processes regulated by PLD activity, however off-target effects are possible that may be attributed to PLD.[17] The ability of butanol to inhibit *C. trachomatis* cell invasion and survival was used as evidence that PZ-PLDs function as true PLDs without characterization of hydrolysis of transphosphatidylation activity.[55] These effects of butanol could be ascribed to off-target effects by unknown mechanisms. Here, I show that primary alcohols do not efficiently inhibit PtdOH production by PldA (Figure 8). This was somewhat unexpected as the catalytic sites of PldA and hPLD are significantly homologous.

Based on these substrate preference and transphosphatidylation studies with PldA, caution should be taken not to assume that general properties ascribed to highly characterized PLD apply to all PLD superfamily members. HKD-containing PLD superfamily members generally assumed to possess PLD activity, however endonuclease activity is also possible, but seems to rarely be considered. This is another reason why biochemical characterization is important for PLD enzymes.

Substrate kinetic analysis was intended to be a significant component of this project, however calculating kinetic parameters, such as V_{max} and K_M, for PldA proved challenging. As PldA is an interfacial enzyme, simple Michealis-Menten kinetics do not accurately describe the kinetic properties of PldA. Instead substrate dilution kinetics must be used, which require a suitable detergent or neutral, non-substrate, phospholipid to form an aggregated membrane in which substrate concentrations can be varied.[176] Identification of a suitable bulk diluent was unsuccessful for PldA. In the course of these studies, an apparent PC substrate inhibition was observed (Figure 9). The physiological significance is unclear, however as PldA interacts with and hydrolyzes eukaryotic cellular membranes, most of which are composed primarily of PC[165], this is a potentially relevant phenomenon.

Interestingly, PldA was found to contain a partially conserved PIP_2 binding motif[190, 195] found in eukaryotic PLD (Figure 56). All phosphoinositides found in eukaryotic cells were capable of stimulating PldA hydrolysis with the most profound effect on PC hydrolysis and binding. (Figures 12-16) Phosphoinositides are major regulators of hPLD localization and activity[187, 188, 190, 195] and conservation of this PIP_2 binding site and stimulation by phosphoinositides suggest that PldA function within eukaryotic cells can be regulated similarly. Through conserving this interaction, PldA localization and signaling may mimic that of the host PLD more effectively.

Future studies are needed to address whether PldA localization is determined by phosphoinositides. Identification of the phosphoinositide binding site and subsequent mutagenesis and imaging studies can be used to answer this question.

PldA is not the first example of a secreted bacterial protein that targets eukaryotic cells that interact with phosphoinositides. Phosphoinositides, including PIP_2, were shown to directly bind and stimulate PLA2 activity of the *P. aeruginosa* T3SS effector, ExoU.[230] The *V. cholera* T6SS effector, VasX, also interacts with an array of phosphoinositides. Along with PldA and PldB of *P. aeruginosa*, VasX is a T6SS effector that targets bacterial and eukaryotic cells.[80, 231] What is still to be determined for all three of these effectors is what role if any phosphoinositides play in terms of effector function during infection.

The conservation of the PIP_2 binding motif and the structural similarity between PldA and hPLD isoforms also prompted me to investigate whether the upstream protein activators of hPLD also modulated PldA activity (Figure 11). PKC nor the small GTPase, Cdc42 and Arf1, stimulated PldA activity even when present at several fold excess of PldA. Upon further examination, regions of homology between hPLD and PldA do not overlap the mapped regions of interaction for these activators with hPLD.[159, 190, 195] Since these interactions were not conserved, I used a Scansite 3 search[197] to look for other putative protein-protein binding motifs and phosphorylation sites within the PldA protein sequence. Several interesting high and medium stringency sites were identified, including high conservation the Akt phosphorylation motif. PldA was confirmed as an *in vitro* substrate for Akt using a non-radioactive Akt kinase assay (Figure 17). Previous published accounts demonstrated binding of Akt with PldA.[52] Although I was not able to co-immunoprecipitate Akt with PldA from cellular lysates, this *in vitro* kinase assay also confirms an interaction between PldA and Akt, although the significance

of this phosphorylation is unknown. Threonine 55 is the predicted phosphorylation site. Determining whether phosphorylation at T55 occurs within human cells and whether this affects cell invasion are important next steps.

The effects of PldA on *P. aeruginosa* lipids have been previously investigated and published,[57] however investigation of the eukaryotic cellular substrates and products of a PLD virulence factor has never been performed. In Chapter III, I continue analysis by assessing PldA activity in bacterial and lung epithelial cells. Understanding of the parallels between the human isoforms of PLD and PldA in this work have contributed to predictions of its regulation and function as a virulence factor. This work details the first characterization of the eukaryotic cellular substrates hydrolyzed and products generated by PldA. Intriguingly the lipid product species generated by PldA in A549 cells closely resembled those of hPLD (Figure 27), despite the difference in substrate preference of these two enzymes. The PldA-activity dependent changes to endosomal lipids (Figure 27) and intracellular stability (Figures 24) prompted an examination of the intracellular localization of PldA over time. Visually PldA labeled puncta, likely intracellular vesicles translocated across the cell body over time in an activity dependent manner (Figure 25), resembling vesicular trafficking, a cellular process commonly associated with eukaryotic PLD activity.[14, 159, 194] Conservation of regulatory mechanisms, such as phosphoinositide stimulation, and interactions with human Akt may be involved in these vesicular trafficking events and are points of interest for future study.

I was able to recapitulate the previous observation of PldA stimulation of Akt phosphorylation[52] with exogenously applied PldA (Figure 29). Despite the difference in delivery, uptake from the medium versus direct translocation by the T6SS, PldA could induce Akt activation. Akt regulates a wide array of downstream signaling pathways that control cellular

metabolism, cell migration and invasion, and cell survival, growth, and proliferation.[232] Further experiments are needed to map out the specific downstream pathways important for inducing cell invasion of *P. aeruginosa*. The molecular mechanism by which PldA stimulates Akt phosphorylation has not been determined. PtdOH by hPLD2 has been shown to increase PIP_3 binding and membrane recruitment of Akt, which in turn leads to increased phosphorylation and activation.[163] Another anionic lipid, PS has been shown to increase Akt-PIP_3 membrane binding and promote increased Akt phosphorylation through changes in Akt conformation upon PS binding.[198] hPLD can also modulate phosphoinositide synthesis to promote endocytosis, summarized in Figure 42. PtdOH produced by hPLD stimulates the lipid kinase, PI4P5K, which phosphorylates PI(4)P to generate $PI(4,5)P_2$.[233, 234] hPLD is then recruited and stimulated by PIP_2 and Arf6 to generate more PtdOH creating a feed forward loop to increase PIP_2 levels.[235] PIP_2 serves to recruit an array of proteins needed to induce endocytosis and actin rearrangement.[193, 236, 237] PI3K can then phosphorylate $PI(4,5)P_2$ to generate PIP_3 which recruits Akt to membranes for phosphorylation. Akt can then also participate in cytoskeletal rearrangement and endocytosis.

I propose two mechanisms by which PldA may increase Akt activation upon *P. aeruginosa* cell infection. (1) PldA is co-localized to PIP_3 rich membranes and PtdOH produced by PldA by direct binding increases Akt membrane recruitment and binding to PIP_3; and (2) PIP_2 binding by PldA serves to localize PldA to the plasma membrane setting off a feed forward cascade of PIP_2 and PIP_3 accumulation resulting in Akt membrane recruitment. Both of these hypotheses are consistent with the observed requirement for PI3K activity[52] for cell invasion and are not mutually exclusive.

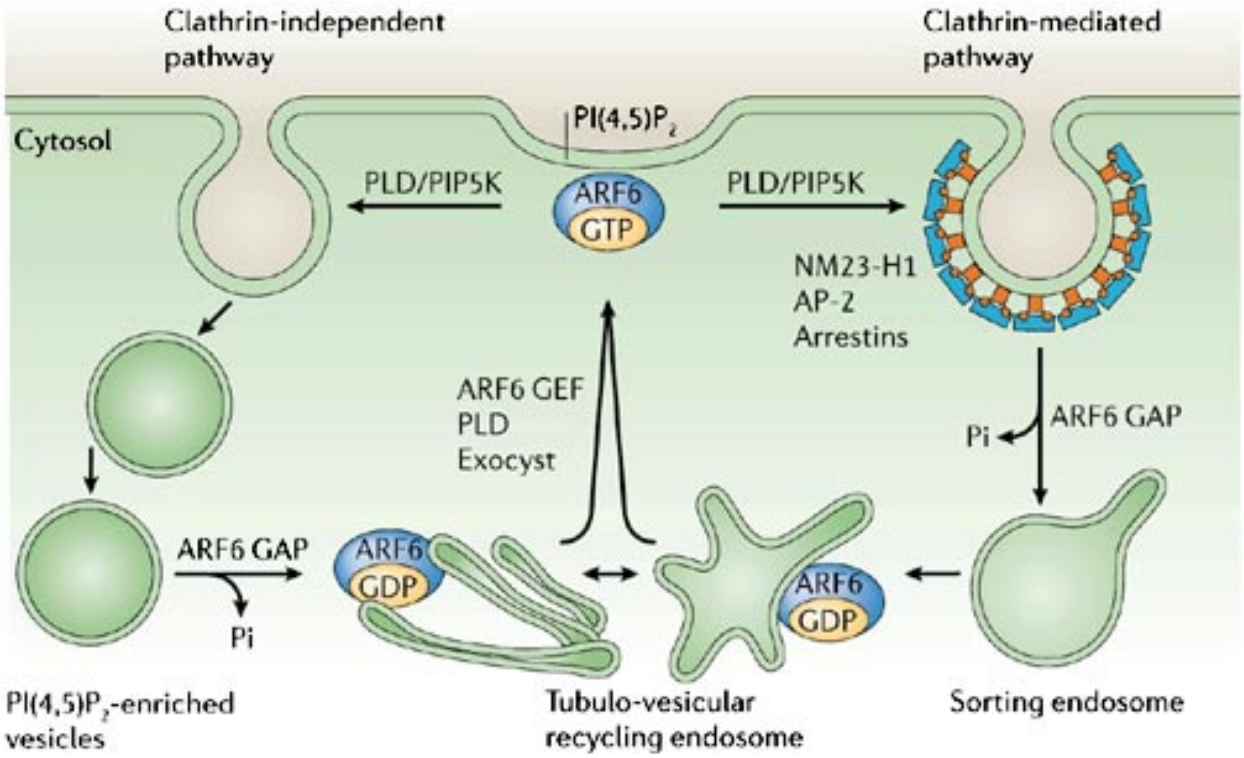


Figure 42. PLD/PI4P5K/PIP_2 feed forward loop increases $PI(4,5)P_2$ to mediate endocytosis. PtdOH produced by hPLD is a cofactor for PI4P5K activation causing $PI(4,5)P_2$ to accumulate at the plasma membrane. $PI(4,5)P_2$ then recruits cellular components of both clathrin-dependent and clathrin-independent endocytosis. Reproduced with permission from D'Souza-Schorey, et al.[237]

As the mechanism of PldA delivery in these experiments with A549 cells was not the physiologically relevant means of entry, further studies are needed to confirm whether changes in phospholipids or vesicular trafficking observed here are the same during infection with *P. aeruginosa*. Many of the changes in PldA-associated vesicle localization occurred over several hours (Figure 25). It would be interesting to assess the fate of *P. aeruginosa* and cellular vesicles upon *P. aeruginosa* infection over an extended time period, and whether those changes are PldA

specific. More work is also needed to identify the intracellular organelles co-localized with PldA. Are those structures of the endolysosomal pathway or perhaps autophagosomes or other organelles? Co-localization studies using confocal microscopy and a range of organelle markers can be used to address this question. As PldA co-localized puncta move throughout the cell over time, the mechanism by which this occurs is also unknown. Likely significant cytoskeletal changes occur. Endocytosis of *P. aeruginosa* also requires cytoskeletal changes. Effects of PldA on actin rearrangement still need to be examined.

Most of this work was conducted using recombinant PldA allowing for recognition of factors that directly modulated PldA activity. One limitation of these *in vitro* studies was the lack of studies using a more physiologically relevant system. The primary downstream cellular consequence of interest identified so far is cell invasion. To fully understand the relevance of the findings presented here, they must be confirmed within the context of cell infectivity. *P. aeruginosa* potentially secretes multiple effectors along with PldA that may interact or influence PldA activity. Other factors may have combinatorial effects on targeted cells with PldA that cannot be assessed in these studies. For example, the full significance of the *Legionella* PLD, LpdA, could not be properly evaluated without the presence of a corresponding effector, LecE. The data suggested that LpdA induced PtdOH generation served to further exacerbate an increase in DAG production by LecE. DAG accumulation was proposed to be the relevant effect of interest. LpdA functional relevance is intrinsically tied to another bacterial effector. Although PldA is necessary, it is likely not the only factor that influences cell invasion of *P. aeruginosa*; multiple bacterial elements likely work in tandem to facilitate this effect.

Targeting of virulence factors is an emerging trend in antibacterial drug discovery. Many human pathogenic bacteria have acquired multi-drug resistance and with a lack of next

generation bactericidal agents in the drug pipeline, virulence factors provide a previously untapped source of new therapeutic targets. As virulence factors are not required for cell viability, but are significant contributors to pathogenesis, development of drug resistance to virulence inhibitors may occur more slowly than bactericidal compounds due to less selective pressure. The first limitation to targeting virulence mechanisms is a lack of small-molecule modulators. Chapter IV details identification of the first published inhibitors of a PLD virulence factor. Our starting points were SERM compounds, which have good pharmacokinetics, and for which extensive preclinical and clinical data exists. Not only could these SERM analogs inhibit PldA, they were found to be pan-PLD inhibitors of hPLD1, hPLD2, and human NAPE-PLD, a non-HKD PLD. This study generated a lead series for further, focused optimization efforts towards potent PldA inhibitors to study the viability of targeting PldA in *in vivo* infection studies.

This was a successful preliminary study to identify PldA inhibitors, however significantly more work is necessary to vet PldA as a viable therapeutic target, but this study is a necessary initial step. Improved solubility is needed as many analogues had poor water solubility and frequently precipitated at concentrations above 40 μM. Potency is another area that needs improvement, as no sub-micromolar inhibitors were identified (Table 3). We did not identify PldA-selective compounds, which would be highly advantageous. hPLD in leukocytes is associated with microbial killing[32-34], so inhibition of the host PLD may negate any positive benefits of inhibiting PldA during infection and convolute interpretations of efficacy. Once more potent, selective PldA inhibitors are developed; the compounds must be tested in various *in vitro* and *in vivo* infection assays to confirm a PldA-dependent inhibitor mechanism.

Significantly more analysis of how PldA-mediated bacterial competition *in vivo* is also needed to understand how PldA inhibition could affect infections. What roles does PldA-mediated bacterial competition play in colonization by *P. aeruginosa*? Although PldA has been shown to induce killing of bacterial competitors in a laboratory setting, is this competition relevant during infection? Does PldA bacterial competition contribute to the evolution of *P. aeruginosa* as the primary bacterial colonizer in the lungs of CF patients? Are PldA expressing strains more or less virulent and do they outcompete comparatively more or less virulent strains? Would PldA inhibition lead to colonization with strains resulting in more severe infections? One issue of virulence inhibitors is also narrow spectrum of activity, which is also a potential problem with targeting PldA. Only 30% of environmental and clinical isolates of *P. aeruginosa* were found to contain the PldA gene[56], and PldA expression may only occur during specific stages of infection, thereby potentially limiting efficacy of a PldA inhibitor.

REFERENCES

[1] Ponting, C. P., and Kerr, I. D. (1996) A novel family of phospholipase D homologues that includes phospholipid synthases and putative endonucleases: Identification of duplicated repeats and potential active site residues, *Protein Sci. 5*, 914-922.

[2] Okamoto, Y., Morishita, J., Tsuboi, K., Tonai, T., and Ueda, N. (2004) Molecular Characterization of a Phospholipase D Generating Anandamide and Its Congeners, *J. Biol. Chem. 279*, 5298-5305.

[3] Yabu, T., Imamura, S., Yamashita, M., and Okazaki, T. (2008) Identification of Mg(2+)-dependent Neutral Sphingomyelinase 1 as a Mediator of Heat Stress-induced Ceramide Generation and Apoptosis, *J. Biol. Chem. 283*, 29971-29982.

[4] Raikwar, Nandita S., Bowen, Rosario F., and Deeg, Mark A. (2005) Mutating His(29), His(125), His(133) or His(158) abolishes glycosylphosphatidylinositol-specific phospholipase D catalytic activity, *Biochem. J. 391*, 285-289.

[5] Tokumura, A., Majima, E., Kariya, Y., Tominaga, K., Kogure, K., Yasuda, K., and Fukuzawa, K. (2002) Identification of Human Plasma Lysophospholipase D, a Lysophosphatidic Acid-producing Enzyme, as Autotaxin, a Multifunctional Phosphodiesterase, *J. Biol. Chem. 277*, 39436-39442.

[6] Leiros, I., Secundo, F., Zambonelli, C., Servi, S., and Hough, E. (2000) The first crystal structure of a phospholipase D, *Structure 8*, 655-667.

[7] Leiros, I., McSweeney, S., and Hough, E. (2004) The Reaction Mechanism of Phospholipase D from *Streptomyces* sp. Strain PMF. Snapshots along the Reaction Pathway Reveal a Pentacoordinate Reaction Intermediate and an Unexpected Final Product, *J. Mol. Biol. 339*, 805-820.

[8] Rudolph, A. E., Stuckey, J. A., Zhao, Y., Matthews, H. R., Patton, W. A., Moss, J., and Dixon, J. E. (1999) Expression, Characterization, and Mutagenesis of the *Yersinia pestis* Murine Toxin, a Phospholipase D Superfamily Member, *J. Biol. Chem. 274*, 11824-11831.

[9] Stuckey, J. A., and Dixon, J. E. (1999) Crystal structure of a phospholipase D family member, *Nat. Struct. Mol. Biol. 6*, 278-284.

[10] Gottlin, E. B., Rudolph, A. E., Zhao, Y., Matthews, H. R., and Dixon, J. E. (1998) Catalytic mechanism of the phospholipase D superfamily proceeds via a covalent phosphohistidine intermediate, *Proc. Natl. Acad. Sci. 95*, 9202-9207.

[11] Zhang, D., and Aravind, L. (2010) Identification of Novel Families and Classification of the C2 domain Superfamily Elucidate the Origin and Evolution of Membrane Targeting Activities in Eukaryotes, *Gene 469*, 18-30.

[12] Seet, L.-F., and Hong, W. (2006) The Phox (PX) domain proteins and membrane traffic, *BBA Mol. Cell Biol. Lipids 1761*, 878-896.

[13] Lemmon, M. A. (2007) Pleckstrin Homology (PH) domains and phosphoinositides, *Biochem. Soc. Sympos. 74*, 81-93.

[14] Roth, M. G. (2008) Molecular mechanisms of PLD function in membrane traffic, *Traffic 9*, 1233-1239.

[15] Gomez-Cambronero, J. (2014) Phosphatidic acid, phospholipase D and tumorigenesis, *Adv. Biol. Regul. 54*, 197-206.

[16] Bruntz, R. C., Lindsley, C. W., and Brown, H. A. (2014) Phospholipase D Signaling Pathways and Phosphatidic Acid as Therapeutic Targets in Cancer, *Pharm. Rev. 66*, 1033-1079.

[17] Selvy, P. E., Lavieri, R. R., Lindsley, C. W., and Brown, H. A. (2011) Phospholipase D: Enzymology, Functionality, and Chemical Modulation, *Chem. Rev. 111*, 6064-6119.

[18] Kang, H. K., Lee, H. Y., Lee, Y.-N., Jo, E. J., Kim, J. I., Kim, G.-Y., Park, Y. M., Min, D. S., Yano, A., Kwak, J.-Y., and Bae, Y.-S. (2004) Up-regulation of phospholipase Cγ1 and phospholipase D during the differentiation of human monocytes to dendritic cells, *Int. Immunopharm. 4*, 911-920.

[19] Iyer, S. S., Barton, J. A., Bourgoin, S., and Kusner, D. J. (2004) Phospholipases D1 and D2 Coordinately Regulate Macrophage Phagocytosis, *J. Immunol. 173*, 2615-2623.

[20] Minnicozzi, M., Anthes, J. C., Siegel, M. I., Billah, M. M., and Egan, R. W. (1990) Activation of phospholipase D in normodense human eosinophils, *Biochem. Biophysic. Res. Commun. 170*, 540-547.

[21] Kumada, T., Miyata, H., and Nozawa, Y. (1993) Involvement of Tyrosine Phosphorylation in IgE Receptor-Mediated Phospholipase D Activation in Rat Basophilic Leukemia (RBL-2H3) Cells, *Biochem. Biophysic. Res. Commun. 191*, 1363-1368.

[22] Lee, H.-Y., Kang, H. K., Jo, E. J., Kim, J. I., Lee, Y.-N., Lee, S. H., Park, Y. M., Ryu, S. H., Kwak, J.-Y., and Bae, Y.-S. (0000) Trp-Lys-Tyr-Met-Val-Met stimulates phagocytosis via phospholipase D-dependent signaling in mouse dendritic cells, *Exper. Mol. Med. 36*, 135-144.

[23] Diaz, O., Mébarek-Azzam, S., Benzaria, A., Dubois, M., Lagarde, M., Némoz, G., and Prigent, A.-F. (2005) Disruption of Lipid Rafts Stimulates Phospholipase D Activity in Human Lymphocytes: Implication in the Regulation of Immune Function, *J. Immunol. 175*, 8077-8086.

[24] Milella, M., Gismondi, A., Roncaioli, P., Palmieri, G., Morrone, S., Piccoli, M., Frati, L., Cifone, M. G., and Santoni, A. (1999) β1 Integrin Cross-Linking Inhibits CD16-Induced Phospholipase D and Secretory Phospholipase A2 Activity and Granule Exocytosis in Human NK cells: Role of Phospholipase D in CD16-Triggered Degranulation, *J. Immunol. 162*, 2064-2072.

[25] Serrander, L., Fällman, M., and Stendahl, O. (1996) Activation of phospholipase D is an early event in integrin-mediated signalling leading to phagocytosis in human neutrophils, *Inflammation 20*, 439-450.

[26] Corrotte, M., Chasserot-Golaz, S., Huang, P., Du, G., Ktistakis, N. T., Frohman, M. A., Vitale, N., Bader, M.-F., and Grant, N. J. (2006) Dynamics and Function of Phospholipase D and Phosphatidic Acid During Phagocytosis, *Traffic 7*, 365-377.

[27] Lisboa, F. A., Peng, Z., Combs, C. A., and Beaven, M. A. (2009) Phospholipase D Promotes Lipid Microdomain-Associated Signaling Events in Mast Cells. *J. Immunol. 183*, 5104-5112.

[28] Choi, W. S., Kim, Y. M., Combs, C., Frohman, M. A., and Beaven, M. A. (2002) Phospholipases D1 and D2 Regulate Different Phases of Exocytosis in Mast Cells, *J. Immunol. 168*, 5682-5689.

[29] Peng, Z., and Beaven, M. A. (2005) An Essential Role for Phospholipase D in the Activation of Protein Kinase C and Degranulation in Mast Cells, *J. Immunol. 174*, 5201-5208.

[30] Yasui, K., and Komiyama, A. (2001) Roles of phosphatidylinositol 3-kinase and phospholipase D in temporal activation of superoxide production in FMLP-stimulated human neutrophils, *Cell Biochem. Funct. 19*, 43-50.

[31] Palicz, A., Foubert, T. R., Jesaitis, A. J., Marodi, L., and McPhail, L. C. (2001) Phosphatidic Acid and Diacylglycerol Directly Activate NADPH Oxidase by Interacting with Enzyme Components, *J. Biol. Chem. 276*, 3090-3097.

[32] Kusner, D. J., and Barton, J. A. (2001) ATP Stimulates Human Macrophages to Kill Intracellular Virulent *Mycobacterium tuberculosis* Via Calcium-Dependent Phagosome-Lysosome Fusion, *J. Immunol. 167*, 3308-3315.

[33] Kusner, D. J., and Adams, J. (2000) ATP-Induced Killing of Virulent *Mycobacterium tuberculosis* Within Human Macrophages Requires Phospholipase D, *J. Immunol. 164*, 379-388.

[34] Fairbairn, I. P., Stober, C. B., Kumararatne, D. S., and Lammas, D. A. (2001) ATP-Mediated Killing of Intracellular Mycobacteria by Macrophages Is a P2X7-Dependent Process Inducing Bacterial Death by Phagosome-Lysosome Fusion, *J. Immunol. 167*, 3300-3307.

[35] Gomez-Cambronero, J., Di Fulvio, M., and Knapek, K. (2007) Understanding phospholipase D (PLD) using leukocytes: PLD involvement in cell adhesion and chemotaxis, *J. Leukocyte Biol. 82*, 272-281.

[36] Lehman, N., Di Fulvio, M., McCray, N., Campos, I., Tabatabaian, F., and Gomez-Cambronero, J. (2006) Phagocyte cell migration is mediated by phospholipases PLD1 and PLD2, *Blood 108*, 3564-3572.

[37] Karathanassis, D., Stahelin, R. V., Bravo, J., Perisic, O., Pacold, C. M., Cho, W., and Williams, R. L. (2002) Binding of the PX domain of p47(phox) to phosphatidylinositol 3,4-bisphosphate and phosphatidic acid is masked by an intramolecular interaction, *EMBO Journal 21*, 5057-5068.

[38] Viner, R., Chetrit, D., Ehrlich, M., and Segal, G. (2012) Identification of Two *Legionella pneumophila* Effectors that Manipulate Host Phospholipids Biosynthesis, *PLoS Pathog 8*, e1002988.

[39] Zhu, W., Banga, S., Tan, Y., Zheng, C., Stephenson, R., Gately, J., and Luo, Z.-Q. (2011) Comprehensive Identification of Protein Substrates of the Dot/Icm Type IV Transporter of *Legionella pneumophila*, *PLoS ONE 6*, e17638.

[40] Darville, T., Welter-Stahl, L., Cruz, C., Sater, A. A., Andrews, C. W., and Ojcius, D. M. (2007) Effect of the Purinergic Receptor P2X7 on Chlamydia Infection in Cervical Epithelial Cells and Vaginally Infected Mice, *J. Immunol. 179*, 3707-3714.

[41] Benagiano, M., Munari, F., Ciervo, A., Amedei, A., Paccani, S. R., Mancini, F., Ferrari, M., Della Bella, C., Ulivi, C., D'Elios, S., Baldari, C. T., Prisco, D., de Bernard, M., and D'Elios, M. M. (2012) *Chlamydophila pneumoniae* phospholipase D (CpPLD) drives Th17 inflammation in human atherosclerosis, *Proc. Natl. Acad. Sci. 109*, 1222-1227.

[42] Renesto, P., Dehoux, P., Gouin, E., Touqui, L., Cossart, P., and Raoult, D. (2003) Identification and Characterization of a Phospholipase D–Superfamily Gene in *Rickettsiae*, *J. Infect. Dis. 188*, 1276-1283.

[43] Edwards, J. L., and Apicella, M. A. (2006) *Neisseria gonorrhoeae* PLD directly interacts with Akt kinase upon infection of primary, human, cervical epithelial cells, *Cell. Microbiol. 8*, 1253-1271.

[44] Edwards, J. L., Entz, D. D., and Apicella, M. A. (2003) Gonococcal Phospholipase D Modulates the Expression and Function of Complement Receptor 3 in Primary Cervical Epithelial Cells, *Infect. Immun. 71*, 6381-6391.

[45] Jacobs, A. C., Hood, I., Boyd, K. L., Olson, P. D., Morrison, J. M., Carson, S., Sayood, K., Iwen, P. C., Skaar, E. P., and Dunman, P. M. (2010) Inactivation of Phospholipase D Diminishes *Acinetobacter baumannii* Pathogenesis, *Infect. Immun. 78*, 1952-1962.

[46] Hinnebusch, J., Cherepanov, P., Du, Y., Rudolph, A., Dixon, J. D., Schwan, T., and Forsberg, Å. (2000) Murine toxin of *Yersinia pestis* shows phospholipase D activity but is not required for virulence in mice, *Int. J. Med. Microbiol. 290*, 483-487.

[47] Hinnebusch, B. J., Rudolph, A. E., Cherepanov, P., Dixon, J. E., Schwan, T. G., and Forsberg, Å. (2002) Role of *Yersinia* Murine Toxin in Survival of Yersinia pestis in the Midgut of the Flea Vector, *Science 296*, 733-735.

[48] Edwards, J. L., Brown, E. J., Ault, K. A., and Apicella, M. A. (2001) The role of complement receptor 3 (CR3) in *Neisseria gonorrhoeae* infection of human cervical epithelia, *Cell. Microbiol. 3*, 611-622.

[49] Burnham, C.-A. D., Shokoples, S. E., and Tyrrell, G. J. (2007) Invasion of HeLa cells by group B streptococcus requires the phosphoinositide-3-kinase signalling pathway and modulates phosphorylation of host-cell Akt and glycogen synthase kinase-3, *Microbiol. 153*, 4240-4252.

[50] Sidhu, G., Li, W., Laryngakis, N., Bishai, E., Balla, T., and Southwick, F. (2005) Phosphoinositide 3-Kinase Is Required for Intracellular *Listeria monocytogenes* Actin-based Motility and Filopod Formation, *J. Biol. Chem. 280*, 11379-11386.

[51] Kierbel, A., Gassama-Diagne, A., Mostov, K., and Engel, J. N. (2005) The Phosphoinositol-3-Kinase–Protein Kinase B/Akt Pathway Is Critical for *Pseudomonas aeruginosa* Strain PAK Internalization, *Mol. Biol. Cell 16*, 2577-2585.

[52] Jiang, F., Waterfield, Nicholas R., Yang, J., Yang, G., and Jin, Q. (2014) A *Pseudomonas aeruginosa* Type VI Secretion Phospholipase D Effector Targets Both Prokaryotic and Eukaryotic Cells, *Cell Host Microbe 15*, 600-610.

[53] Knodler, L. A., Finlay, B. B., and Steele-Mortimer, O. (2005) The *Salmonella* Effector Protein SopB Protects Epithelial Cells from Apoptosis by Sustained Activation of Akt, *J. Biol. Chem. 280*, 9058-9064.

[54] Hubber, A., and Roy, C. R. (2010) Modulation of Host Cell Function by *Legionella pneumophila* Type IV Effectors, *Annu. Rev. Cell. Dev. Bio. 26*, 261-283.

[55] Nelson, D. E., Crane, D. D., Taylor, L. D., Dorward, D. W., Goheen, M. M., and Caldwell, H. D. (2006) Inhibition of *Chlamydiae* by Primary Alcohols Correlates with the Strain-Specific Complement of Plasticity Zone Phospholipase D Genes, *Infect. Immun. 74*, 73-80.

[56] Wilderman, P. J., Vasil, A. I., Johnson, Z., and Vasil, M. L. (2001) Genetic and biochemical analyses of a eukaryotic-like phospholipase D of *Pseudomonas aeruginosa* suggest horizontal acquisition and a role for persistence in a chronic pulmonary infection model, *Mol. Microbiol. 39*, 291-304.

[57] Russell, A. B., LeRoux, M., Hathazi, K., Agnello, D. M., Ishikawa, T., Wiggins, P. A., Wai, S. N., and Mougous, J. D. (2013) Diverse type VI secretion phospholipases are functionally plastic antibacterial effectors, *Nature 496*, 508-512.

[58] Mougous, J. D., Cuff, M. E., Raunser, S., Shen, A., Zhou, M., Gifford, C. A., Goodman, A. L., Joachimiak, G., Ordoñez, C. L., Lory, S., Walz, T., Joachimiak, A., and Mekalanos, J. J. (2006) A Virulence Locus of *Pseudomonas aeruginosa* Encodes a Protein Secretion Apparatus, *Science 312*, 1526-1530.

[59] Pukatzki, S., Ma, A. T., Sturtevant, D., Krastins, B., Sarracino, D., Nelson, W. C., Heidelberg, J. F., and Mekalanos, J. J. (2006) Identification of a conserved bacterial protein secretion system in *Vibrio cholerae* using the Dictyostelium host model system, *Proc. Natl. Acad. Sci. 103*, 1528-1533.

[60] Lesic, B., Starkey, M., He, J., Hazan, R., and Rahme, L. G. (2009) Quorum sensing differentially regulates *Pseudomonas aeruginosa* type VI secretion locus I and homologous loci II and III, which are required for pathogenesis, *Microbiol. 155*, 2845-2855.

[61] Sana, T. G., Hachani, A., Bucior, I., Soscia, C., Garvis, S., Termine, E., Engel, J., Filloux, A., and Bleves, S. (2012) The Second Type VI Secretion System of *Pseudomonas aeruginosa* Strain PAO1 Is Regulated by Quorum Sensing and Fur and Modulates Internalization in Epithelial Cells, *J. Biol. Chem. 287*, 27095-27105.

[62] Bingle, L. E. H., Bailey, C. M., and Pallen, M. J. (2008) Type VI secretion: a beginner's guide, *Curr. Opin. Microbio. 11*, 3-8.

[63] Boyer, F., Fichant, G., Berthod, J., Vandenbrouck, Y., and Attree, I. (2009) Dissecting the bacterial type VI secretion system by a genome wide in silico analysis: what can be learned from available microbial genomic resources?, *BMC Genomics 10*, 104.

[64] Zoued, A., Brunet, Y. R., Durand, E., Aschtgen, M.-S., Logger, L., Douzi, B., Journet, L., Cambillau, C., and Cascales, E. (2014) Architecture and assembly of the Type VI secretion system, *BBA Mol. Cell Res. 1843*, 1664-1673.

[65] Pell, L. G., Kanelis, V., Donaldson, L. W., Lynne Howell, P., and Davidson, A. R. (2009) The phage λ major tail protein structure reveals a common evolution for long-tailed phages and the type VI bacterial secretion system, *Proc. Natl. Acad. Sci. 106*, 4160-4165.

[66] Leiman, P. G., Basler, M., Ramagopal, U. A., Bonanno, J. B., Sauder, J. M., Pukatzki, S., Burley, S. K., Almo, S. C., and Mekalanos, J. J. (2009) Type VI secretion apparatus and phage tail-associated protein complexes share a common evolutionary origin, *Proc. Natl. Acad. Sci. 106*, 4154-4159.

[67] Bönemann, G., Pietrosiuk, A., Diemand, A., Zentgraf, H., and Mogk, A. (2009) Remodelling of VipA/VipB tubules by ClpV-mediated threading is crucial for type VI protein secretion, *EMBO J. 28*, 315-325.

[68] Basler, M., Pilhofer, M., Henderson, P. G., Jensen, J. G., and Mekalanos, J. (2012) Type VI secretion requires a dynamic contractile phage tail-like structure, *Nature 483*, 182-186.

[69] Ho, Brian T., Dong, Tao G., and Mekalanos, John J. (2014) A View to a Kill: The Bacterial Type VI Secretion System, *Cell Host Microbe 15*, 9-21.

[70] Hsu, F., Schwarz, S., and Mougous, J. D. (2009) TagR Promotes PpkA-Catalyzed Type VI Secretion Activation in Pseudomonas aeruginosa, *Mol. Microbiol. 72*, 1111-1125.

[71] Mougous, J. D., Gifford, C. A., Ramsdell, T. L., and Mekalanos, J. J. (2007) Threonine phosphorylation post-translationally regulates protein secretion in *Pseudomonas aeruginosa*, *Nat. Cell. Biol. 9*, 797-803.

[72] Murdoch, S. L., Trunk, K., English, G., Fritsch, M. J., Pourkarimi, E., and Coulthurst, S. J. (2011) The Opportunistic Pathogen *Serratia marcescens* Utilizes Type VI Secretion To Target Bacterial Competitors, *J. Bacteriol. 193*, 6057-6069.

[73] Schwarz, S., West, T. E., Boyer, F., Chiang, W.-C., Carl, M. A., Hood, R. D., Rohmer, L., Tolker-Nielsen, T., Skerrett, S. J., and Mougous, J. D. (2010) *Burkholderia* Type VI Secretion Systems Have Distinct Roles in Eukaryotic and Bacterial Cell Interactions, *PLoS Pathog. 6*, e1001068.

[74] Bernard, C. S., Brunet, Y. R., Gueguen, E., and Cascales, E. (2010) Nooks and Crannies in Type VI Secretion Regulation, *J. Bacteriol. 192*, 3850-3860.

[75] Bordi, C., Lamy, M.-C., Ventre, I., Termine, E., Hachani, A., Fillet, S., Roche, B., Bleves, S., Méjean, V., Lazdunski, A., and Filloux, A. (2010) Regulatory RNAs and the HptB/RetS signalling pathways fine-tune *Pseudomonas aeruginosa* pathogenesis, *Mol. Microbiol. 76*, 1427-1443.

[76] Goodman, A. L., Kulasekara, B., Rietsch, A., Boyd, D., Smith, R. S., and Lory, S. (2004) A Signaling Network Reciprocally Regulates Genes Associated with Acute Infection and Chronic Persistence in *Pseudomonas aeruginosa*, *Dev. Cell 7*, 745-754.

[77] Venturi, V. (2006) Regulation of quorum sensing in *Pseudomonas*, *FEMS Microbiol. Rev. 30*, 274-291.

[78] Miyata, S. T., Bachmann, V., and Pukatzki, S. (2013) Type VI secretion system regulation as a consequence of evolutionary pressure, *J. Med. Microbiol. 62*, 663-676.

[79] Ma, A. T., McAuley, S., Pukatzki, S., and Mekalanos, J. J. (2009) Translocation of a *Vibrio cholerae* Type VI Secretion Effector Requires Bacterial Endocytosis by Host Cells, *Cell Host Microbe 5*, 234-243.

[80] Miyata, S. T., Kitaoka, M., Brooks, T. M., McAuley, S. B., and Pukatzki, S. (2011) *Vibrio cholerae* Requires the Type VI Secretion System Virulence Factor VasX To Kill Dictyostelium discoideum, *Infect. Immun. 79*, 2941-2949.

[81] MacIntyre, D. L., Miyata, S. T., Kitaoka, M., and Pukatzki, S. (2010) The *Vibrio cholerae* type VI secretion system displays antimicrobial properties, *Proc. Natl. Acad. Sci. 107*, 19520-19524.

[82] Potvin, E., Lehoux, D. E., Kukavica-Ibrulj, I., Richard, K. L., Sanschagrin, F., Lau, G. W., and Levesque, R. C. (2003) In vivo functional genomics of *Pseudomonas aeruginosa* for high-throughput screening of new virulence factors and antibacterial targets, *Environ. Microbiol. 5*, 1294-1308.

[83] de Pace, F., Nakazato, G., Pacheco, A., Boldrin de Paiva, J., Sperandio, V., and Dias da Silveira, W. (2010) The Type VI Secretion System Plays a Role in Type 1 Fimbria Expression and Pathogenesis of an Avian Pathogenic *Escherichia coli* Strain, *Infect. Immun. 78*, 4990-4998.

[84] Zheng, J., and Leung, K. Y. (2007) Dissection of a type VI secretion system in *Edwardsiella tarda*, *Mol. Microbiol. 66*, 1192-1206.

[85] Bleumink-Pluym, N. M. C., van Alphen, L. B., Bouwman, L. I., Wösten, M. M. S. M., and van Putten, J. P. M. (2013) Identification of a Functional Type VI Secretion System in *Campylobacter jejuni* Conferring Capsule Polysaccharide Sensitive Cytotoxicity, *PLoS Pathog. 9*, e1003393.

[86] Schell, M. A., Ulrich, R. L., Ribot, W. J., Brueggemann, E. E., Hines, H. B., Chen, D., Lipscomb, L., Kim, H. S., Mrázek, J., Nierman, W. C., and DeShazer, D. (2007) Type VI secretion is a major virulence determinant in *Burkholderia mallei*, *Mol. Microbiol. 64*, 1466-1485.

[87] Burtnick, M. N., Brett, P. J., Harding, S. V., Ngugi, S. A., Ribot, W. J., Chantratita, N., Scorpio, A., Milne, T. S., Dean, R. E., Fritz, D. L., Peacock, S. J., Prior, J. L., Atkins, T. P., and DeShazer, D. (2011) The Cluster 1 Type VI Secretion System Is a Major Virulence Determinant in *Burkholderia pseudomallei*, *Infect. Immun. 79*, 1512-1525.

[88] Burtnick, M. N., DeShazer, D., Nair, V., Gherardini, F. C., and Brett, P. J. (2010) *Burkholderia mallei* Cluster 1 Type VI Secretion Mutants Exhibit Growth and Actin Polymerization Defects in RAW 264.7 Murine Macrophages, *Infect. Immun. 78*, 88-99.

[89] Suarez, G., Sierra, J. C., Erova, T. E., Sha, J., Horneman, A. J., and Chopra, A. K. (2010) A Type VI Secretion System Effector Protein, VgrG1, from *Aeromonas hydrophila* That Induces Host Cell Toxicity by ADP Ribosylation of Actin, *J. Bacteriol. 192*, 155-168.

[90] Suarez, G., Sierra, J. C., Sha, J., Wang, S., Erova, T. E., Fadl, A. A., Foltz, S. M., Horneman, A. J., and Chopra, A. K. (2008) Molecular characterization of a functional type VI secretion system from a clinical isolate of *Aeromonas hydrophila*, *Microb. Pathogenesis 44*, 344-361.

[91] Brooks, T. M., Unterweger, D., Bachmann, V., Kostiuk, B., and Pukatzki, S. (2013) Lytic Activity of the *Vibrio cholerae* Type VI Secretion Toxin VgrG-3 Is Inhibited by the Antitoxin TsaB, *J. Biol. Chem. 288*, 7618-7625.

[92] Pukatzki, S., Ma, A. T., Revel, A. T., Sturtevant, D., and Mekalanos, J. J. (2007) Type VI secretion system translocates a phage tail spike-like protein into target cells where it cross-links actin, *Proc. Natl. Acad. Sci. 104*, 15508-15513.

[93] Schwarz, S., Singh, P., Robertson, J. D., LeRoux, M., Skerrett, S. J., Goodlett, D. R., West, T. E., and Mougous, J. D. (2014) VgrG-5 Is a *Burkholderia* Type VI Secretion System-Exported Protein Required for Multinucleated Giant Cell Formation and Virulence, *Infect. Immun. 82*, 1445-1452.

[94] Toesca, I. J., French, C. T., and Miller, J. F. (2014) The Type VI Secretion System Spike Protein VgrG5 Mediates Membrane Fusion during Intercellular Spread by *Pseudomallei* Group *Burkholderia* Species, *Infect. Immun. 82*, 1436-1444.

[95] Barret, M., Egan, F., Fargier, E., Morrissey, J. P., and O'Gara, F. (2011) Genomic analysis of the type VI secretion systems in *Pseudomonas* spp.: novel clusters and putative effectors uncovered, *Microbiol. 157*, 1726-1739.

[96] De Maayer, P., Venter, S., Kamber, T., Duffy, B., Coutinho, T., and Smits, T. (2011) Comparative genomics of the type VI secretion systems of *Pantoea* and *Erwinia* species reveals the presence of putative effector islands that may be translocated by the VgrG and Hcp proteins, *BMC Genomics 12*, 576.

[97] Hachani, A., Allsopp, L. P., Oduko, Y., and Filloux, A. (2014) The VgrG Proteins Are "à la Carte" Delivery Systems for Bacterial Type VI Effectors, *J. Biol. Chem. 289*, 17872-17884.

[98] Russell, Alistair B., Wexler, Aaron G., Harding, Brittany N., Whitney, John C., Bohn, Alan J., Goo, Young A., Tran, Bao Q., Barry, Natasha A., Zheng, H., Peterson, S. B., Chou, S., Gonen, T., Goodlett, David R., Goodman, Andrew L., and Mougous, Joseph D. (2014) A Type VI Secretion-Related Pathway in Bacteroidetes Mediates Interbacterial Antagonism, *Cell Host Microbe 16*, 227-236.

[99] Li, M., Le Trong, I., Carl, M. A., Larson, E. T., Chou, S., De Leon, J. A., Dove, S. L., Stenkamp, R. E., and Mougous, J. D. (2012) Structural Basis for Type VI Secretion Effector Recognition by a Cognate Immunity Protein, *PLoS Pathog. 8*, e1002613.

[100] Benz, J., Reinstein, J., and Meinhart, A. (2013) Structural Insights into the Effector – Immunity System Tae4/Tai4 from *Salmonella typhimurium*, *PLoS ONE 8*, e67362.

[101] Zhang, H., Zhang, H., Gao, Z.-Q., Wang, W.-J., Liu, G.-F., Xu, J.-H., Su, X.-D., and Dong, Y.-H. (2013) Structure of the Type VI Effector-Immunity Complex (Tae4-Tai4)

Provides Novel Insights into the Inhibition Mechanism of the Effector by Its Immunity Protein, *The J. Biol. Chem. 288*, 5928-5939.

[102] Hood, R. D., Singh, P., Hsu, F., Güvener, T., Carl, M. A., Trinidad, R. R. S., Silverman, J. M., Ohlson, B. B., Hicks, K. G., Plemel, R. L., Li, M., Schwarz, S., Wang, W. Y., Merz, A. J., Goodlett, D. R., and Mougous, J. D. (2010) A Type VI Secretion System of *Pseudomonas aeruginosa* Targets a Toxin to Bacteria, *Cell Host Microbe 7*, 25-37.

[103] Vollmer, W., Blanot, D., and De Pedro, M. A. (2008) Peptidoglycan structure and architecture, *FEMS Microbiol. Rev. 32*, 149-167.

[104] Russell, Alistair B., Singh, P., Brittnacher, M., Bui, Nhat K., Hood, Rachel D., Carl, Mike A., Agnello, Danielle M., Schwarz, S., Goodlett, David R., Vollmer, W., and Mougous, Joseph D. (2012) A Widespread Bacterial Type VI Secretion Effector Superfamily Identified Using a Heuristic Approach, *Cell Host Microbe 11*, 538-549.

[105] Whitney, J. C., Chou, S., Russell, A. B., Biboy, J., Gardiner, T. E., Ferrin, M. A., Brittnacher, M., Vollmer, W., and Mougous, J. D. (2013) Identification, Structure, and Function of a Novel Type VI Secretion Peptidoglycan Glycoside Hydrolase Effector-Immunity Pair, *J. Biol. Chem. 288*, 26616-26624.

[106] Sana, T. G., Soscia, C., Tonglet, C. M., Garvis, S., and Bleves, S. (2013) Divergent Control of Two Type VI Secretion Systems by RpoN in *Pseudomonas aeruginosa PLoS ONE 8*, e76030.

[107] Dong, T. G., Ho, B. T., Yoder-Himes, D. R., and Mekalanos, J. J. (2013) Identification of T6SS-dependent effector and immunity proteins by Tn-seq in *Vibrio cholerae*, *Proc. Natl. Acad. Sci. 110*, 2623-2628.

[108] Lery, L., Frangeul, L., Tomas, A., Passet, V., Almeida, A., Bialek-Davenet, S., Barbe, V., Bengoechea, J., Sansonetti, P., Brisse, S., and Tournebize, R. (2014) Comparative analysis of *Klebsiella pneumoniae* genomes identifies a phospholipase D family protein as a novel virulence factor, *BMC Biol. 12*, 41.

[109] Koskiniemi, S., Lamoureux, J. G., Nikolakakis, K. C., t'Kint de Roodenbeke, C., Kaplan, M. D., Low, D. A., and Hayes, C. S. (2013) Rhs proteins from diverse bacteria mediate intercellular competition, *Proc. Natl. Acad. Sci. 110*, 7032-7037.

[110] Salomon, D., Kinch, L. N., Trudgian, D. C., Guo, X., Klimko, J. A., Grishin, N. V., Mirzaei, H., and Orth, K. (2014) Marker for type VI secretion system effectors, *Proc. Natl. Acad. Sci. 111*, 9271-9276.

[111] Ma, L.-S., Hachani, A., Lin, J.-S., Filloux, A., and Lai, E.-M. (2014) *Agrobacterium tumefaciens* Deploys a Superfamily of Type VI Secretion DNase Effectors as Weapons for Interbacterial Competition In Planta, *Cell Host Microbe 16*, 94-104.

[112] Basler, M., and Mekalanos, J. J. (2012) Type 6 Secretion Dynamics Within and Between Bacterial Cells, *Science 337*, 815.

[113] Durand, E., Derrez, E., Audoly, G., Spinelli, S., Ortiz-Lombardia, M., Raoult, D., Cascales, E., and Cambillau, C. (2012) Crystal Structure of the VgrG1 Actin Cross-linking Domain of the *Vibrio cholerae* Type VI Secretion System, *J. Biol. Chem. 287*, 38190-38199.

[114] Satchell, K. J. F. (2009) Bacterial Martyrdom: Phagocytes Disabled by Type VI Secretion after Engulfing Bacteria, *Cell Host Microbe 5*, 213-214.

[115] Lyczak, J. B., Cannon, C. L., and Pier, G. B. (2000) Establishment of *Pseudomonas aeruginosa* infection: lessons from a versatile opportunist, *Microbes Infect. 2*, 1051-1060.

[116] Sadikot, R. T., Blackwell, T. S., Christman, J. W., and Prince, A. S. (2005) Pathogen–Host Interactions in Pseudomonas aeruginosa Pneumonia, *Am. J. Res. Crit. Care Med. 171*, 1209-1223.

[117] Folkesson, A., Jelsbak, L., Yang, L., Johansen, H. K., Ciofu, O., Hoiby, N., and Molin, S. (2012) Adaptation of *Pseudomonas aeruginosa* to the cystic fibrosis airway: an evolutionary perspective, *Nat. Rev. Micro. 10*, 841-851.

[118] Riordan, J. R. (2008) CFTR Function and Prospects for Therapy, *Ann. Rev. Biochem. 77*, 701-726.

[119] Smith, K. M., Bu, Y., and Suga, H. (2003) Induction and Inhibition of *Pseudomonas aeruginosa* Quorum Sensing by Synthetic Autoinducer Analogs, *Chem. Biol. 10*, 81-89.

[120] Hogardt, M., Hoboth, C., Schmoldt, S., Henke, C., Bader, L., and Heesemann, J. (2007) Stage-Specific Adaptation of Hypermutable *Pseudomonas aeruginosa* Isolates during Chronic Pulmonary Infection in Patients with Cystic Fibrosis, *J. Infect. Dis. 195*, 70-80.

[121] Mena, A., Smith, E. E., Burns, J. L., Speert, D. P., Moskowitz, S. M., Perez, J. L., and Oliver, A. (2008) Genetic Adaptation of *Pseudomonas aeruginosa* to the Airways of Cystic Fibrosis Patients Is Catalyzed by Hypermutation, *J. Bacteriol. 190*, 7910-7917.

[122] Tingpej, P., Smith, L., Rose, B., Zhu, H., Conibear, T., Al Nassafi, K., Manos, J., Elkins, M., Bye, P., Willcox, M., Bell, S., Wainwright, C., and Harbour, C. (2007) Phenotypic Characterization of Clonal and Nonclonal *Pseudomonas aeruginosa* Strains Isolated from Lungs of Adults with Cystic Fibrosis, *J. Clin. Microbiol.45*, 1697-1704.

[123] Mowat, E., Paterson, S., Fothergill, J. L., Wright, E. A., Ledson, M. J., Walshaw, M. J., Brockhurst, M. A., and Winstanley, C. (2011) *Pseudomonas aeruginosa* Population

Diversity and Turnover in Cystic Fibrosis Chronic Infections, *Am. J. Res. Crit. Care Med. 183*, 1674-1679.

[124] Wolfgang, M. C., Jyot, J., Goodman, A. L., Ramphal, R., and Lory, S. (2004) *Pseudomonas aeruginosa* regulates flagellin expression as part of a global response to airway fluid from cystic fibrosis patients, *Proc. Natl. Acad. Sci. 101*, 6664-6668.

[125] Burrows, L. L. (2012) *Pseudomonas aeruginosa* Twitching Motility: Type IV Pili in Action, *Ann. Rev. Microbiol. 66*, 493-520.

[126] Köhler, T., Curty, L. K., Barja, F., van Delden, C., and Pechère, J.-C. (2000) Swarming of *Pseudomonas aeruginosa* Is Dependent on Cell-to-Cell Signaling and Requires Flagella and Pili, *J. Bacteriol. 182*, 5990-5996.

[127] Adamo, R., Sokol, S., Soong, G., Gomez, M. I., and Prince, A. (2004) *Pseudomonas aeruginosa* Flagella Activate Airway Epithelial Cells through asialoGM1 and Toll-Like Receptor 2 as well as Toll-Like Receptor 5, *Am. J. Res. Cell Mol. Biol. 30*, 627-634.

[128] Sriramulu, D. D., Lünsdorf, H., Lam, J. S., and Römling, U. (2005) Microcolony formation: a novel biofilm model of *Pseudomonas aeruginosa* for the cystic fibrosis lung, *J. Med. Microbiol. 54*, 667-676.

[129] Hauser, A. R. (2009) The type III secretion system of *Pseudomonas aeruginosa*: infection by injection, *Nat. Rev. Micro. 7*, 654-665.

[130] Pederson, K. J., Vallis, A. J., Aktories, K., Frank, D. W., and Barbieri, J. T. (1999) The amino-terminal domain of *Pseudomonas aeruginosa* ExoS disrupts actin filaments via small-molecular-weight GTP-binding proteins, *Mol. Microbiol. 32*, 393-401.

[131] Goehring, U.-M., Schmidt, G., Pederson, K. J., Aktories, K., and Barbieri, J. T. (1999) The N-terminal Domain of *Pseudomonas aeruginosa* Exoenzyme S Is a GTPase-activating Protein for Rho GTPases, *J. Biol. Chem. 274*, 36369-36372.

[132] Schulert, G. S., Feltman, H., Rabin, S. D. P., Martin, C. G., Battle, S. E., Rello, J., and Hauser, A. R. (2003) Secretion of the Toxin ExoU Is a Marker for Highly Virulent *Pseudomonas aeruginosa* Isolates Obtained from Patients with Hospital-Acquired Pneumonia, *J. Infect. Dis. 188*, 1695-1706.

[133] Fleiszig, S. M., Wiener-Kronish, J. P., Miyazaki, H., Vallas, V., Mostov, K. E., Kanada, D., Sawa, T., Yen, T. S., and Frank, D. W. (1997) *Pseudomonas aeruginosa*-mediated cytotoxicity and invasion correlate with distinct genotypes at the loci encoding exoenzyme S, *Infect. Immun. 65*, 579-586.

[134] Schuster, M., Joseph Sexton, D., Diggle, S. P., and Peter Greenberg, E. (2013) Acyl-Homoserine Lactone Quorum Sensing: From Evolution to Application, *Ann. Rev. Microbiol. 67*, 43-63.

[135] Nadal Jimenez, P., Koch, G., Thompson, J. A., Xavier, K. B., Cool, R. H., and Quax, W. J. (2012) The Multiple Signaling Systems Regulating Virulence in *Pseudomonas aeruginosa*, *Microbiol. Mol. Biol. Rev. 76*, 46-65.

[136] Juhas, M., Eberl, L., and Tümmler, B. (2005) Quorum sensing: the power of cooperation in the world of *Pseudomonas*, *Environ. Microbiol. 7*, 459-471.

[137] Stewart, P. S., and William Costerton, J. (2001) Antibiotic resistance of bacteria in biofilms, *Lancet 358*, 135-138.

[138] Döring, G., Meisner, C., Stern, M., and Group, f. t. F. V. T. S. (2007) A double-blind randomized placebo-controlled phase III study of a *Pseudomonas aeruginosa* flagella vaccine in cystic fibrosis patients, *Proc. Natl. Acad. Sci. 104*, 11020-11025.

[139] Hertle, R., Mrsny, R., and Fitzgerald, D. J. (2001) Dual-Function Vaccine for *Pseudomonas aeruginosa*: Characterization of Chimeric Exotoxin A-Pilin Protein, *Infect. Immun. 69*, 6962-6969.

[140] Matsumoto, T., Tateda, K., Furuya, N., Miyazaki, S., Ohno, A., Ishii, Y., Hirakata, Y., and Yamaguchi, K. (1998) Efficacies of alkaline protease, elastase and exotoxin A toxoid vaccines against gut-derived *Pseudomonas aeruginosa* sepsis in mice, *J. Med. Microbiol. 47*, 303-308.

[141] Brackman, G., and Coenye, T. (2014) Quorum sensing inhibitors as anti-biofilm agents, *Curr. Pharm. Design 21*, 5-11.

[142] Geske, G. D., Mattmann, M. E., and Blackwell, H. E. (2008) Evaluation of a focused library of N-aryl L-homoserine lactones reveals a new set of potent quorum sensing modulators, *Bioorgan. Med. Chem. Lett. 18*, 5978-5981.

[143] Geske, G. D., Wezeman, R. J., Siegel, A. P., and Blackwell, H. E. (2005) Small Molecule Inhibitors of Bacterial Quorum Sensing and Biofilm Formation, *J. Am. Chem. Soc. 127*, 12762-12763.

[144] Ishida, T., Ikeda, T., Takiguchi, N., Kuroda, A., Ohtake, H., and Kato, J. (2007) Inhibition of Quorum Sensing in Pseudomonas aeruginosa by N-Acyl Cyclopentylamides, *Applied Enivron. Microbiol. 73*, 3183-3188.

[145] Wu, H., Song, Z., Hentzer, M., Andersen, J. B., Molin, S., Givskov, M., and Høiby, N. (2004) Synthetic furanones inhibit quorum-sensing and enhance bacterial clearance in

Pseudomonas aeruginosa lung infection in mice, *J. Antimicro. Chemother. 53*, 1054-1061.

[146] Hentzer, M., Wu, H., Andersen, J. B., Riedel, K., Rasmussen, T. B., Bagge, N., Kumar, N., Schembri, M. A., Song, Z., Kristoffersen, P., Manefield, M., Costerton, J. W., Molin, S., Eberl, L., Steinberg, P., Kjelleberg, S., Høiby, N., and Givskov, M. (2003) Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors, *EMBO J. 22*, 3803-3815.

[147] Girennavar, B., Cepeda, M. L., Soni, K. A., Vikram, A., Jesudhasan, P., Jayaprakasha, G. K., Pillai, S. D., and Patil, B. S. (2008) Grapefruit juice and its furocoumarins inhibits autoinducer signaling and biofilm formation in bacteria, *Int. J. Food Microbiol. 125*, 204-208.

[148] Rasmussen, T. B., Bjarnsholt, T., Skindersoe, M. E., Hentzer, M., Kristoffersen, P., Köte, M., Nielsen, J., Eberl, L., and Givskov, M. (2005) Screening for Quorum-Sensing Inhibitors (QSI) by Use of a Novel Genetic System, the QSI Selector, *J. Bacteriol. 187*, 1799-1814.

[149] Bjarnsholt, T., Jensen, P. Ø., Rasmussen, T. B., Christophersen, L., Calum, H., Hentzer, M., Hougen, H.-P., Rygaard, J., Moser, C., Eberl, L., Høiby, N., and Givskov, M. (2005) Garlic blocks quorum sensing and promotes rapid clearing of pulmonary *Pseudomonas aeruginosa* infections, *Microbiol. 151*, 3873-3880.

[150] Keyser, P., Elofsson, M., Rosell, S., and Wolf-Watz, H. (2008) Virulence blockers as alternatives to antibiotics: type III secretion inhibitors against Gram-negative bacteria, *J. Int. Med. 264*, 17-29.

[151] Lee, V. T., Pukatzki, S., Sato, H., Kikawada, E., Kazimirova, A. A., Huang, J., Li, X., Arm, J. P., Frank, D. W., and Lory, S. (2007) Pseudolipasin A is a specific inhibitor for phospholipase A2 activity of *Pseudomonas aeruginosa* cytotoxin ExoU, *Infect Immun 75*, 1089-1098.

[152] Aiello, D., Williams, J. D., Majgier-Baranowska, H., Patel, I., Peet, N. P., Huang, J., Lory, S., Bowlin, T. L., and Moir, D. T. (2010) Discovery and Characterization of Inhibitors of *Pseudomonas aeruginosa* Type III Secretion, *Antimicrob. Agents Ch. 54*, 1988-1999.

[153] Bowlin, N. O., Williams, J. D., Knoten, C. A., Torhan, M. C., Tashjian, T. F., Li, B., Aiello, D., Mecsas, J., Hauser, A. R., Peet, N. P., Bowlin, T. L., and Moir, D. T. (2014) Mutations in the *Pseudomonas aeruginosa* Needle Protein Gene pscF Confer Resistance to Phenoxyacetamide Inhibitors of the Type III Secretion System, *Antimicrob. Agents Ch. 58*, 2211-2220.

[154] Zetterström, C. E., Hasselgren, J., Salin, O., Davis, R. A., Quinn, R. J., Sundin, C., and Elofsson, M. (2013) The Resveratrol Tetramer (-)-Hopeaphenol Inhibits Type III Secretion in the Gram-Negative Pathogens *Yersinia pseudotuberculosis* and *Pseudomonas aeruginosa*, *PLoS ONE 8*, e81969.

[155] Hung, D. T., Shakhnovich, E. A., Pierson, E., and Mekalanos, J. J. (2005) Small-molecule inhibitor of Vibrio cholerae virulence and intestinal colonization, *Science 310*, 670-674.

[156] Arnoldo, A., Curak, J., Kittanakom, S., Chevelev, I., Lee, V. T., Sahebol-Amri, M., Koscik, B., Ljuma, L., Roy, P. J., Bedalov, A., Giaever, G., Nislow, C., Merrill, R. A., Lory, S., and Stagljar, I. (2008) Identification of Small Molecule Inhibitors of *Pseudomonas aeruginosa* Exoenzyme S Using a Yeast Phenotypic Screen, *PLoS Genetics 4*, e1000005.

[157] Pinkner, J. S., Remaut, H., Buelens, F., Miller, E., Åberg, V., Pemberton, N., Hedenström, M., Larsson, A., Seed, P., Waksman, G., Hultgren, S. J., and Almqvist, F. (2006) Rationally designed small compounds inhibit pilus biogenesis in uropathogenic bacteria, *Proc. Natl. Acad. Sci. 103*, 17897-17902.

[158] Svensson, A., Larsson, A., Emtenäs, H., Hedenström, M., Fex, T., Hultgren, S. J., Pinkner, J. S., Almqvist, F., and Kihlberg, J. (2001) Design and Evaluation of Pilicides: Potential Novel Antibacterial Agents Directed Against Uropathogenic *Escherichia coli*, *Chem. Bio. Chem. 2*, 915-918.

[159] Morris, A. J. (2007) Regulation of phospholipase D activity, membrane targeting and intracellular trafficking by phosphoinositides, *Biochem Soc Symp*, 247-257.

[160] Ivanova, P. T., Milne, S. B., Byrne, M. O., Xiang, Y., and Brown, H. A. (2007) Glycerophospholipid Identification and Quantitation by Electrospray Ionization Mass Spectrometry, In *Methods in Enzymology* (Brown, H. A., Ed.), pp 21-57, Academic Press.

[161] Brown, H. A., Henage, L. G., Preininger, A. M., Xiang, Y., and Exton, J. H. (2007) Biochemical Analysis of Phospholipase D, In *Methods in Enzymology* (Brown, H. A., Ed.), pp 49-87, Academic Press.

[162] Henage, L. G., Exton, J. H., and Brown, H. A. (2006) Kinetic Analysis of a Mammalian Phospholipase D, *J. Biol. Chem. 281*, 3408-3417.

[163] Bruntz, R. C., Taylor, H. E., Lindsley, C. W., and Brown, H. A. (2014) Phospholipase D2 Mediates Survival Signaling through Direct Regulation of Akt in Glioblastoma Cells, *J. Biol. Chem. 289*, 600-616.

[164] Ogino, C., Kanemasu, M., Hayashi, Y., Kondo, A., Shimizu, N., Tokuyama, S., Tahara, Y., Kuroda, S., Tanizawa, K., and Fukuda, H. (2004) Over-expression system for secretory phospholipase D by *Streptomyces lividans*, *Appl. Microbiol. Biotechnol. 64*, 823-828.

[165] van Meer, G., Voelker, D. R., and Feigenson, G. W. (2008) Membrane lipids: where they are and how they behave, *Nat. Rev. Mol. Cell. Biol. 9*, 112-124.

[166] Wu, Y., Xu, Z., Henderson, F. C., Ryan, A. J., Yahr, T. L., and Mallampalli, R. K. (2007) Chronic *Pseudomonas aeruginosa* infection reduces surfactant levels by inhibiting its biosynthesis, *Cell. Microbiol. 9*, 1062-1072.

[167] Vanderzwan, J., McCaig, L., Mehta, S., Joseph, M., Whitsett, J., McCormack, D., and Lewis, J. (1998) Characterizing alterations in the pulmonary surfactant system in a rat model of *Pseudomonas aeruginosa* pneumonia, *Euro. Res. J. 12*, 1388-1396.

[168] Veldhuizen, R., Nag, K., Orgeig, S., and Possmayer, F. (1998) The role of lipids in pulmonary surfactant, *BBA Mol. Basis Dis. 1408*, 90-108.

[169] Ulbrich-Hofmann, R., Lerchner, A., Oblozinsky, M., and Bezakova, L. (2005) Phospholipase D and its application in biocatalysis, *Biotechnol. Lett. 27*, 535-544.

[170] Ella, K. M., Meier, K. E., Kumar, A., Zhang, Y., and Meier, G. P. (1997) Utilization of alcohols by plant and mammalian phospholipase D, *IUBMB Life 41*, 715-724.

[171] Tang, X., Waksman, M., Ely, Y., and Liscovitch, M. (2002) Characterization and regulation of yeast Ca2+-dependent phosphatidylethanolamine-phospholipase D activity, *Euro. J. Biochem. 269*, 3821-3830.

[172] Wang, X., Xu, L., and Zheng, L. (1994) Cloning and expression of phosphatidylcholine-hydrolyzing phospholipase D from *Ricinus communis* L, *J. Biol. Chem. 269*, 20312-20317.

[173] Mayr, J. A., Kohlwein, S. D., and Paltauf, F. (1996) Identification of a novel, Ca2+-dependent phospholipase D with preference for phosphatidylserine and phosphatidylethanolamine in *Saccharomyces cerevisiae*, *FEBS Letters 393*, 236-240.

[174] Mander, P., Simkhada, J., Cho, S., Park, S., Choi, H., Lee, H., Sohng, J., and Yoo, J. (2009) A novel Ca-dependent phospholipase D from *Streptomyces tendae* possessing only hydrolytic activity, *Arch. Pharm. Res. 32*, 1461-1467.

[175] Nakazawa, Y., Sagane, Y., Kikuchi, T., Uchino, M., Nagai, T., Sato, H., Toeda, K., and Takano, K. (2010) Purification, Biochemical Characterization, and Cloning of Phospholipase D from *Streptomyces racemochromogenes* Strain 10-3, *Protein J. 29*, 598-608.

[176] Carman, G. M., Deems, R. A., and Dennis, E. A. (1995) Lipid Signaling Enzymes and Surface Dilution Kinetics, *J. Biol. Chem. 270*, 18711-18714.

[177] Chalifa, V., Möhn, H., and Liscovitch, M. (1990) A neutral phospholipase D activity from rat brain synaptic plasma membranes. Identification and partial characterization, *J. Biol. Chem. 265*, 17512-17519.

[178] Okamura, S., and Yamashita, S. (1994) Purification and characterization of phosphatidylcholine phospholipase D from pig lung, *J. Biol. Chem. 269*, 31207-31213.

[179] Wang, X. (2000) Multiple forms of phospholipase D in plants: the gene family, catalytic and regulatory properties, and cellular functions, *Prog. Lipid Res. 39*, 109-149.

[180] Berridge, M. J., Lipp, P., and Bootman, M. D. (2000) The versatility and universality of calcium signalling, *Nat. Rev. Mol. Cell. Biol. 1*, 11-21.

[181] Cockcroft, S., Thomas, G. M. H., Fensome, A., Geny, B., Cunningham, E., Gout, I., Hiles, I., Totty, N. F., Truong, O., and Hsuan, J. J. (1994) Phospholipase D: A Downstream Effector of ARF in Granulocytes, *Science 263*, 523-526.

[182] Brown, H. A., Gutowski, S., Moomaw, C. R., Slaughter, C., and Sternwels, P. C. (1993) ADP-ribosylation factor, a small GTP-dependent regulatory protein, stimulates phospholipase D activity, *Cell 75*, 1137-1144.

[183] Sung, T.-C., Altshuller, Y. M., Morris, A. J., and Frohman, M. A. (1999) Molecular Analysis of Mammalian Phospholipase D2, *J. Biol. Chem. 274*, 494-502.

[184] Kook, S., and Exton, J. H. (2005) Identification of interaction sites of protein kinase Cα on phospholipase D1, *Cell. Signal. 17*, 1423-1432.

[185] Cai, S., and Exton, J. H. (2001) Determination of interaction sites of phospholipase D1 for RhoA, *Biochem. J .355*, 779-785.

[186] Peng, H.-J., Henkels, K. M., Mahankali, M., Dinauer, M. C., and Gomez-Cambronero, J. (2011) Evidence for Two CRIB Domains in Phospholipase D2 (PLD2) That the Enzyme Uses to Specifically Bind to the Small GTPase Rac2, *J. Biol. Chem. 286*, 16308-16320.

[187] Pappan, K., Austin-Brown, S., Chapman, K. D., and Wang, X. (1998) Substrate Selectivities and Lipid Modulation of Plant Phospholipase Dα, -β, and -γ, *Arch. Biochem. Biophysics 353*, 131-140.

[188] Liscovitch, M., Chalifa, V., Pertile, P., Chen, C. S., and Cantley, L. C. (1994) Novel function of phosphatidylinositol 4,5-bisphosphate as a cofactor for brain membrane phospholipase D, *J. Biol. Chem. 269*, 21403-21406.

[189] Harkins, A. L., Yuan, G., London, S. D., and Dolan, J. W. (2010) An oleate-stimulated, phosphatidylinositol 4,5-bisphosphate-independent phospholipase D in *Schizosaccharomyces pombe*, *FEMS Yeast Res. 10*, 717-726.

[190] Sciorra, V. A., Rudge, S. A., Wang, J., McLaughlin, S., Engebrecht, J., and Morris, A. J. (2002) Dual role for phosphoinositides in regulation of yeast and mammalian phospholipase D enzymes, *J. Cell Biol. 159*, 1039-1049.

[191] Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J., and Higgins, D. G. (2007) Clustal W and Clustal X version 2.0, *Bioinformatics 23*, 2947-2948.

[192] Sciorra, V. A., Rudge, S. A., Prestwich, G. D., Frohman, M. A., Engebrecht, J., and Morris, A. J. (1999) Identification of a phosphoinositide binding motif that mediates activation of mammalian and yeast phospholipase D isoenzymes, *EMBO J. 18*, 5911-5921.

[193] Kutateladze, T. G. (2010) Translation of the phosphoinositide code by PI effectors, *Nat. Chem. Biol. 6*, 507-513.

[194] Du, G., Altshuller, Y. M., Vitale, N., Huang, P., Chasserot-Golaz, S., Morris, A. J., Bader, M. F., and Frohman, M. A. (2003) Regulation of phospholipase D1 subcellular cycling through coordination of multiple membrane association motifs, *J. Cell. Biol. 162*, 305-315.

[195] Hodgkin, M. N., Masson, M. R., Powner, D., Saqib, K. M., Ponting, C. P., and Wakelam, M. J. O. (2000) Phospholipase D regulation and localisation is dependent upon a phosphatidylinositol 4,5-bisphosphate-specific PH domain, *Curr. Biol. 10*, 43-46.

[196] Fayard, E., Xue, G., Parcellier, A., Bozulic, L., and Hemmings, B. (2011) Protein Kinase B (PKB/Akt), a Key Mediator of the PI3K Signaling Pathway, In *Phosphoinositide 3-kinase in Health and Disease* (Rommel, C., Vanhaesebroeck, B., and Vogt, P. K., Eds.), pp 31-56, Springer Berlin Heidelberg.

[197] Obenauer, J. C., Cantley, L. C., and Yaffe, M. B. (2003) Scansite 2.0: proteome-wide prediction of cell signaling interactions using short sequence motifs, *Nucleic Acids Res. 31*, 3635-3641.

[198] Huang, B. X., Akbar, M., Kevala, K., and Kim, H.-Y. (2011) Phosphatidylserine is a critical modulator for Akt activation, *J. Cell Biol. 192*, 979-992.

[199] Buser, C., and McLaughlin, S. (1998) Ultracentrifugation Technique for Measuring the Binding of Peptides and Proteins to Sucrose-Loaded Phospholipid Vesicles, In *Transmembrane Signaling Protocols* (Bar-Sagi, D., Ed.), pp 267-281, Humana Press.

[200] Bleves, S., Viarre, V., Salacha, R., Michel, G. P. F., Filloux, A., and Voulhoux, R. (2010) Protein secretion systems in *Pseudomonas aeruginosa*: A wealth of pathogenic weapons, *Int. J. Med. Microbiol. 300*, 534-543.

[201] Das, K. C., Lewis-Molock, Y., and White, C. W. (1997) Elevation of Manganese Superoxide Dismutase Gene Expression by Thioredoxin, *Am. J. Res. Cell Mol. Biol. 17*, 713-726.

[202] Kondo, N., Ishii, Y., Kwon, Y. W., Tanito, M., Sakakura-Nishiyama, J., Mochizuki, M., Maeda, M., Suzuki, S., Kojima, M., Kim, Y. C., Son, A., Nakamura, H., and Yodoi, J. (2007) Lipid raft-mediated uptake of cysteine-modified thioredoxin-1: apoptosis enhancement by inhibiting the endogenous thioredoxin-1, *Antioxid. Redox Sign. 9*, 1439-1448.

[203] Angus, A. A., Lee, A. A., Augustin, D. K., Lee, E. J., Evans, D. J., and Fleiszig, S. M. J. (2008) Pseudomonas aeruginosa Induces Membrane Blebs in Epithelial Cells, Which Are Utilized as a Niche for Intracellular Replication and Motility, *Infect. Immun. 76*, 1992-2001.

[204] Baxt, L. A., Garza-Mayers, A. C., and Goldberg, M. B. (2013) Bacterial Subversion of Host Innate Immune Pathways, *Science 340*, 697-701.

[205] Hullin-Matsuda, F., Kawasaki, K., Delton-Vandenbroucke, I., Xu, Y., Nishijima, M., Lagarde, M., Schlame, M., and Kobayashi, T. (2007) De novo biosynthesis of the late endosome lipid, bis(monoacylglycero)phosphate, *J. Lipid Res. 48*, 1997-2008.

[206] Hullin-Matsuda, F., Luquain-Costaz, C., Bouvier, J., and Delton-Vandenbroucke, I. (2009) Bis(monoacylglycero)phosphate, a peculiar phospholipid to control the fate of cholesterol: Implications in pathology, *Prostag. Leukotr. Ess. 81*, 313-324.

[207] Pettitt, T. R., Martin, A., Horton, T., Liossis, C., Lord, J. M., and Wakelam, M. J. O. (1997) Diacylglycerol and Phosphatidate Generated by Phospholipases C and D, Respectively, Have Distinct Fatty Acid Compositions and Functions, *J. Biol. Chem. 272*, 17354-17359.

[208] Pettitt, T. R., McDermott, M., Saqib, K. M., Shimwell, N., and Wakelam, M. J. (2001) Phospholipase D1b and D2a generate structurally identical phosphatidic acid species in mammalian cells, *Biochem. J. 360*, 707-715.

[209] Lewis, J. A., Scott, S. A., Lavieri, R., Buck, J. R., Selvy, P. E., Stoops, S. L., Armstrong, M. D., Brown, H. A., and Lindsley, C. W. (2009) Design and synthesis of isoform-selective phospholipase D (PLD) inhibitors. Part I: Impact of alternative halogenated privileged structures for PLD1 specificity, *Bioorgan. Med. Chem. Lett. 19*, 1916-1920.

[210] Lavieri, R., Scott, S. A., Lewis, J. A., Selvy, P. E., Armstrong, M. D., Brown, H. A., and Lindsley, C. W. (2009) Design and synthesis of isoform-selective phospholipase D (PLD) inhibitors. Part II. Identification of the 1,3,8-triazaspiro[4,5]decan-4-one privileged structure that engenders PLD2 selectivity, *Bioorgan. Med. Chem. Lett. 19*, 2240-2243.

[211] Lavieri, R. R., Scott, S. A., Selvy, P. E., Kim, K., Jadhav, S., Morrison, R. D., Daniels, J. S., Brown, H. A., and Lindsley, C. W. (2010) Design, Synthesis and Biological Evaluation of Halogenated N-(2-(4-Oxo-1-phenyl-1,3,8-triazaspiro[4.5]decan-8-yl)ethylbenzamides: Discovery of an Isoform-Selective Small Molecule Phospholipase D2 (PLD2) Inhibitor, *J. Med. Chem. 53*, 6706-6719.

[212] Scott, S. A., Selvy, P. E., Buck, J. R., Cho, H. P., Criswell, T. L., Thomas, A. L., Armstrong, M. D., Arteaga, C. L., Lindsley, C. W., and Brown, H. A. (2009) Design of isoform-selective phospholipase D inhibitors that modulate cancer cell invasiveness, *Nat. Chem. Biol. 5*, 108-117.

[213] Oliveira, T. G., and Di Paolo, G. (2010) Phospholipase D in brain function and Alzheimer's disease, *Biochem. Biophysica Acta 1801*, 799-805.

[214] Pacher, P., and Kunos, G. (2013) Modulating the endocannabinoid system in human health and disease: successes and failures, *The FEBS journal 280*, 1918-1943.

[215] Oh, M.-K., Yang, H., and Roberts, M. F. (2003) Using O-(n-alkyl)-N-(N,N′ -dimethylethyl)phosphoramidates to investigate the role of Ca2+ and interfacial binding in a bacterial phospholipase D, *BBA Proteins and Proteom. 1649*, 146-153.

[216] Egertová, M., Simon, G. M., Cravatt, B. F., and Elphick, M. R. (2008) Localization of N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD) expression in mouse

brain: A new perspective on N-acylethanolamines as neural signaling molecules, *The J. Comp. Neurol. 506*, 604-615.

[217] Guo, L., Gragg, S. D., Chen, Z., Zhang, Y., Amarnath, V., and Davies, S. S. (2013) Isolevuglandin-modified phosphatidylethanolamine is metabolized by NAPE-hydrolyzing phospholipase D, *J. Lipid Res. 54*, 3151-3157.

[218] Eisen, S. F., and Brown, H. A. (2002) Selective Estrogen Receptor (ER) Modulators Differentially Regulate Phospholipase D Catalytic Activity in ER-Negative Breast Cancer Cells, *Mol. Pharm. 62*, 911-920.

[219] Tou, J.-s., and Urbizo, C. (2001) Resveratrol inhibits the formation of phosphatidic acid and diglyceride in chemotactic peptide- or phorbol ester-stimulated human neutrophils, *Cell. Signal. 13*, 191-197.

[220] Tou, J.-s., and Urbizo, C. (2008) Diethylstilbestrol inhibits phospholipase D activity and degranulation by stimulated human neutrophils, *Steroids 73*, 216-221.

[221] Garcia, A., Zheng, Y., Zhao, C., Toschi, A., Fan, J., Shraibman, N., Brown, H. A., Bar-Sagi, D., Foster, D. A., and Arbiser, J. L. (2008) Honokiol Suppresses Survival Signals Mediated by Ras-Dependent Phospholipase D Activity in Human Cancer Cells, *Clin. Cancer Res. 14*, 4267-4274.

[222] LEVY, B. D., FOKIN, V. V., CLARK, J. M., WAKELAM, M. J. O., PETASIS, N. A., and SERHAN, C. N. (1999) Polyisoprenyl phosphate (PIPP) signaling regulates phospholipase D activity: a 'stop' signaling switch for aspirin-triggered lipoxin A4, *FASEB J. 13*, 903-911.

[223] Levy, B. D., Hickey, L., Morris, A. J., Larvie, M., Keledjian, R., Petasis, N. A., Bannenberg, G., and Serhan, C. N. (2005) Novel polyisoprenyl phosphates block

phospholipase D and human neutrophil activation in vitro and murine peritoneal inflammation in vivo, *Brit. J. Pharm. 146*, 344-351.

[224] Monovich, L., Mugrage, B., Quadros, E., Toscano, K., Tommasi, R., LaVoie, S., Liu, E., Du, Z., LaSala, D., Boyar, W., and Steed, P. (2007) Optimization of Halopemide for Phospholipase D2 inhibition, *Bioorgan. Med. Chem. Lett. 17*, 2310-2311.

[225] Su, W., Yeku, O., Olepu, S., Genna, A., Park, J.-S., Ren, H., Du, G., Gelb, M. H., Morris, A. J., and Frohman, M. A. (2009) 5-Fluoro-2-indolyl des-chlorohalopemide (FIPI), a Phospholipase D Pharmacological Inhibitor That Alters Cell Spreading and Inhibits Chemotaxis, *Mol. Pharm. 75*, 437-446.

[226] Perry, R., Kang, Y., and Greaves, B. (1995) Effects of tamoxifen on growth and apoptosis of estrogen-dependent and -independent human breast cancer cells, *Ann. Surg. Oncol. 2*, 238-245.

[227] Cho, C.-H., Jung, D.-I., Neuenswander, B., and Larock, R. C. (2011) Parallel Synthesis of a Desketoraloxifene Analogue Library via Iodocyclization/Palladium-Catalyzed Coupling, *ACS Comb. Sci. 13*, 501-510.

[228] Grese, T. A., Sluka, J. P., Bryant, H. U., Cullinan, G. J., Glasebrook, A. L., Jones, C. D., Matsumoto, K., Palkowitz, A. D., Sato, M., Termine, J. D., Winter, M. A., Yang, N. N., and Dodge, J. A. (1997) Molecular determinants of tissue selectivity in estrogen receptor modulators, *Proc. Natl. Acad. Sci. 94*, 14105-14110.

[229] Scott, S. A., Spencer, C. T., O'Reilly, M. C., Brown, K. A., Lavieri, R. R., Cho, C.-H., Jung, D.-I., Larock, R. C., Brown, H. A., and Lindsley, C. W. (2014) Discovery of Desketoraloxifene Analogues as Inhibitors of Mammalian, *Pseudomonas aeruginosa*, and NAPE Phospholipase D Enzymes, *ACS Chem. Biol.* in press

[230] Tyson, G. H., and Hauser, A. R. (2013) Phosphatidylinositol 4,5-Bisphosphate Is a Novel Coactivator of the *Pseudomonas aeruginosa* Cytotoxin ExoU, *Infect. Immun. 81*, 2873-2881.

[231] Miyata, S. T., Unterweger, D., Rudko, S. P., and Pukatzki, S. (2013) Dual Expression Profile of Type VI Secretion System Immunity Genes Protects Pandemic *Vibrio cholerae*, *PLoS Pathog. 9*, 18.

[232] Manning, B. D., and Cantley, L. C. (2007) AKT/PKB Signaling: Navigating Downstream, *Cell 129*, 1261-1274.

[233] Moritz, A., De Graan, P. N., Gispen, W. H., and Wirtz, K. W. (1992) Phosphatidic acid is a specific activator of phosphatidylinositol-4-phosphate kinase, *J. Biol. Chem. 267*, 7207-7210.

[234] Jarquin-Pardo, M., Fitzpatrick, A., Galiano, F. J., First, E. A., and Davis, J. N. (2007) Phosphatidic acid regulates the affinity of the murine phosphatidylinositol 4-phosphate 5-kinase-Iβ for phosphatidylinositol-4-phosphate, *J. Cell. Biochem. 100*, 112-128.

[235] Cockcroft, S. (2009) Phosphatidic acid regulation of phosphatidylinositol 4-phosphate 5-kinases, *BBA Mol. Cell Biol. Lipids 1791*, 905-912.

[236] Di Paolo, G., and De Camilli, P. (2006) Phosphoinositides in cell regulation and membrane dynamics, *Nature 443*, 651-657.

[237] D'Souza-Schorey, C., and Chavrier, P. (2006) ARF proteins: roles in membrane traffic and beyond, *Nat. Rev. Mol. Cell. Biol. 7*, 347-358.