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Using Chemoproteomic and Metabolomic Platforms to Identify Nodal Metabolic Pathways Important to Inflammation

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By

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ABSTRACT

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There are an increasing number of human pathologies that have been associated with altered metabolism, including obesity, diabetes, cancer, atherosclerosis, and neurodegenerative diseases. Most attention on metabolism has been focused on well-understood metabolic pathways and has largely ignored most of the biochemical pathways that operate in physiological and pathophysiological settings. This is, in part, because of the vast landscape of uncharacterized and yet-undiscovered enzymes and metabolites that operate in metabolism. One technology that has arisen to address this challenge is activity-based protein profiling (ABPP). ABPP uses activity-based chemical probes to broadly assess the functional states of characterized and uncharacterized enzymes alike across entire enzyme classes. ABPP, when coupled with inhibitor discovery platforms and functional metabolomic technologies has led to discoveries that increase our definition of known biochemical pathways to expand our knowledge of metabolism in human health and disease.

Being able to identify key nodal metabolic pathways will undoubtedly lead to new therapeutic strategies for combating diseases associated with metabolism. We are particularly interested in studying inflammatory metabolism, because chronic, low-grade inflammation is increasingly associated with many human pathologies. Although there are several successfully marketed small molecule anti-inflammatory drugs such as cyclooxygenase inhibitors and glucocorticoids, many of these compounds are also associated with various adverse cardiovascular or immunosuppressive effects. Thus, identifying novel anti-inflammatory small molecules and their biological targets is critical for developing safer and more effective treatment strategies for inflammatory diseases.

We conducted a chemical genetics screen to identify small molecules that suppress the release of the pro-inflammatory cytokine TNFα from stimulated macrophages. We have used an enzyme class-directed chemical library for our screening efforts to facilitate subsequent target identification using ABPP. Using this strategy, we have found that KIAA1363 is a novel target for lowering certain pro-inflammatory cytokines through affecting key ether lipid metabolism pathways. This study highlights the application of combining chemical genetics with chemoproteomic and metabolomic approaches toward identifying and characterizing anti-inflammatory small molecules and their targets.

DEDICATION

I would like to thank my framily for their unconditional love and support, and for always reminding me where home is. Without you all, the last five years would not have been possible. Also to my advisor, Dan Nomura, thank you for giving me a second chance and for your continued mentorship and guidance. Lastly, to all my mentors, past and present: thank you for always believing in me.

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LIST OF ABBREVIATIONS

ABPP	activity-based protein profiling
MudPIT	multidimensional protein identification technology
NIMS	nanostructure initiator mass spectrometry
DMP	discover metabolite profiling
MGLL	monoacylglycerol lipase
FAAH	fatty acid amide hydrolase
ABHD12	alpha/beta hydrolase domain-containing protein 12
PAFAH	platelet activating factor acetyl hydrolase
LIPE	hormone sensitive lipase
PLA2G15	phospholipase A2, group XV
PLA2G7	phospholipase A2, group VII
PNPLA6	patatin-like phospholipase domain containing 6
COX	cyclooxygenase
NSAID	nonsteroidal anti-inflammatory drug
BMDM	bone marrow-derived macrophage
TNFα	tumor necrosis factor alpha
IL12	interleukin 12
IL1α	interleukin 1 alpha
IL6	interleukin 6
G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte macrophage colony stimulating factor
LPS	lipopolysaccharide or lysophosphatidyl serine

FP	fluorophosphonate
PPARγ	peroxisome proliferator-activated receptor gamma
LC/MS	liquid chromatography/mass spectrometry
NAE	N-acylethanolamine
NAT	N-acyltaurine
2-acetyl-MAGe	2-acetyl monoalkylglycerol ether
MAGe	monoalkylglycerol ether
PAF	platelet activating factor
LPCe	lysophosphatidylcholine ether (aka lyso PAF)
LPCp	lysophosphatidylcholine plasmalogen
LPAe	lysophosphatidic acid ether
PCp	phosphatidylcholine plasmalogen
PIe	phosphatidylinositol ether
PGe	phosphatidylglycerol ether
MAG	monoacylglycerol
DAG	diacylglycerol
FFA	free fatty acid
PE	phosphatidyl ethanolamine
PA	phosphatidic acid
PI	phosphatidyl inositol
LPC	lysophosphatidylcholine
LPE	lysophosphatidylethanolamine
LPI	lysophosphatidylinositol

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CHAPTER ONE

Chemoproteomic and Metabolomic Platforms for Mapping Dysregulated Metabolic Pathways in Disease

Introduction

Advancements in the genome sequencing effort have revealed a huge number of predicted but previously unknown proteins. Thus, in the post-genomic era, scientists are faced with the daunting task of deciphering the biochemical, physiological, and pathophysiological functions of the vast landscape of poorly understood or uncharacterized enzymes (1,2). The incorporation of these proteins into our incomplete knowledge of metabolism is the broad goal of modern proteomic and metabolomic technologies. Understanding the biological functions of these uncharacterized enzymes will undoubtedly lead to an expansion of our knowledge of metabolic pathways and to novel therapeutic targets that can be manipulated to treat metabolic diseases. Indeed, a large number of complex human pathologies are associated with dysregulated metabolism that now includes obesity, diabetes, cancer, and inflammatory diseases, but most research has focused on well-established biochemical or regulatory pathways, largely ignoring the majority of poorly understood or uncharacterized networks in metabolism (3). Being able to identify key nodal metabolic pathways, not only in the well-characterized metabolic realm but also in the undiscovered biochemical networks, will undoubtedly lead to new therapeutic strategies for combating diseases associated with metabolism.

Powerful proteomic and metabolomic platforms in combination with advancements in chemical tools have emerged to address this challenge. These chemical proteomic (chemoproteomic) and metabolomic technologies can be incorporated into an integrated workflow to identify and characterize previously unannotated enzymes in complex physiology and disease and to develop potent and selective small molecule chemical inhibitors for these enzymes. This chapter focuses on such chemical approaches that have emerged to investigate metabolism to provide insights into enzyme and metabolite functions in complex physiological or pathophysiological settings.

Broad Profiling of Enzyme Activities

Traditional systems-level approaches for studying gene expression, like microarray and high throughput RNAi, have led to significant advancements in our understanding of physiological and pathophysiological processes alike (4,5). One notable shortcoming in such genomic technologies is the reliance on survey and manipulation of gene expression to assume protein function, and as a result, post-translational control of protein activity often goes unaccounted for. These technologies do not provide information on the functional state of enzymes in complex living systems. The last decade has seen the emergence of powerful chemoproteomic and mass spectrometry-based approaches that facilitate the assessment of enzyme activities or protein hyper-reactivities *en masse*.

One such chemoproteomic platform is called activity-based protein profiling (ABPP), which uses active-site directed chemical probes to assess enzyme activities in complex biological samples (6–8). An activity-based probe consists of a chemical reactive group that covalently reacts with the active sites of enzymes, coupled to an analytical handle

to read-out enzyme activities by SDS/PAGE and fluorescence (e.g. probes coupled to rhodamine) (gel-based ABPP) or enrichment and mass spectrometry-based proteomic platforms (e.g. probes coupled to biotin) (ABPP-Multidimensional Protein Identification Technology (ABPP-MudPIT)) **(Figure 1-1)** (7,8). Thus, these probes facilitate the detection and enrichment of entire families of enzymes that are united by common catalytic mechanisms (e.g., kinases, phosphatases, proteases, histone deacetylases, and hydrolases) (6,7). Also in recent years, activity-based probes have been developed for other types of enzymes such as glycoside hydrolase enzymes, ATP-binding enzymes, caspases, and cysteine proteases **(Table 1-1)**. Unique to ABPP platforms is the ability of these probes to assess the functional state of uncharacterized enzymes in the proteome, since the chemical probes react with the active sites based on reactivity and not on the state of functional annotation. ABPP also enables the detection of changes in enzyme activities that occur without changes in abundance at the mRNA or protein level and facilitates the functional assessment of very low abundance enzymes, which can be enriched with activity-based probes for subsequent proteomic analysis (9). Additionally, native proteomes are compatible with ABPP, eliminating the need to recombinantly overexpress, knockdown, or mutate enzymes of interest in living systems to elucidate their biological role.

Two analytical platforms for exploring activity-labeled proteomes are commonly used. The first, gel-based ABPP, involves the separation of probe-labeled proteomes by SDS-PAGE followed by in-gel fluorescence scanning which facilitates the rapid comparative analysis of multiple proteomes in parallel **(Figure 1-1 A)**. This platform, of course, does not reveal the identities of labeled enzymes. To overcome this limitation, researchers have established liquid chromatography/mass spectrometry (LC/MS)-based ABPP, in which biotinylated probe-labeled enzymes are first enriched with avidin, subjected to on-bead tryptic digest, then resolved, identified, and quantified by multidimensional MS (9) **(Figure 1-1 B)**.

ABPP has been previously used to identify many dysregulated enzyme activities that underlie human diseases or enzyme activities that can be used for industrial applications. There are numerous successful examples of ABPP platforms used to identify unique and novel metabolic enzymes that drive cancer pathogenesis that may represent promising targets for cancer therapy. Using the serine hydrolase-directed fluorophosphonate (FP) activity-based probe, Cravatt, Nomura, and colleagues have shown enzyme activities such as KIAA1363 and monoacylglycerol lipase (MGLL) as upregulated in aggressive human cancer cells and primary human tumors and were critical nodal enzymes in driving malignant and tumorigenic features of cancer (10,11). These probes have also been used to identify the enzymes urokinase (uPA) and tissue plasminogen activator (tPA), as highly secreted enzymes in aggressive human breast cancer cells (12,13). Quigley and colleagues showed that active extracellular uPA, but not total uPA levels, were upregulated in high-intravasating variants of human fibrosacroma HT-1080 cells and that blocking uPA inhibited invasion *in vitro* and intravasation and metastasis *in vivo* (14), indicating increased proteolytic processes contribute to cancer pathogenicity. Using the serine hydrolase probe, Cheresh and colleagues profiled primary human ductal adenocarcinomas and identified

retinoblastoma-binding protein 9 (RBBP9) as a tumor-associated serine hydrolase that promotes anchorage-independent growth *in vitro* as well as pancreatic carcinogenesis *in vivo* through overcoming TGF-b-mediated antiproliferative signaling by reducing Smad2/3 phosphorylation (15).

ABPP has also been used to identify nodal or dysregulated enzyme activities in bacteria or in viral infections. Pezacki used ABPP to identify carboxylesterase 1 (CES1) as an upregulated enzyme activity in hepatitis C virus (HCV)-infected hepatoma cells that was also critical in maintaining viral replication (16). The same group used a non-directed phenyl sulfonate ester probe to target a broad range of enzyme families and showed that HCV infection led to dysregulation of several protein activities that may be relevant to HCV replication (17). Wright and colleagues recently used a cysteine-reactive sulfonate ester probe and the serine hydrolase probe to identify several dysregulated enzyme activities in *Aspergillus fumigatus,* the primary pathogen causing the devastating pulmonary disease Invasive Aspergillosis (18). The same group also developed activity-based probes for cellulose degrading enzymes in *Clostridium thermocellum* which may have applications in biofuel development (19).

ABPP has also been used to identify important enzymes involved in the development of insulin resistance and the metabolic syndrome. Wright and colleagues developed a chemical probe for ATP-binding proteins by incorporating reactive acyl phosphate moieties that directly acylate the lysine e-amino residues of ATP-binding proteins such as ATPases, kinases, and nucleotide-binding proteins. This probe facilitated the identification of altered citric acid cycle enzymes, oxidative phosphorylation, and lipid metabolism enzymes in mitochondria isolated from the skeletal muscle of high-fat diet fed mice (20). Cravatt and Barglow used an a-chloroacetamide dipeptide probe library and serine hydrolase probes to profile enzyme activities in obese *ob/ob* mice and identified multiple dysregulated metabolic activities including fatty acid synthase, hydroxypyruvate reductase, MGLL, malic enzyme, and liver carboxylesterase (21).

ABPP platforms have also been successfully used as imaging agents for imaging dysregulated metabolism in cancer cells. Bogyo and colleagues have developed a suite of chemical probes for cysteine proteases and caspases and have successfully used these probes for *in vivo* imaging of tumors whose formation, growth, and invasiveness are promoted by activation of cathepsins (22–28). These probes can potentially be used in the clinic to define tumor margins, diagnose tumor grade, assess drug-target occupancy, and monitor tumor apoptosis *in vivo*. Cravatt and colleagues also developed an imaging probe for the cancer-associated serine hydrolase KIAA1363 to provide temporal and spatial tracking of KIAA1363 in aggressive human cancer cells (29).

There have also been pioneering efforts to perform high-throughput screening (HTS) of enzyme activity assays to facilitate the identification and characterization of enzymes with desired enzyme activities or for inhibitor discovery efforts (to be covered below). ABPP is amenable to HTS strategies using fluorescent-tagged activity-based probes and fluorescence polarization (fluopol) screening (30). Siuzdak and Northen have also developed innovative HTS enzyme activity assays based on a Nanostructure-Initiator

Mass Spectrometry (NIMS). NIMS offers superior resolution and sensitivity to MALDI and allows for spatially defined mass analysis of peptide microarrays, single cells, or even tissues (Northen et al., 2007). The NIMS-based enzymatic (Nimzyme) assay immobilizes enzyme substrates on a “soft” (noncovalent) mass spectrometry surface by fluorous-phase interactions and enzyme products are then detected by desorption/ionization. This technique allows surface washing steps to reduce signal suppression in complex biological samples, is sensitive to very low abundance enzymes (500 femptograms), and works with a wide range of pHs and temperatures (31). While there are natural or artificial substrate assays that can be performed to assess the activities of enzymes by color or by indirectly measuring product formation by a coupled assay or biosensor, these assays are only applicable to a narrow range of biochemical transformations for which methods have been developed. Mass spectrometry-based assays are more universal but often require lengthy chromatographic separations, reducing throughput. Northen and colleagues have further advanced this platform by combining the NIMs technology with acoustic printing to speed up the liquid-liquid handling process to make this approach even faster (32). *Nimzyme* is a NIMS-based analytical method that detects enzyme activities in complex biological mixtures, circumvents time-intensive chromatographic separations by in situ fluorous affinity purification. In combination with acoustic sample deposition, *Nimzyme* assays are amenable to HTS approaches for optimizing conditions for enzyme activities (e.g. temperature, time, pH, buffer conditions) or testing a library of mutated or evolved enzymes for new functionalities.

Using this approach Suizdak, Northen, and colleagues were able to identify and directly characterize b-1,4-galactosidase activity directly from complex proteomes from a thermophilic microbial community lysate (31). They also applied the *Ninzyme* technology coupled with acoustic printing to characterize glycosyl hydrolases (32). Recently, Cheng et al used the NIMs assay in the thermophilic cellulolytic actinomycete *Thermobispora bispora* to identify optimal growth conditions to maximize b-glucosidase production towards discovering and characterizing enzymes from environmental microbes for industrial and biofuel applications.

Collectively, approaches like ABPP and NIMS are modern technologies that expand our ability to identify and characterize important enzyme activities on a much broader or faster scale to identify important metabolic enzymes in diseases or in industrial applications.

Chemoproteomics for Developing Selective Small Molecule Inhibitors for Metabolic Enzymes

ABPP is, no doubt, a powerful technology for the discovery of novel, uncharacterized, or dysregulated enzyme activity within proteomes. Selective tools with which to perturb enzyme activity are required to determine the biochemical functions of these enzymes in (patho)physiology. Traditional genetic approaches to overexpress, knockout, or mutate enzymes in living systems have shed light on countless biochemical processes but often fail due to organismal death or toxicity, increased compensatory pathways, or

the inability to gain temporal control over protein function. Small molecule chemical inhibitors offer superior tools to assess enzyme function, because they facilitate spatiotemporal control over functionality without changes in protein expression.

Thus, the development of chemical tools to interrogate metabolic enzymes of interest is invaluable for both further investigating their underlying biology and developing small molecules for drug development. Important to the generation of chemical tools is the ability to validate the selectivity and efficacy of the small molecule to not only make certain that the follow-up biology is due to on-target effects, but also to ensure safety of the molecule for follow-up clinical development.

ABPP has emerged as a powerful platform for developing potent and selective small molecule inhibitors for both characterized and uncharacterized enzymes, which have in-turn been used to better understand metabolic pathways in living systems (8,33)**(Figure 1-2)**. Because activity-based probes bind to the active sites of enzymes, inhibitors can be competed against probe binding, facilitating a competitive platform for inhibitor discovery (8,34,35). Furthermore, because the activity-based probes assess enzyme activities of large numbers of enzymes, the selectivity of the small molecules can be tested across entire enzyme class(es). Thus, this competitive ABPP approach can be utilized to develop potent and selective inhibitors for any enzyme, regardless of its state of annotation, if there is a cognate activity-based probe for the enzyme of interest. Selectivity of covalent inhibitors can be further tested across the entire proteome by developing a small molecule mimic of the lead compound that incorporates a bioorthogonal handle (e.g. alkyne and azide) (36–38). This probe can then be reacted with complex proteomes, subjected to click chemistry to append an analytical handle (e.g. biotin or fluorophore) and analyzed by mass-spectrometry or in-gel fluorescence to identify on-target engagement as well as any off-targets.

This competitive ABPP platform can be employed in a medium-throughput gel-based format with fluorescent activity-based probes (competitive gel-based ABPP), a lower throughput but more in-depth mass-spectrometry-based proteomics format with biotin-tagged activity-based probes (competitive ABPP-MudPIT), or an HTS format using fluorescence polarization (competitive fluopol-ABPP) **(Figure 1-3)** and fluorescent activity-based probes against large compound libraries (8,33).

Competitive ABPP screening platforms have lead to the discovery and development of many potent and selective enzyme inhibitors that have been used for in-depth biological characterization of enzymes physiological and pathophysiological settings as well as to ascertain the potential of these enzymes and their inhibitors as therapeutic targets and therapeutics, respectively (36,38–42).

Competitive ABPP platforms have particularly benefited the pharmacological targeting of the endogenous cannabinoid (“endocannabinoid”) system (43,44). The endocannabinoid system consists of two endogenous signaling lipids, 2-arachidonoylglycerol (2-AG) and anandamide which bind to cannabinoid receptors to modulate responses in pain, inflammation, and mood (44–46). Targeting

endocannabinoid degradation and synthesis have been put forth as promising therapeutic strategies for combating a variety of pathologies. Many of these enzymes, such as the 2-AG hydrolyzing enzyme MGLL, anandamide hydrolyzing enzyme fatty acid amide hydrolase (FAAH), and the 2-AG biosynthetic enzyme diacylglycerol lipase (DAGL), all belong to the serine hydrolase enzyme class (44). Inhibitor discovery for the serine hydrolase superfamily of enzymes has benefitted from chemical libraries of electrophilic carbamate and triazole urea scaffolds that specifically target the nucleophilic catalytic mechanism of serine hydrolases (34,35,47). Screening of serine-hydrolase directed chemical libraries, coupled with traditional medicinal chemistry efforts, has facilitated the discovery of potent, selective, and *in vivo*-active inhibitors for many potential therapeutic serine hydrolase targets (43).

MGLL inhibitors found through a competitive ABPP screen of a structurally diverse carbamate library and subsequent medicinal chemistry efforts generated the carbamate JZL184 as the first potent, selective, and *in vivo* active MGLL inhibitor (39). JZL184 has been used extensively to characterize the biochemical function of MGLL using metabolomic technologies (discussed below), and to implicate this enzyme as a therapeutic target for cancer, inflammation and inflammatory diseases, neurodegenerative diseases such as Alzheimer's and Parkinson's diseases, anxiety, and pain (11,39,45,48–50). Many generations of inhibitors for FAAH have been developed and tested for their selectivity using competitive ABPP platforms to elucidate the role of FAAH as the primary degrading enzyme for the endogenous cannabinoid signaling lipid anandamide and the utility of FAAH inhibitors in combating pain and inflammation through heightening anandamide signaling (41,51). FAAH inhibitors are now in clinical trials for treatment of pain and inflammation. DAGL inhibitors were also developed using competitive ABPP platforms and used to show that the DAGL pathway is an important pathway for generation of arachidonic acid precursor pools for eicosanoid synthesis in macrophages to modulate inflammatory responses (52). Gel-based and MudPIT-based competitive ABPP have been used to develop many more inhibitors for various characterized and uncharacterized enzymes that may eventually have therapeutic potential, including the cancer-associated serine hydrolase KIAA1363, acyl peptide hydrolase, alpha/beta hydrolase domain-containing protein 11 (ABHD11), and platelet activating factor acetylhydrolase 2 (PAFAH2) (42,47). Fluopol-ABPP has been used several times to identify inhibitors for many other metabolic enzymes that have therapeutic potential including the potential anti-cancer targets protein methyl esterase 1 (PME1) and RBBP9 and the anti-inflammatory target protein arginine deaminase 4 (PAD4) (30,36,53).

Thus, competitive ABPP platforms are powerful approaches for developing small molecule inhibitors for both characterized and uncharacterized metabolic enzymes, which can be used for expanding our knowledge of metabolism in (patho)physiology, but also to develop chemical tools for subsequent translational development. While NIMS has not yet been used for HTS discovery of enzyme inhibitors, NIMS would also be an attractive strategy for small molecule inhibitor development using mass-spectrometry as the read-out, instead of fluorescence polarization.

Metabolomic Approaches to Define and Map Biochemical Pathways

Modern technologies, such as ABPP and NIMS to assay the activities of enzymes and develop small molecule enzyme inhibitors are powerful strategies that allow us to more broadly assess metabolism beyond well-understood and characterized biochemical pathways. These technologies can then be combined with advanced targeted and untargeted mass spectrometry-based metabolomic approaches to define the endogenous substrate/product relationships as well as the larger metabolic networks controlled by metabolic enzymes. Targeted metabolomics approaches consist of targeting for specific masses and associated parent and fragment ion mass-to-charge ratios (m/z) using mass-spectrometry allowing for the quantification of several hundred known metabolites **(Figure 1-4)**. However, the metabolome is highly physicochemically diverse and likely consists of many metabolites whose structures are yet unknown. Thus, untargeted metabolomic profiling platforms, such as discovery metabolite profiling (DMP) **(Figure 1-5)**, have arisen to capture a much wider metabolomic landscape (7,54–56). While untargeted metabolomics likely still does not capture the entirety of the metabolome, this approach broadly scans detectable ions across a large m/z range using mass-spectrometry platforms and the resulting large datasets are processed by bioinformatic tools to align, integrate, and compare all m/z ion intensities between different biological samples and identify differentially changing ions.

There are several examples of how targeted and DMP-based metabolomics have been successfully applied to discover novel functions of previously characterized enzymes or uncovering the role of completely uncharacterized enzymes, towards understanding the roles of these enzymes in normal physiology and disease. Using these platforms to profile differentially changed metabolites in FAAH-deficient mice, FAAH was found to not only regulate the levels of N-acylethanolamine (NAE), but also N-acyltaurine (NAT) lipid species. While it was known that FAAH regulated arachidonoyl NAE (anandamide) and its action upon cannabinoid receptors and cannabinoid-mediated antinociceptive phenotypes, DMP led to the discovery that FAAH also regulates NAT levels, which activate TRP ion channels giving rise to unique physiological actions mediated by FAAH (57,58).

Chiang et al. had found that the uncharacterized enzyme KIAA1363 was upregulated across multiple types of aggressive human cancer cells. However, the role of this enzyme was completely unknown. Using DMP, Chiang et al. discovered that this enzyme deacetylates the ether lipid 2-acetyl monoalkylglycerol ether (2-acetyl MAGe) to produce MAGe and subsequently the tumor-promoting lipid lysophosphatidic acid-ether (LPAe) to fuel aggressive features of cancer cells (59). Selective KIAA1363 inhibitors, developed through competitive ABPP platforms, have been used to lower MAGE and LPAe, to suppress cancer cell motility and tumorigenesis.

In another example, targeted metabolomics and DMP were also essential in establishing MGLL as a nodal enzyme that not only controls 2-AG and other monoacylglycerols, but also the arachidonic acid pool that generates pro-inflammatory prostaglandins in certain tissues such as brain, liver, and lung (48). While this enzyme

was known to regulate monoacylglycerols, metabolomics led to the unique discovery that this enzyme feeds into the pathway that generates arachidonic acid for the synthesis of pro-inflammatory eicosanoids. This biochemical understanding of MGLL function has in-turn led to the discovery that MGLL inhibitors show potent anti-inflammatory effects and neuroprotection against Parkinson's and Alzheimer's disease as well as inflammatory tissue injury in liver and lung (60–63).

Metabolomics also led to the discovery that MGLL plays a distinct and unique role in aggressive cancer cells in regulating fatty acid levels and a fatty acid network enriched in protumorigenic signaling lipids that drive cancer pathogenicity (10). These findings were unexpected since MGLL does not play a major role in regulating cancer cell free fatty acid levels, and represents a retasked (patho)physiological function of this enzyme in malignant cancer cells. These findings led to understanding the mechanism of action behind MGLL inhibitors and their anti-tumorigenic and anti-pathogenic function in cancer (10,64).

Blankman et al utilized both targeted and DMP-based methods to establish the previously uncharacterized enzyme ABHD12 as a lysophasphatidylserine (LPS) hydrolase and showed that ABHD12-deficiency leads to elevations in brain levels of LPS, which subsequently stimulates toll-like receptor 2 (TLR2) and causes neuroinflammation and auditory and motor deficiencies, recapitulating the human neurodegenerative condition polyneuropathy, hearing loss, ataxia, retinitis pigmentosa, and cataract (PHARC) associated with a loss-of-function ABHD12 mutation (65).

Perhaps one of the most provocative examples of how metabolomics has been used to assign a completely altered and unique function to a well-characterized enzyme in cancer cells has been in elucidating the role of a mutant form of isocitrate dehydrogenase 1 (IDH1) in cancers. IDH1 catalyzes the oxidative decarboxylation of isocitrate to α-ketoglutarate with concomitant reduction of $NADP^+$ to NADPH. Multiple genome-wide analyses of glioma and acute myeloid leukemia patients had identified an arginine 132 to histidine mutation in the active site of IDH1 (66,67). Metabolomic profiling revealed that this IDH1 R132 mutant led to the generation of a novel oncometabolite 2-hydroxyglurarate (2-HG). Surprisingly, the authors discovered that the R132 mutant IDH1 *consumed* NADPH and reduced a-ketoglutarate to 2-HG (68). These studies provided the first evidence for a mutated enzyme in cancer conferring a neomorphic function to yield an unforeseen metabolite. Subsequent studies have shown that 2-HG has also functions as an epigenetic regulator in cancer, primarily through an increase in CpG island methylation (69,70) through acting as a competitive inhibitor of a-ketoglutarate dependent demethylases (71).

In yet another example of how cancer cells rewire their metabolism to fuel their pathogenicity, Ulanovskaya et al. discovered that nicotinamide *N*-methyltransferase (NNMT), which catalyzes the transfer of the methyl group of *S*-adenosyl-methionine (SAM) to nicotinamide, was overexpressed in a variety of tumors. Using metabolomics, the authors showed that NNMT overexpression led to a build up of the stable metabolic product 1-methylnicotinamide, revealing a mechanism by which cancer cells consume

methyl units from SAM and ultimately alter the epigenetic potential of the cell. This included both hypomethylation of histones and cancer related proteins and increased expression of protumorigenic genes (72). SAM metabolism was also found to be coupled to threonine, which provides a large fraction of cellular glycine and acetyl-coenzyme A needed for SAM synthesis. Depletion of threonine from the culture medium of mouse embryonic stem cells decreased SAM accumulation resulting in decreased histone 3 lysine 4 methylation and ultimately slowed growth and increased differentiation (73).

Metabolomic technologies have also been used to study the metabolic effects of viral infection of host cells to provide energy for viral replication. Viral infections are not typically considered metabolic diseases, however viral replication requires massive metabolic demands from the host cell (74). Therefore, infection of adult humans with viruses like herpes simplex virus-1 (HSV-1) or human cytomegalovirus (HCMV) can have profound effects on host cell metabolism and are major causes of human diseases. Metabolomic analysis of HSV-1 infected fibroblasts revealed a shift in central carbon metabolism toward the production of pyrimidine nucleotide metabolites. HCMV infected cells showed enhanced glycolytic flux and TCA cycle to fuel fatty acid biosynthesis (75). Furthermore, reducing the expression of a single metabolic enzyme, argininosuccinate synthetase (AS1), was sufficient to mimic these HSV-1 induced metabolomic changes to improve viral replication (76). These metabolomic approaches can point to potential new sites for antiviral therapy (77).

In summary, targeted and untargeted metabolomic platforms have been successfully and repeatedly used to identify novel functions to previously well-characterized enzymes or to uncharacterized enzymes, which has led to understanding how these enzymes function in regulating metabolism in normal physiology or dysregulated metabolism in diseases such as cancer, neurodegenerative diseases, tissue injury, and infection.

Conclusions

Collectively, we have reviewed how chemical proteomic strategies such as ABPP and metabolomic platforms have arisen to undertake the daunting task of demystifying the undiscovered and uncharacterized aspects of metabolism in (patho)physiological settings, towards identifying unique and nodal metabolic pathways that can be targeted for disease therapy or other commercial applications. We also show how the ABPP platform has been used to develop potent and selective small molecule inhibitors for even previously uncharacterized enzymes, giving rise to chemical tools to further interrogate enzyme function as well as translational development of enzyme inhibitors for disease therapy.

The integration of these technologies, such as ABPP, NIMS, and metabolomic profiling platforms with traditional sequencing and quantitative proteomics approaches will be critical moving forward towards gaining a more complete understanding of how altered enzymatic pathways cause alterations in metabolites which, in turn, may regulate

protein function, signaling pathways, or other aspects of metabolism to fuel disease pathogenesis. While these technological platforms can certainly be advanced and improved to increase throughput, improve sensitivity, increase metabolic coverage, and quicken the process of uncovering novel metabolite and PTM structures, it is no longer necessary for scientists to remain focused on well-understood metabolic pathways. With the increasing sophistication of these modern approaches, we should actively and systematically mine the largely uncharacterized metabolic landscape for unique and novel metabolic networks that can be effectively targeted to treat human diseases.

CHAPTER TWO

Chemical Genetics and Chemoproteomics to Identify Novel Anti-Inflammatory Small Molecules and their Targets

Introduction

Inflammation is normal defense mechanism against infection or tissue injury. However, chronic or non-resolving inflammation can lead to a wide range of pathologies including cancer, neurodegenerative diseases, and diabetes (78–81). Many biochemical pathways have been implicated in driving or suppressing the inflammatory response. Examples include pro-inflammatory prostaglandins and anti-inflammatory resolvins, glucocorticoids, and endocannabinoid signaling molecules (82–85). These metabolites are controlled by their biosynthesizing and degrading enzymes, and exerting control over these biochemical pathways holds great promise for the treatment of inflammation and associated complex diseases. A prominent example is the non-steroidal anti-inflammatory drugs (NSAIDs) (e.g. aspirin and ibuprofen) that target cyclooxygenases (COXs) and are clinically used for pain, inflammation, and arthritis, but have been shown in mouse models to be protective against neurodegenerative diseases, diabetes, and cancer (79,86–90). However, many of these agents also show negative effects that prevent long-term usage that would be necessary for these complex diseases (e.g. cardiovascular or gastrointestinal side effects with COX inhibitors) (90). It is therefore critical to gain a deeper understanding into the metabolic pathways that underlie inflammation.

Chemical genetics represents a powerful approach towards discovery of novel and effective small molecules for treatment of complex diseases (91). Unlike the traditional, target-based screen that relies on a predefined, sometimes poorly validated target, a chemical genetics-based phenotypic screen efficiently interrogates entire metabolic or molecular signaling pathways in an unbiased manner for the most drug- sensitive node. However, the single most significant impediment associated with this approach is the identification of the targets of the efficacious small molecules (91). To address this challenge, we have combined a chemical genetic screen for identifying pro-inflammatory cytokine lowering small molecules with chemoproteomic and metabolomic platforms to enable straightforward identification of lead compounds, their targets, and their mechanisms.

Here, we performed a chemical genetics screen using a serine hydrolase- directed inhibitor library in macrophages to discover new anti-inflammatory small molecules. We coupled this with a functional chemoproteomics platform to identify their biological targets and used metabolomic approaches to characterize the mechanism of anti-inflammatory action. Using this pipeline, we have identified that the serine hydrolase KIAA1363 is a novel anti-inflammatory target and that KIAA1363-selective inhibitors lower key pro-inflammatory cytokines through modulating ether lipid signaling pathways.

Chemical Genetics Screen for Serine Hydrolase Inhibitors that Lower TNFα Release in Macrophages Reveals a Lead Anti-Inflammatory Compound

For our chemical genetics screening strategy, we chose to focus on a small- molecule library directed towards the serine hydrolase superfamily, since several members of this enzyme class have previously been implicated in inflammation, including PLA2G4A,

MGLL, and PLA2G7 (92). Serine hydrolases make up a large class of metabolic enzymes, which include lipases, esterases, hydrolases, proteases, and peptidases that serve vital (patho)physiological functions in numerous biological processes (92). Previous studies have shown that the carbamate, phosphonate, and triazole urea chemotypes are optimal for covalent inhibition of serine hydrolases **(Figure 2-1)** (35,43,47). With diversification of substituents, many studies have shown that selectivity can be attained for specific members of serine hydrolase class (35,40,42,43,47).

We screened a library of 120 compounds to identify small molecules that inhibited lipopolysaccharide (LPS)-induced tumor necrosis factor-alpha (TNFα) secretion from the THP1 human monocyte cell line **(Figure 2-2; Table 2-1)**. This compound library consisted of carbamates, phosphonates, and triazole ureas obtained from the Cravatt and Casida laboratories from previous studies as well as several newly synthesized compounds (35,47,93–95). Among the compounds tested, we identified 12 inhibitors that lowered LPS-induced TNFα secretion in THP1 cells by >50 % **(Figure 2-3)**. While we used THP1 cells for our initial screening efforts, this cell line may not be representative of primary macrophages. We thus performed a counterscreen to identify those inhibitors that also lowered LPS-induced TNFα secretion from primary mouse bone marrow-derived macrophages (BMDMs). While most of the 12 initial leads significantly lowered TNFα levels, only two of the compounds showed >50 % decreases in LPS-stimulated TNFα secretion in this cell type: WWL107 and WWL115 **(Figure 2-4)**. To eliminate any compounds that may be lowering TNFα due to cytotoxicity, we also performed a cell survival counterscreen and found that WWL107 significantly impaired cell viability, leaving WWL115 as our lead compound for further study **(Figure 2-5)**. We show that WWL115 lowers LPS-induced TNFα release in BMDMs in a dose-dependent manner **(Figure 2-6)**.

Chemoproteomic Analysis of WWL115 Reveals Five Significantly Inhibited Serine Hydrolases

Our chemical genetics screen in both THP1 and BMDMs revealed WWL115 as a promising lead anti-inflammatory compound. We next wanted to identify the targets of WWL115 in BMDMs to determine the serine hydrolase(s) responsible for its inflammatory cytokine-lowering effects. To achieve this, we used a chemoproteomic strategy termed activity-based protein profiling (ABPP), a technology that uses active site-directed chemical probes to directly assess the activities of large numbers of enzymes in complex proteomes (7,8,96). Small molecule inhibitors can be competed against activity-based probe binding to enzymes thus enabling identification of functionally inhibited targets of lead compounds that arise from chemical genetic screens **(Figure 2-7)** (8,97,98). Here, we used a competitive ABPP platform using the serine hydrolase activity-based probe, fluorophosphonate-biotin (FP-biotin), to identify the serine hydrolase targets inhibited *in situ* in BMDMs by WWL115. We treated BMDMs with vehicle or WWL115 and subsequently labeled cell lysates with FP-biotin, followed by avidin-enrichment, trypsinization, and analysis of tryptic peptides by Multidimensional Protein Identification Technology (ABPP-MudPIT). Inhibited targets manifested as loss of spectral counts compared with vehicle treatment. Among the 36

serine hydrolases enriched by our activity-based probe, we found 5 lipases that were significantly inhibited by WWL115: KIAA1363, PLA2G15, MGLL, PNPLA6, and LIPE **(Figure 2-8)**.

Characterizing KIAA1363 as a novel anti-cytokine target in BMDMs

Among the 5 targets identified for WWL115, KIAA1363 was the most abundant serine hydrolase in BMDMs. Chang et al. recently developed a highly selective KIAA1363 inhibitor JW480 that irreversibly inhibited this enzyme both *in situ* in cancer cells and *in vivo* in mice (42). To confirm target occupancy and selectivity of this inhibitor in macrophages, we treated BMDMs with JW480 and assessed the selectivity of this inhibitor by competitive ABPP using both FP-rhodamine and FP-biotin for gel-based fluorescence and ABPP-MudPIT analysis, respectively. We confirmed that JW480 was highly selective in BMDMs and only inhibited KIAA1363 among all detectable serine hydrolase activities **(Figure 2-9 and Figure 2-10)**.

We next tested whether JW480 could recapitulate the TNFα-lowering effects of WWL115. We find that JW480 significantly lowers LPS-induced TNFα secretion from BMDMs in a dose-responsive manner to levels comparable to those observed with WWL115, indicating that KIAA1363 was largely responsible for the anti-TNFα effects of this compound **(Figure 2-11)**. We also show that KIAA1363 inhibition by JW480 selectively impairs certain inflammatory cytokines in addition to TNFα, including interleukin-12 (IL12), and granulocyte macrophage colony-stimulating factor (GM-CSF), without affecting other inflammatory cytokines such as IL1α, IL6, and granulocyte stimulating factor (G-CSF) **(Figure 2-12)**. We also tested the contribution of MGLL using the selective MGLL inhibitor JZL184, since MGLL inhibitors have been shown to elicit anti-inflammatory effects in specific paradigms. We find that MGLL inhibition by JZL184 has no effect in lowering LPS- induced TNFα secretion in BMDMs **(Figure 2-13)**.

While we show here that KIAA1363 inhibition is a unique and novel strategy for lowering key LPS-induced pro-inflammatory cytokine levels, we cannot rule out the contribution of the remaining three targets PLA2G15, PNPLA6, and LIPE. We attempted to knockdown the expression of these enzymes using RNA interference approaches, but could not achieve sufficient knockdown in BMDMs and there are a lack of selective pharmacological tools for interrogating the remaining enzymes (data not shown). Nonetheless, LIPE (hormone-sensitive lipase) blockade has been linked to sterility and increased adiposity and PNPLA6 (also known as neuropathy target esterase) blockade causes peripheral neuropathy and demyelination, thus precluding these enzymes as potential therapeutic targets (99,100). PLA2G15 may be of interest since other phospholipase A2 enzymes have been shown to be anti-inflammatory targets.

Metabolomic Profiling Reveals Key Anti-Inflammatory Lipids Regulated by KIAA1363 in BMDMs

We next used untargeted and targeted liquid chromatography/mass spectrometry (LC/MS)-based metabolomic platforms to investigate the mechanism through which

KIAA1363 blockade lowered LPS-induced TNFα release from BMDMs. KIAA1363 was previously characterized as a serine hydrolase that deacetylates the ether lipid 2-acetyl monoalkylglycerol ether (2-acetyl-MAGe or C16:0e/C2:0 MAGe), the penultimate precursor in the *de novo* biosynthesis of platelet activating factor (PAF), to the product monoalkylglycerol ether (MAGe) **(Figure 2-14)** (59,101). Consistent with its role, we show that *in situ* treatment of BMDMs with JW480 inhibits d4-2-acetyl-MAGe hydrolytic activity in BMDMs **(Figure 2-15)**.

Since KIAA1363 is a deacetylase of an ether lipid, we focused our metabolomic profiling efforts on lipid metabolites. We used single-reaction monitoring (SRM)-based targeted approaches to measure >100 lipid metabolites encompassing phospholipids, neutral lipids, sphingolipids, ether lipids, fatty acids, and eicosanoids. We also used untargeted metabolomic methods to profile the levels of an additional ~6000 ions and used XCMSOnline to identify any significantly altered metabolites **(Figure 2-16)**. Combining targeted and untargeted metabolomic data **(Figure 2-17)**, we found the levels of 35 lipids to be significantly changed upon KIAA1363 inhibition with JW480 in BMDMs **(Figure 2-18)**.

While we did not observe changes in 2-acetyl MAGe levels, KIAA1363 blockade reduced MAGe levels and increased the levels of multiple LPCe (also known as lyso-PAF), LPCp, and LPAe species, suggesting that these ether lipid species may be downstream metabolic products of 2-acetyl MAGe and PAF, rather than downstream of MAGe. Consistent with this premise, d4-2-acetyl-MAGe isotopic incorporation studies in BMDMs revealed reduced d4-incorporation into MAGe and increased d4-incorporation into LPCe (lyso-PAF) and LPAe **(Figure 2-19)**. These results are in contrast to previous studies in cancer cells showing that LPCe and LPAe were downstream of MAGe metabolism. We also identified changes in multiple other ether lipid species including phosphatidylcholine-plasmalogen (PCp), phosphatidylinositol-ether (PIe), and phosphatidylglycerol-ether (PGe), likely due to network wide alterations stemming from 2-acetyl MAGe or MAGe metabolism. Interestingly, we also observed changes in additional lipid metabolism pathways including neutral lipids monoacylglycerols (MAG) and diacylglycerols (DAG), free fatty acids (FFA), N-acyl ethanolamines (NAEs), and phospholipids phosphatidyl ethanolamine (PE), phosphatidic acids (PA), phosphatidyl inositols (PI), lysophosphatidylcholines (LPC), lysophosphatidylethanolamine (LPE), and lysophosphatidylserines (LPS), lysophosphatidylinositols (LPI), sphingolipids ceramide and sphingosine, indicating that KIAA1363 may directly or indirectly regulate broader metabolic pathways in lipid metabolism **(Figure 2-18)**.

We next wanted to determine whether these changes in specific lipid species might be driving the TNFα-lowering effects observed upon KIAA1363 inhibition. We screened representative lipid species altered by JW480 treatment for TNFα-lowering effects and found that LPCe, LPAe, and C20:4 FFA significantly reduced LPS-induced TNFα secretion in macrophages **(Figure 2-20)**. While the specific receptor for LPCe is unknown, LPAe is known to stimulate LPA receptors and C20:4 FFA is an agonist of the peroxisome proliferator-activated receptor-γ (PPARγ) (102,103). We show that the TNFα- lowering effects of JW480 are partially reversed by treatment with an LPA

receptor antagonist, but not by a PPARγ or PAF receptor antagonist, indicating that enhanced LPAe and LPA receptor signaling may be responsible for the JW480 effects **(Figure 2-21)**.

Collectively, our results show that KIAA1363 may serve as a unique metabolic node between ether lipids and other signaling lipids to drive the inflammatory response in macrophages.

Conclusions

Here, we have coupled an enzyme class-directed chemical genetics screen with ABPP platforms to identify pro-inflammatory cytokine-lowering compounds and their targets in stimulated macrophages. Using this strategy, we identified KIAA1363 and its inhibitors as a novel metabolic target that influences LPS-stimulated TNFα release. Through metabolomic profiling, we further revealed that KIAA1363 modulates inflammatory cytokine release in part through affecting LPAe and potentially other ether lipid pathways. This enzyme has also been shown to be important in driving aggressive features of cancer cells. KIAA1363 blockade in cancer cells leads to a reduction in the levels of LPAe which leads to reduced motility and tumor growth. In cancer cells, LPAe is downstream of the KIAA1363 product MAGe, whereas our studies in BMDMs suggest that LPAe is downstream of LPCe, a metabolite arising from PAF hydrolysis (59). Thus, our data indicate that the KIAA1363-ether lipid pathway may be wired differently in these two different contexts. Holly et al. also discovered that KIAA1363 regulates platelet aggregation, thrombus growth, RAP1 and PKC activation, ether lipid metabolism, and fibrinogen binding to platelets and megakaryocytes (98). Thus, KIAA1363 inhibitors potentially possess multiple biological activities through manipulating ether lipid signaling pathways and show multiple potential therapeutic avenues.

Our study underscores the utility of combining chemical genetics with chemical systems biology platforms such as ABPP and functional metabolomic profiling towards identifying and characterizing anti-inflammatory small molecules and their targets.

Materials and Methods

Materials. The THP1 cell line was purchased from ATCC. Mouse colony stimulating factor (M-CSF) was purchased from Cell Signaling Technologies. d4-PAF was purchased from Cayman Chemical. Internal standards and metabolite standards were purchased from Sigma, Cayman Chemicals, or Avanti Polar Lipids. The carbamate, phosphonate, and triazole urea inhibitors were obtained from Professor Benjamin Cravatt at The Scripps Research Institute and Professor John Casida at the University of California, Berkeley or were synthesized. The synthesis and characterization of the materials obtained from the Cravatt and Casida labs are described previously (35,47,93–95). Synthetic methods and characterization of lead compounds that were synthesized in our lab are described in Supplemental Methods. The KIAA1363 inhibitor

JW480 was purchased from Cayman Chemicals. FP-biotin was synthesized as previously described (104).

Cell culture conditions. THP1 cells were cultured in RPMI supplemented with 10% FBS, L-glutamine, and β-mercaptoethanol and maintained in a humidified, 37 °C at 5% CO2 incubator. BMDMs were cultured in DMEM supplemented with 10% FBS, L- glutamine, and 20ng/mL M-CSF and maintained in a humidified 37 °C at 8% CO2 incubator.

Isolation of murine bone marrow-derived macrophages. Bone marrow was isolated from the femurs and tibias of male C57BL/6 mice (10-12 week) using a mortar and pestle in complete media containing DMEM supplemented with 10% FBS, L-glutamine, and 20ng/mL M-CSF. Particulate matter was removed by slow-speed centrifugation. Bone marrow cells were then pelleted by centrifugation, resuspended, and plated in complete media on non-treated plastic. Medium was replaced every 2-3 days. On day 7, adherent cells were washed and incubated at 4°C for 20 min. Cells were gently scraped, isolated by centrifugation, counted, and plated for experiments.

Cytokine quantification. THP-1 (1 million per well) or BMDMs (100,000-200,000 cells/well in 24-well plates) were plated. Cells were switched to serum-free media and inhibitors, lipids, and/or antagonists added for 1 hour. After stimulation with 100ng/mL LPS for 6 hours, media was collected and TNFα levels were quantified by ELISA per the manufacturer's instructions (Qiagen).

Survival assays. Cell survival analysis was performed using the Hoechst 33342 nuclear stain (Invitrogen). Briefly, 20,000 were seeded into 96-well plates in a volume of 100μL for 0, 24, and 48 hours in the presence of inhibitors in serum-free DMEM. Cells were washed, fixed, and stained according to the manufacturer's protocol. Plates were scanned using the fluorescence excitation/emission wavelengths for Hoechst 33342 (350nm and 461nm, respectively).

d4-2-acetyl MAGe synthesis. d4-C16:0 2-acetyl MAGe was prepared from [d4-C16:0e] PAF by incubation with 20 units phospholipase C from *Bacillus cereus* (Sigma Aldrich) in PBS for 45 min at room temperature as described previously (101). Completion of the reaction was confirmed by LC/MS. The product was extracted in 2:1 chloroform:methanol and the organic layer was dried under N2 and resuspended in 2:1 chloroform:methanol to desired concentration.

KIAA1363 activity assays. BMDMs were treated with JW480 (5 μM in DMSO) or DMSO for 4 hours. BMDM cell lysates (25 μg) were incubated with d4-2-acetyl MAGe (100μM final concentration) for 30 min at room temperature in PBS (200μL total volume). To quench the reaction, 1:1 ethyl acetate:hexanes was added (600μL), followed by vortexing and addition of internal standards. After centrifugation, the organic layer was removed for analysis of d4-MAGe formation by LC/MS.

Lipidomic profiling of macrophages. BMDMs were plated (3x106 cell/well of 6-well plate) and allowed to adhere overnight. Cells were washed with PBS and switched to serum-

free media containing JW480 (5 μM) or DMSO control for 1 hour, then stimulated with LPS (100 ng/mL) for 6 hours. Cells were washed with PBS, harvested by scraping, and isolated by centrifugation. Cell pellets were flash frozen and stored at -80° until extraction.

Nonpolar lipid metabolites were extracted and analyzed by targeted and untargeted metabolomic methods using previously described procedures (105,106).
Briefly, lipid metabolites were extracted in a 2:1:1 chloroform:methanol:PBS with addition of internal standards C12:0 dodecylglycerol (10 nmol) and pentadecanoic acid (10 nmol). Organic and aqueous layers were separated by centrifugation at 1000xg for 5 min, and the organic layer collected. The aqueous layer was acidified by the addition of 0.1% formic acid followed by the addition of 2mL chloroform, vortexing, and centrifugation.

The organic layers were combined, dried under N2, and resuspended in 120 μL chloroform. An aliquot (10 μL) was analyzed by single-reaction monitoring (SRM)-based LC-MS/MS. LC separation was achieved with a Luna reverse-phase C5 column (Phenomenex). Mobile phase A was composed of 95:5 water:methanol, and mobile phase B consisted of 60:35:5 isopropanol:methanol:water. Solvent modifiers 0.1% formic acid with 5 mM ammonium formate and 0.1% ammonium hydroxide were used to assist ion formation and to improve the LC resolution in both positive and negative ionization modes, respectively. The flow rate for each run started at 0.1 mL/min for 5 min, to alleviate backpressure associated with injecting chloroform. The gradient started at 0% B and increased linearly to 100% B over the course of 45 min with a flow rate of 0.4 mL/min, followed by an isocratic gradient of 100% B for 17 min at 0.5 mL/min before equilibrating for 8 min at 0% B with a flow rate of 0.5 mL/min.

MS analysis was performed with an electrospray ionization sourse (ESI) on an Agilent 6430 QQQ LC-MS/MS. Lipid metabolites were quantified by SRM of the precursor to product ion transition at associated collision energies as previously described (105,106). Quantification was achieved by integrating the area under the peak and expressed as a percent of control after normalizing to the internal standard.

ABPP analysis of macrophages. For gel-based ABPP experiments, BMDMs were treated with inhibitor (5 μM in DMSO) or DMSO control for 4 hours, harvested by scraping, and pelleted by centrifugation. BMDM cell lysate proteomes (50 μg) were labeled with FP-rhodamine (2 μM) for 30 min at room temperature, quenched with 4x SDS/PAGE loading buffer, heated at 95o C for 5 min, and separated by 10% SDS/PAGE as previously described. Gels were scanned using a Typhoon flatbed fluorescence scanner (GE Healthcare).

ABPP-MudPIT analysis was performed using previously established methods. BMDMs were treated with inhibitor (5 μM in DMSO) or DMSO control for 4 hours, harvested by scraping, and pelleted by centrifugation. Briefly, BMDM proteome (1 mg) was labeled with FP-biotin (5 μM) in 1 mL PBS for 1 hour, solubilized in 1% Triton-X100 for 1 hour, and denatured. Labeled enzymes were enriched using avidin beads, reduced, alkylated,

and trypsinized as previously described (10). Tryptic peptides were loaded on to a strong cation exchange/reverse phase capillary column and analyzed by two-dimensional LC-LC-MS/MS also known as Multidimensional Protein Identification Technology (MudPIT) as previously described (10). Resulting ms2 datafiles were then analyzed by Integrated Proteomics Pipeline.

d4-2-acetyl MAGe isotopic labeling. BMDMs (2 x 106 cells) were plated and allowed to adhere overnight. Cells were pre-treated in serum free media with JW480 (5 μM in DMSO) or DMSO control for 1 hour, then stimulated with LPS (100 ng/ml) and d4-2-acetyl MAGe (10 μM) for 15 min. Cells were washed, scraped on ice, and immediately extracted in 2:1 chloroform:methanol as described above. Isotopic incorporation was detected and quantified using targeted LC/MS using SRM transitions based on previously derived optimized transitions of nonisotopic standards.

CHAPTER THREE

Conclusions

Chemoproteomic and Metabolomic Platforms for Mapping Dysregulated Metabolic Pathways in Disease

A large number of complex human pathologies are associated with dysregulated metabolism that now includes obesity, diabetes, cancer, and inflammatory diseases, but most research has focused on well-established biochemical or regulatory pathways, largely ignoring the majority of poorly understood or uncharacterized networks in metabolism. Being able to identify key nodal metabolic pathways, not only in the well-characterized metabolic realm but also in the undiscovered biochemical networks, will undoubtedly lead to new therapeutic strategies for combating diseases associated with metabolism. Powerful proteomic and metabolomic platforms in combination with advancements in chemical tools have emerged to address this challenge. These chemical proteomic (chemoproteomic) and metabolomic technologies can be incorporated into an integrated workflow to identify and characterize previously unannotated enzymes in complex physiology and disease and to develop potent and selective small molecule chemical inhibitors for these enzymes.

The last decade has seen the emergence of powerful chemoproteomic and mass spectrometry-based approaches that facilitate the assessment of enzyme activities or protein hyper-reactivities on a broad scale. One such approach is activity-based protein profiling (ABPP), which uses active site-directed chemical probes to study enzyme activities in biological samples. In gel-based ABPP, probe-labeled proteomes are separated by SDS-PAGE followed by in-gel fluorescence, which facilitates comparative analysis of multiple proteomes in parallel. To uncover the identity of the labeled enzymes, a biotinylated probe in combination with LC/MS-based ABPP can be used. Recently, several pioneering efforts to adapt this technology to more high-throughput platforms have been made, including fluorescence polarization (fluopol) screening and the NIMS-based enzymatic (Nimzyme) assay. These modern technologies expand our ability to identify and characterize important enzyme activities on a much broader and faster scale to identify important metabolic enzymes in diseases or in industrial applications.

The development of small molecule chemical tools to investigate metabolic enzymes is critical, because traditional genetic approaches often fail due to toxicity, compensatory pathways, and the inability to achieve temporal control over protein function. Important to the generation of such chemical tools is the ability to validate the selectivity and efficacy of the small molecule to not only make certain that the follow-up biology is due to on-target effects, but also to ensure safety of the molecule for follow-up clinical development. One key adaptation of ABPP, competitive-ABPP, can be employed for the discovery of potent and selective small molecule inhibitors of enzyme. Inhibitors are identified by their ability to compete against activity-based probe binding, facilitating a competitive platform for inhibitor discovery. This strategy is amenable to gel-based ABPP for medium-throughput screening of inhibitors, LC/MS-based ABPP for a lower-throughput but more in-depth proteomics analysis, and high-throughput formats including fluopol-ABPP for the screening of large compound libraries. Thus, competitive

ABPP platforms are powerful approaches for developing small molecule inhibitors for metabolic enzymes, which can be used for expanding our knowledge of metabolism in (patho)physiology, but also to develop chemical tools for subsequent translational development.

Modern technologies, such as ABPP and NIMS to assay the activities of enzymes and develop small molecule enzyme inhibitors are powerful strategies that allow us to more broadly assess metabolism beyond well-understood and characterized biochemical pathways. These technologies can then be combined with advanced targeted and untargeted mass spectrometry-based metabolomic approaches to define the endogenous substrate/product relationships as well as the larger metabolic networks controlled by metabolic enzymes. Targeted metabolomics approaches consist of targeting for specific masses and associated parent and fragment ion mass-to-charge ratios (m/z) using mass-spectrometry allowing for the quantification of several hundred known metabolites. However, the metabolome is highly physicochemically diverse and likely consists of many metabolites whose structures are yet unknown. Thus, untargeted metabolomic profiling platforms, such as discovery metabolite profiling (DMP), have arisen to capture a much wider metabolomic landscape. While untargeted metabolomics likely still does not capture the entirety of the metabolome, this approach broadly scans detectable ions across a large m/z range using mass-spectrometry platforms and the resulting large datasets are processed by bioinformatic tools to align, integrate, and compare all m/z ion intensities between different biological samples and identify differentially changing ions.

The integration of these technologies, such as ABPP, NIMS, and metabolomic profiling platforms with traditional sequencing and quantitative proteomics approaches will be critical moving forward towards gaining a more complete understanding of how altered enzymatic pathways cause alterations in metabolites which, in turn, may regulate protein function, signaling pathways, or other aspects of metabolism to fuel disease pathogenesis. While these technological platforms can certainly be advanced and improved to increase throughput, improve sensitivity, increase metabolic coverage, and quicken the process of uncovering novel metabolite and PTM structures, it is no longer necessary for scientists to remain focused on well-understood metabolic pathways. With the increasing sophistication of these modern approaches, we should actively and systematically mine the largely uncharacterized metabolic landscape for unique and novel metabolic networks that can be effectively targeted to treat human diseases.

In summary, targeted and untargeted metabolomic platforms have been successfully and repeatedly used to identify novel functions to previously well-characterized enzymes or to uncharacterized enzymes, which has led to understanding how these enzymes function in regulating metabolism in normal physiology or dysregulated metabolism in diseases such as cancer, neurodegenerative diseases, tissue injury, and infection.

Chemical Genetics and Chemoproteomics to Identify Novel Anti-Inflammatory Small Molecules and their Targets

Inflammation is normal defense mechanism against infection or tissue injury. Yet, chronic and low-grade inflammation is a hallmark of many diseases including cancer, diabetes, and arthritis. Currently available treatments for inflammatory diseases show many negative side effects that prevent any long-term use that would be required for the treatment of these complex diseases. Thus, new therapeutics are needed.

Chemical genetics represents a powerful approach towards discovery of novel and effective small molecules for treatment of complex diseases. Unlike the traditional, target-based screen that relies on a predefined, sometimes poorly validated target, a chemical genetics-based phenotypic screen efficiently interrogates entire metabolic or molecular signaling pathways in an unbiased manner for the most drug-sensitive node. However, the single most significant impediment associated with this approach is the identification of the targets of the efficacious small molecules. To address this challenge, we have combined a chemical genetic screen for identifying pro-inflammatory cytokine lowering small molecules with chemoproteomic and metabolomic platforms to enable straightforward identification of lead compounds, their targets, and their mechanisms.

For our chemical genetics screening strategy, we chose to focus on a small-molecule library directed towards the serine hydrolase superfamily, since several members of this enzyme class have previously been implicated in inflammation. Previous studies have shown that the carbamate, phosphonate, and triazole urea chemotypes are optimal for covalent inhibition of serine hydrolases. With diversification of substituents, many studies have shown that selectivity can be attained for specific members of serine hydrolase class. A selection of inhibitors was identified based on the ability to lower TNFα secretion from primary mouse bone marrow-derived macrophages (BMDMs). The compound WWL115 was selected as our lead compounds because it was the most efficacious, non-cytotoxic inhibitor.

To identify the biological targets of WWL115, we used competitive ABPP. Specifically, we competed WWL115 against the serine hydrolase fluorosphosphonate activity-based probe to identify the functionally inhibited enzymes by ABPP-MudPIT. Among the 36 serine hydrolases enriched by our activity-based probe, we found 5 lipases that were significantly inhibited by WWL115: KIAA1363, PLA2G15, MGLL, PNPLA6, and LIPE. JW480 is an inhibitor previously shown to be selective for KIAA1363, the most abundant serine hydrolase identified as inhibited by WWL115. We confirmed JW480 selectivity in BMDMs and showed it is able to recapitulate the same anti-inflammatory phenotype as WWL115, indicating KIAA1363 inhibition could largely be responsible for the observed phenotype, but we cannot yet rule out the contribution of the remaining 4 targets.

We next used untargeted and targeted liquid chromatography/mass spectrometry (LC/MS)-based metabolomic platforms to investigate the mechanism through which KIAA1363 blockade lowered LPS-induced TNFα release from BMDMs. KIAA1363 was previously characterized as a serine hydrolase that deacetylates the ether lipid 2-acetyl monoalkylglycerol ether (2-acetyl-MAGe or C16:0e/C2:0 MAGe), the penultimate

precursor in the *de novo* biosynthesis of platelet activating factor (PAF), to the product monoalkylglycerol ether (MAGe).

Since KIAA1363 is a deacetylase of an ether lipid, we focused our metabolomic profiling efforts on lipid metabolites. We used single-reaction monitoring (SRM)-based targeted approaches to measure >100 lipid metabolites encompassing phospholipids, neutral lipids, sphingolipids, ether lipids, fatty acids, and eicosanoids, and found the levels of 35 lipids to be significantly changed upon KIAA1363 inhibition with JW480 in BMDMs. This included members from many lipid classes, including phosphatidylcholine-plasmalogen (PCp), phosphatidylinositol-ether (PIe), and phosphatidylglycerol-ether (PGe), neutral lipids monoacylglycerols (MAG) and diacylglycerols (DAG), free fatty acids (FFA), N-acyl ethanolamines (NAEs), and phospholipids phosphatidyl ethanolamine (PE), phosphatidic acids (PA), phosphatidyl inositols (PI), lysophosphatidylcholines (LPC), lysophosphatidylethanolamine (LPE), and lysophosphatidylserines (LPS), lysophosphatidylinositols (LPI), sphingolipids ceramide and sphingosine, revealing broad metabolic alterations as a result of KIAA1363 inhibition.

Finally, we showed that LPAe, through LPA receptor signaling may be responsible for the anti-inflammatory effect seen with KIAA1363 inhibition. Thus, our results show that KIAA1363 may serve as a unique metabolic node between ether lipids and other signaling lipids to drive the inflammatory response in macrophages. Our study underscores the utility of combining chemical genetics with chemical systems biology platforms such as ABPP and functional metabolomic profiling towards identifying and characterizing anti-inflammatory small molecules and their targets.

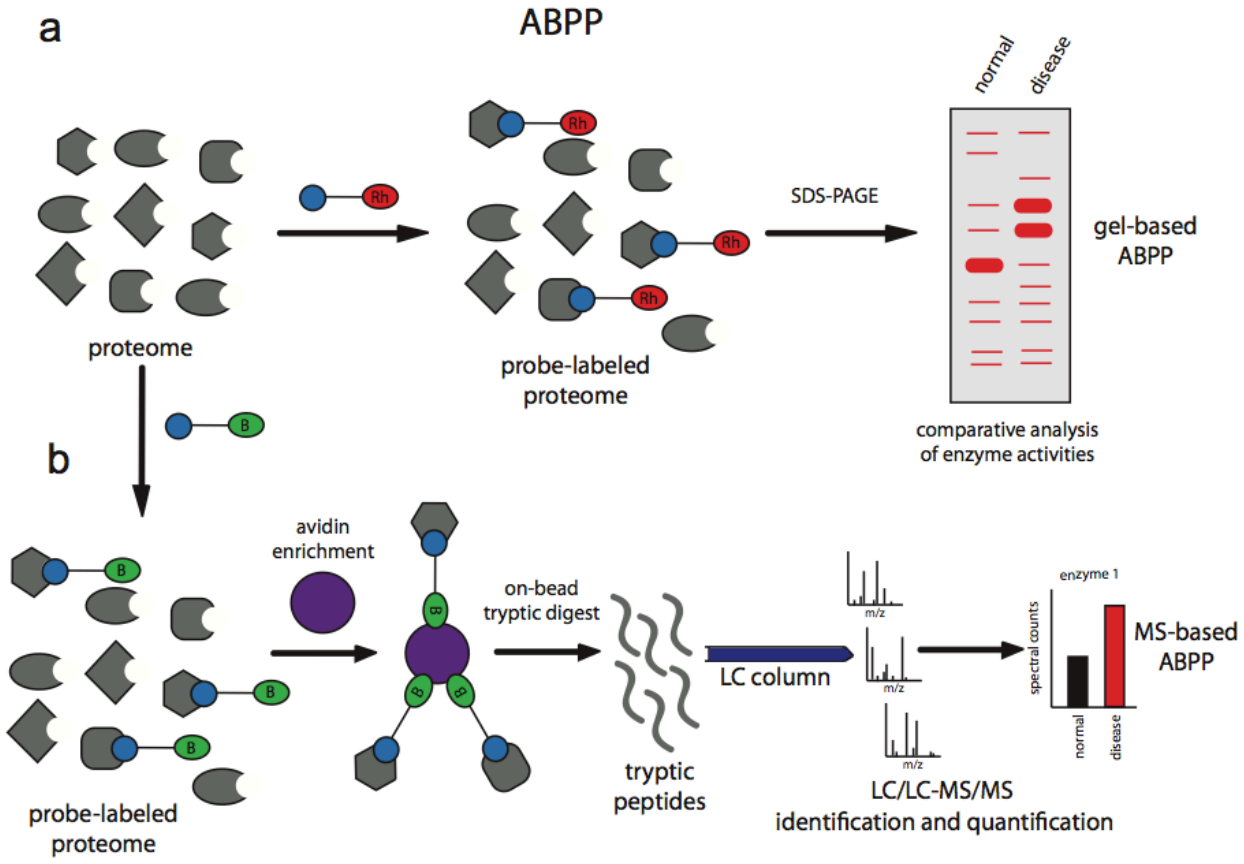


Figure 1-1. Activity-based protein profiling. ABPP uses active site-directed chemical probes to broadly assess the functional state of enzymes across enzyme families. These probes consist of a reactive group and a detection handle, most commonly rhodamine (Rh) or biotin (B). A) In gel-based ABPP, native proteomes are reacted with the probe and proteins are separated by SDS-PAGE and visualized by fluorescent scanning. B) MS-based ABPP facilitates the identification and quantification of enzyme activities following avidin enrichment, on-bead tryptic digest, and resolution by Multidimensional Protein Identification Technology (MudPIT).

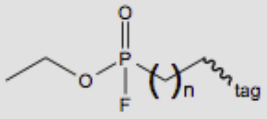
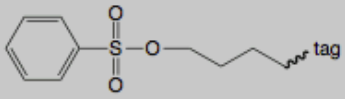
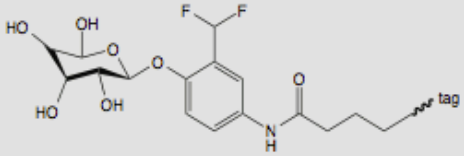
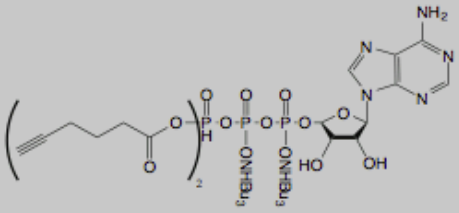
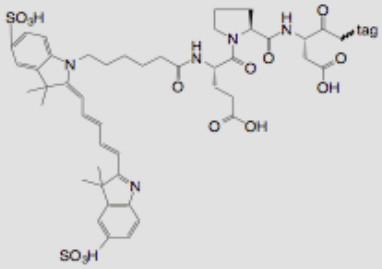
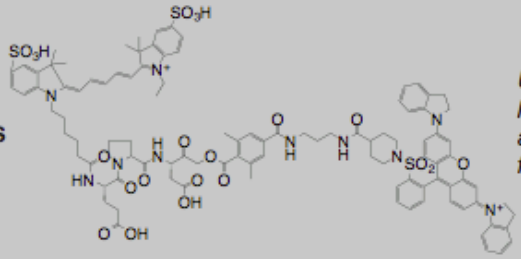
Probe target	Structure	Applications
Serine hydrolases		*Identified upregulated KIAA1363 and MAGL activities (10, 11) and increased secreted uPA and tPA in aggressive cancer (12, 13). Identified increased RBBP9 activity in pancreatic carcinomas (15).*
Phenyl sulfonate esters		*Targeted several enzyme families and showed that infection by hepatitis C leads to dysregulation of several protein activities (17).*
Glycoside hydrolase enzymes		*Used to profile functional cellulose-degrading enzyme activity in bacteria applicable to the development of biofuels (19).*
ATP-binding enzymes		*Identification of altered TCA cycle, oxidative phosphorylation, and lipid metabolism enzymes in skeletal muscle mitochondria of high fat-fed mice (20).*
Caspases		*Visualization of apoptosis in colon tumors of mice treated with Apomab (24).*
Cysteine proteases		*Used to identify highly upregulated legumain activity in activated macrophages and for* in vivo *imaging of tumor formation (26).*

Table 1-1. Representative activity-based probes and their applications (CHECK REFERENCE NUMBERS)

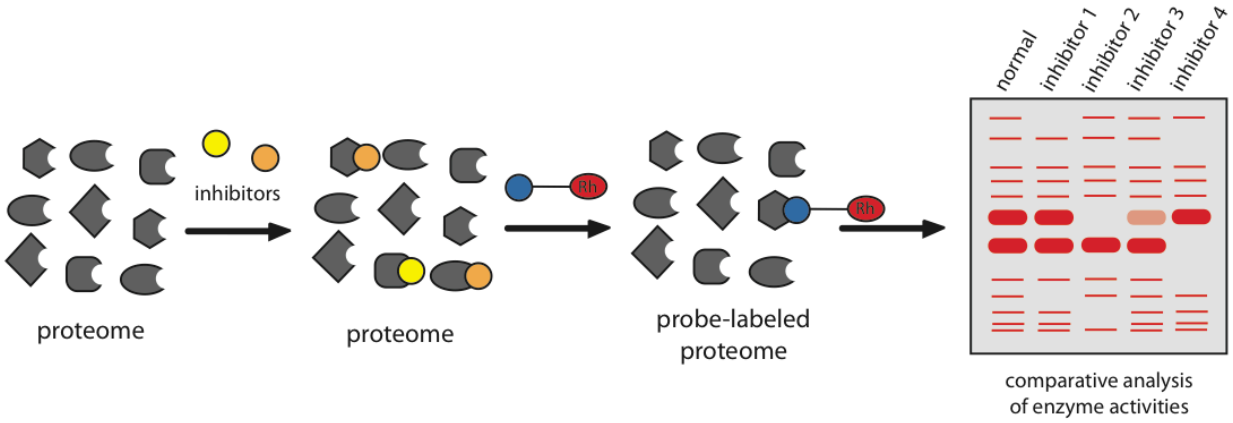


Figure 1-2. Competitive ABPP. Competitive ABPP assess the potency and selectivity of small molecule inhibitors in native proteomes by competing with the ability of the activity-based probes to bind. Enzyme inhibition is indicated by a loss of fluorescent intensity by gel or by a loss of spectral counts by mass spectrometry.

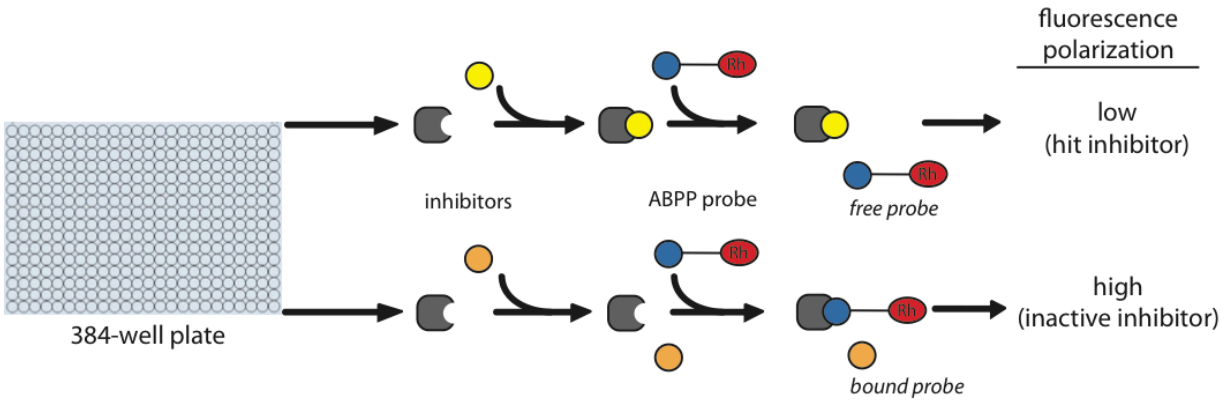


Figure 1-3. Fluorescence polarization ABPP. Fluopol ABPP is a HTS version of competitive ABPP conducted with pure or recombinant protein. Fluorescence polarization is high if enzyme activity is high (inactive inhibitor) and low if enzyme activity is low (active inhibitor).

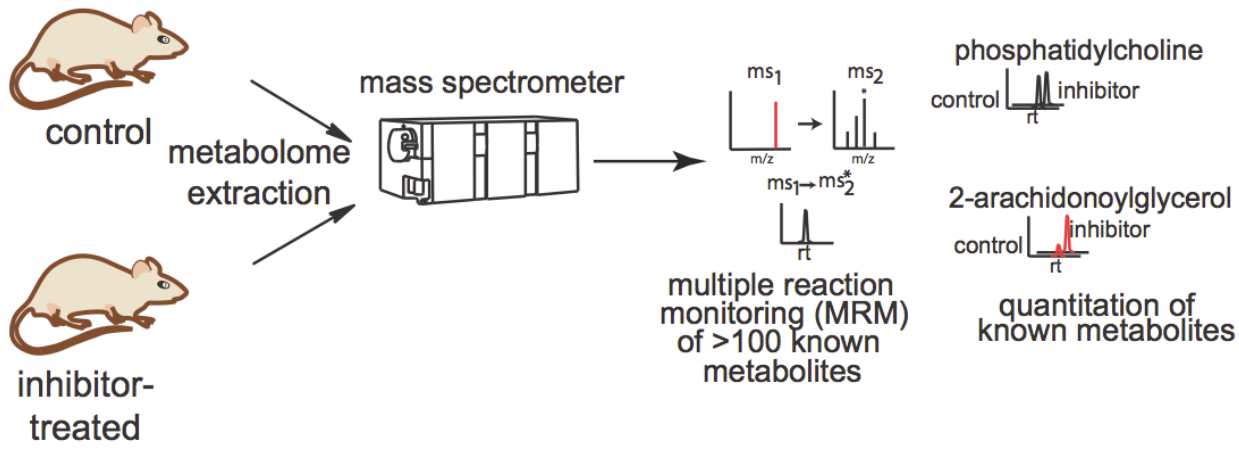


Figure 1-4. Targeted metabolomics. In targeted metabolomics, specific sets of known metabolites are selected for analysis by targeting for their associated parent and fragment ion mass-to-charge ratios (m/z) using LC/MS.

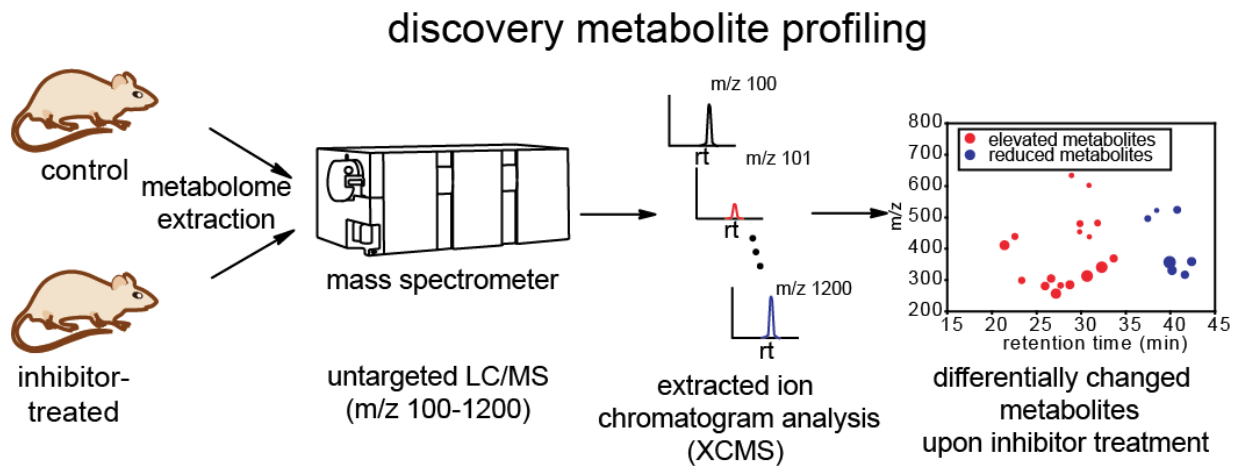


Figure 1-5. Discovery metabolite profiling. In untargeted metabolomics, the mass spectrometer scans a large mass range (m/z 100–1200) for known and unknown metabolites. Datasets are then analyzed by bioinformatics platforms which align, quantify, and identify metabolites that are significantly altered between treatment groups.

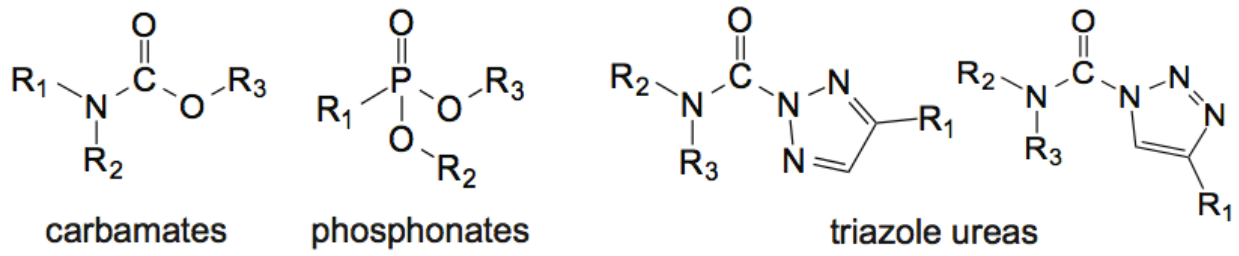
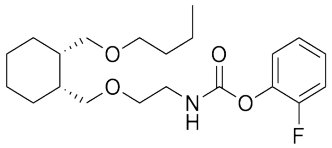
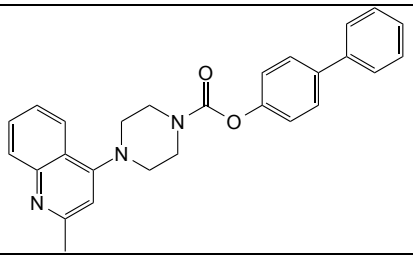
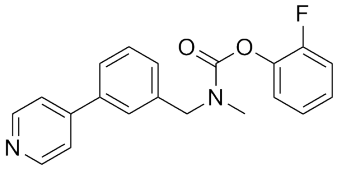
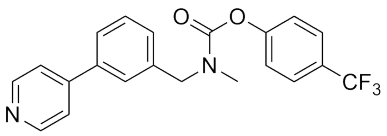
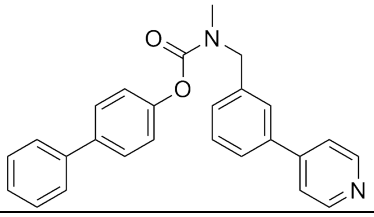
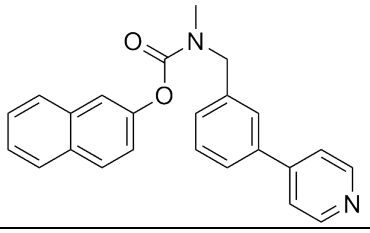
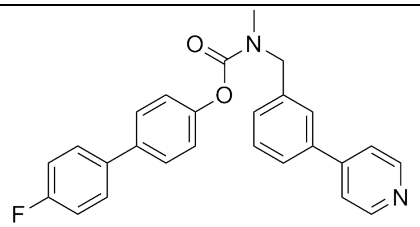
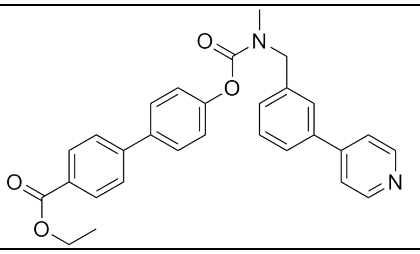
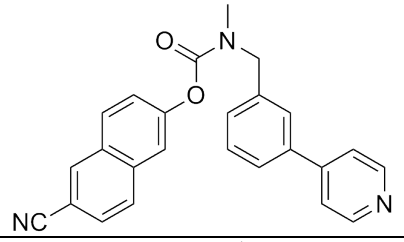
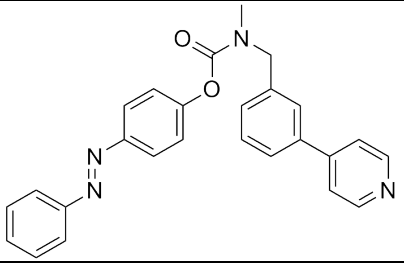
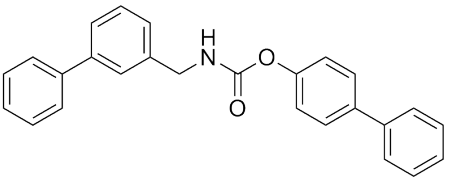
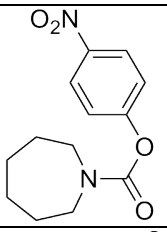
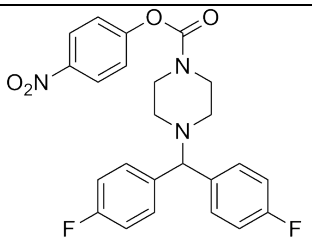
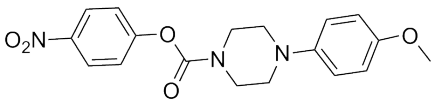
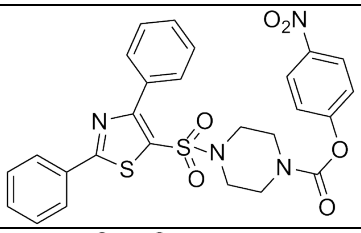
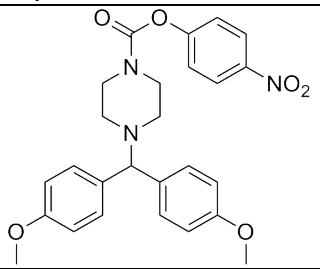
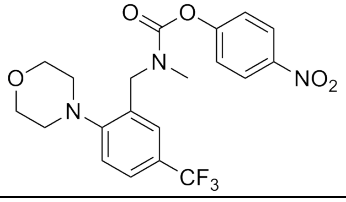
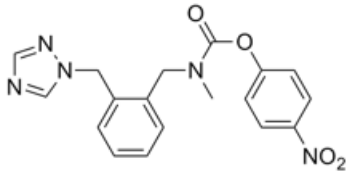
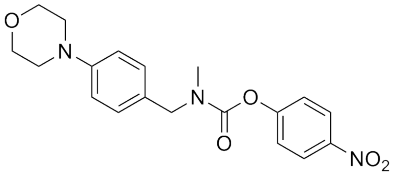
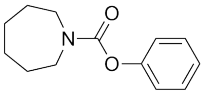
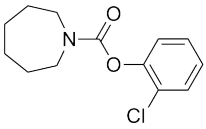
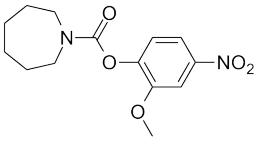
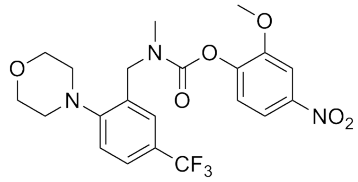
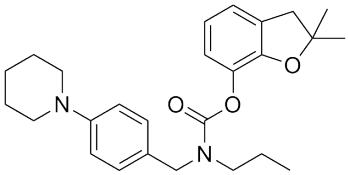
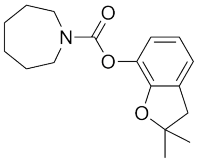
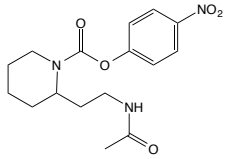
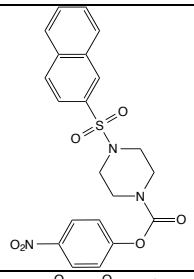
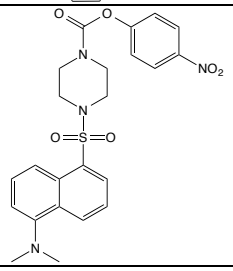


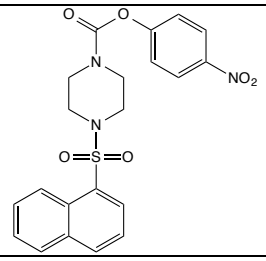
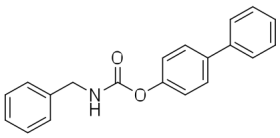
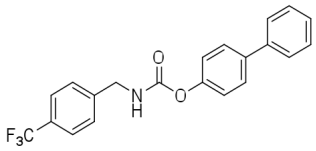
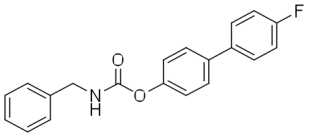
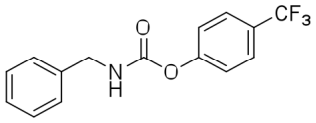
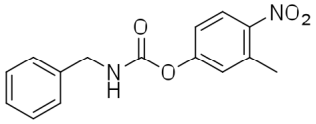
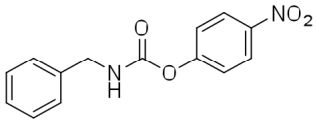
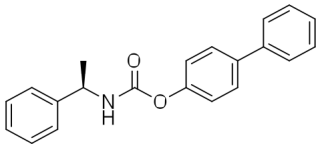
Figure 2-1. Chemotypes for serine hydrolase inhibitors. We screened a library of small molecules based on known serine hydrolase inhibitor scaffolds: carbamates, phosphonates, and triazole ureas. R groups represent diversification points on the small molecules.

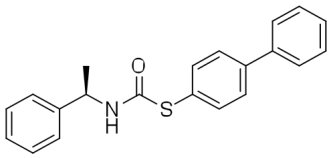
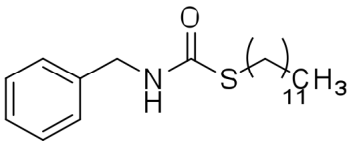
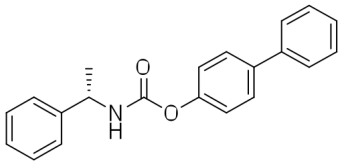
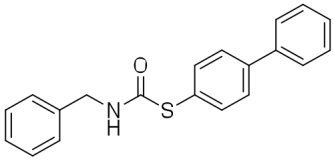
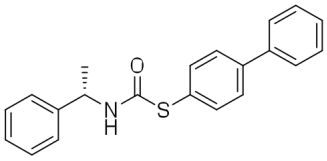
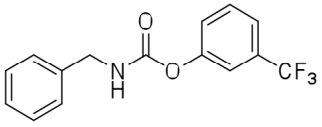
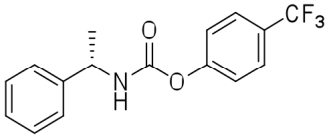
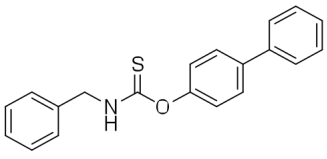
Compound	Structure
AS115	
JMN4	
WWL101	
WWL102	
WWL103	
WWL107	

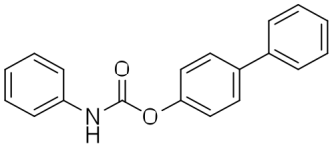
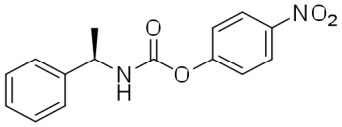
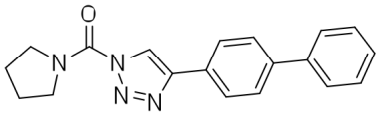
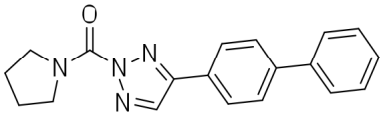
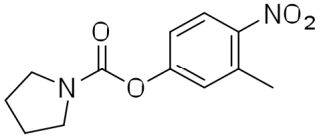
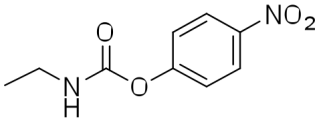
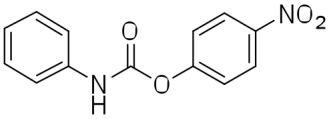
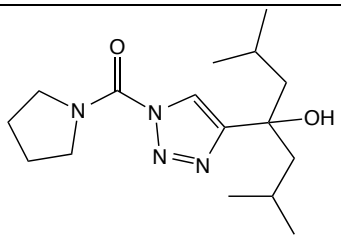
WWL108	
WWL113	
WWL115	
WWL116	
WWL121	
WWL151	
WWL152	

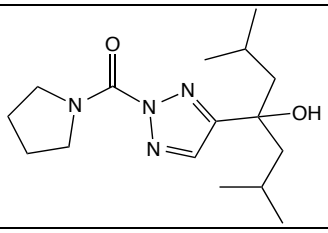
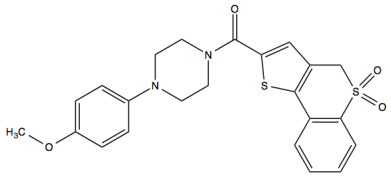
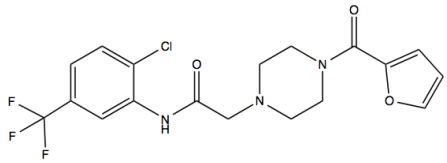
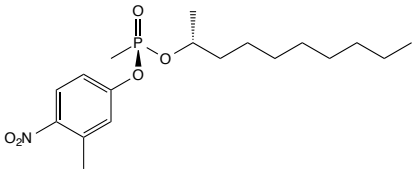
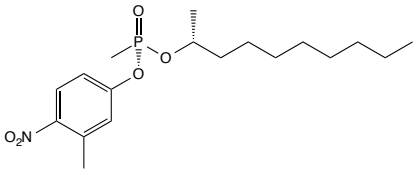
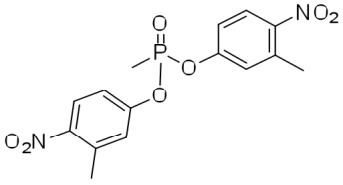
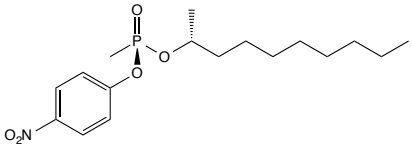
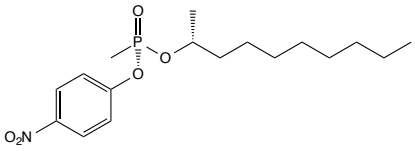
WWL154	
WWL156	
WWL162	
WWL201	
WWL203	
WWL208	
WWL209	
WWL210	

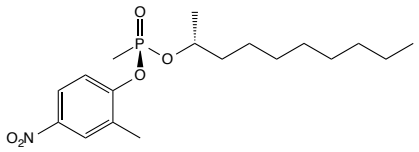
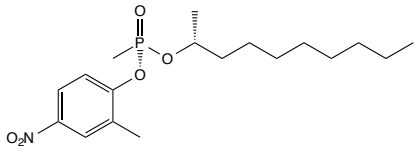
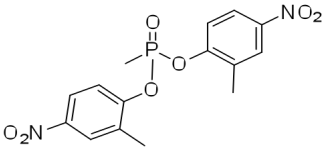
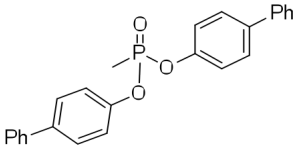
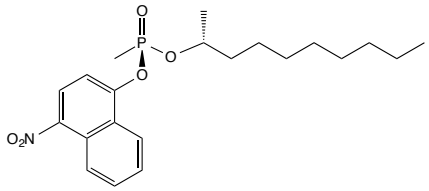
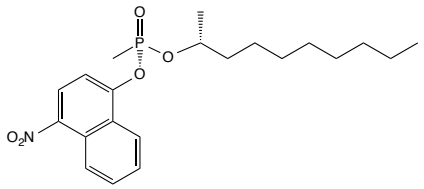
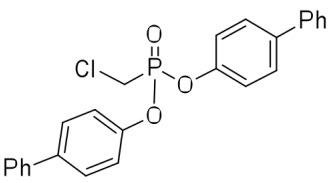
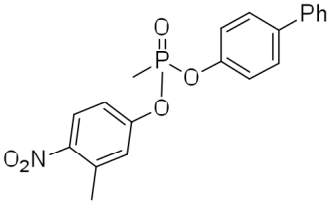
WWL211	
WWL212	
WWL213	
WWL214	
WWL228	
WWL240	
WWL241	

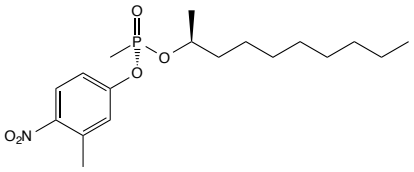
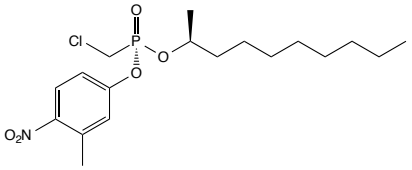
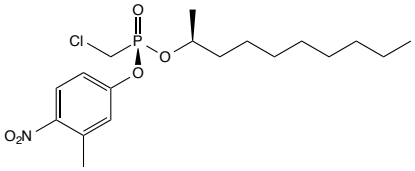
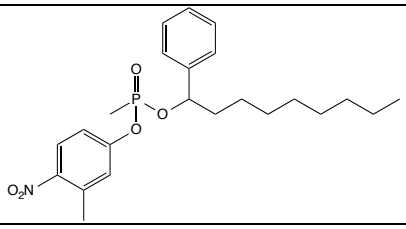
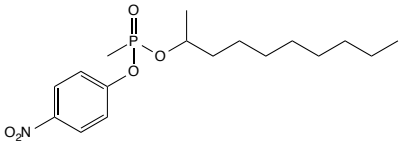
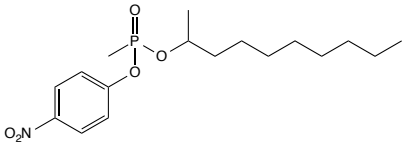
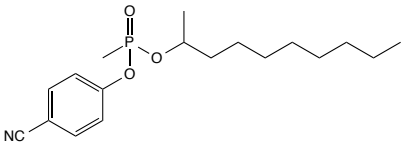
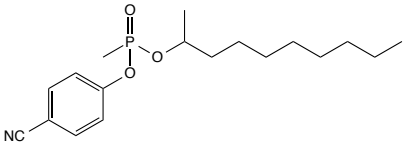
WWL242	
PJM 1-11	
PJM 1-31	
PJM 1-37	
PJM 1-49	
PJM 1-51	
PJM 1-53	
PJM 1-57	

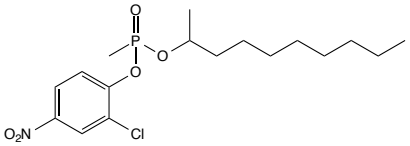
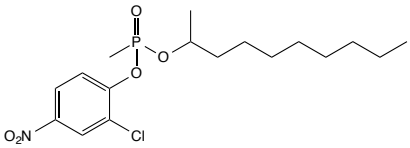
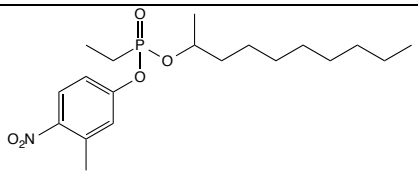
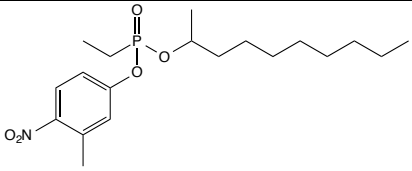
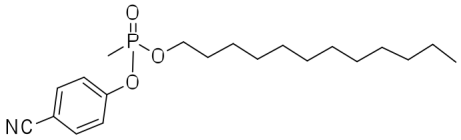
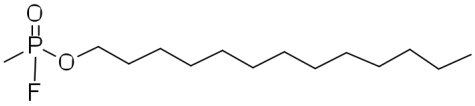
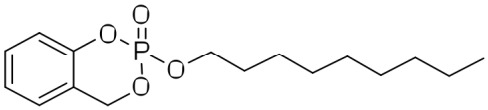
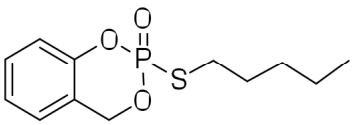
PJM 1-63	
PJM 1-65	
PJM 1-67	
PJM 1-69	
PJM 1-73	
YA 1-9	
YA 1-13	
YA 1-14	

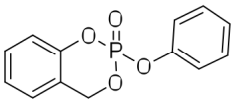
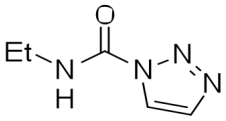
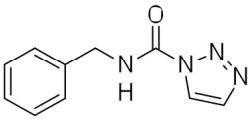
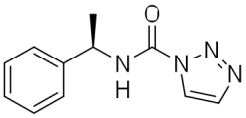
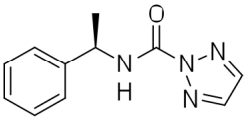
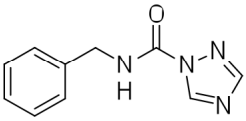
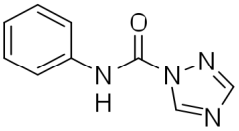
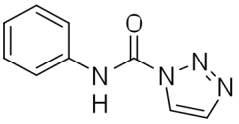
YA 1-15	
YA 1-17	
PJM 1-79-1	
PJM 1-79-2	
PJM 1-89	
YA 1-18	
YA 1-19	
PJM 1-101-1	

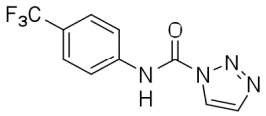
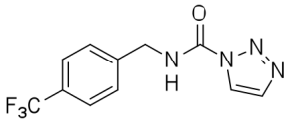
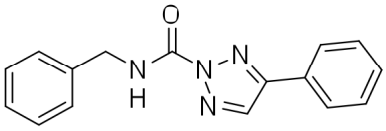
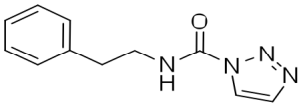
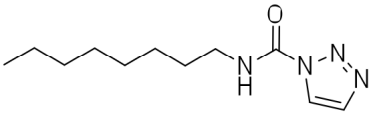
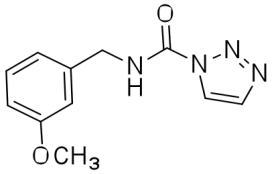
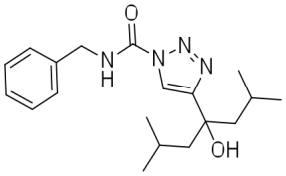
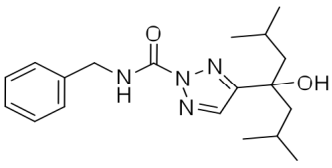
PJM 1-101-2	
Lypla2 inhibitor	
Lypla1 inhibitor	
PJM 1-95-1	
PJM 1-95-2	
PJM 1-95-3	
PJM 1-97-1	
PJM 1-97-2	

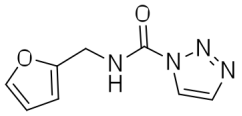
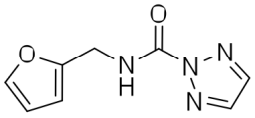
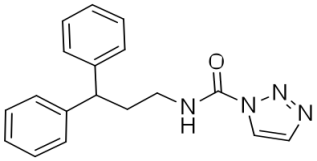
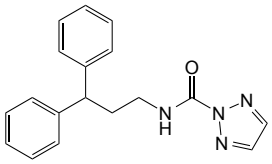
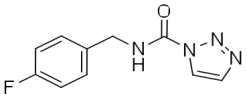
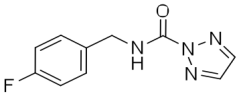
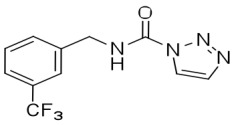
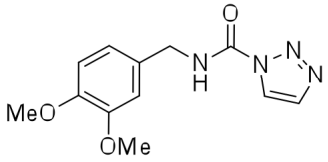
PJM 1-107-1	
PJM 1-107-2	
PJM 1-107-3	
PJM 1-109	
PJM 1-111-1	
PJM 1-111-2	
PJM 1-113	
PJM 1-115-2	

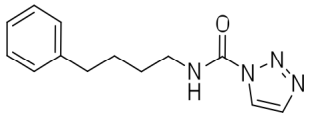
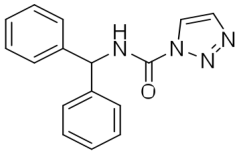
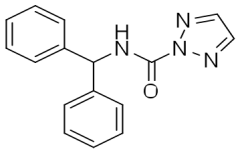
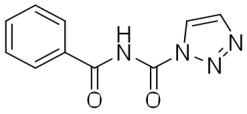
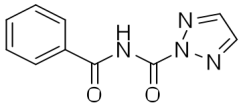
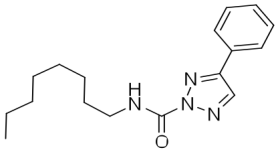
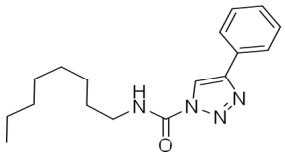
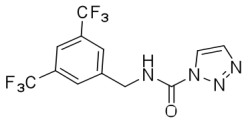
PJM 1-119-1	
PJM 2-5-1	
PJM 2-5-2	
PJM 2-7-1	
PJM 2-11-1	 Diastereomer 1
PJM 2-11-2	 Diastereomer 2
PJM 2-13-1	 Diastereomer 1
PJM 2-13-2	 Diastereomer 2

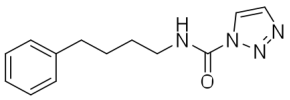
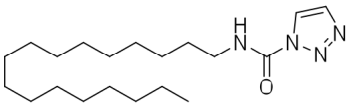
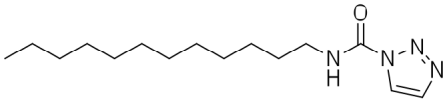
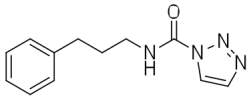
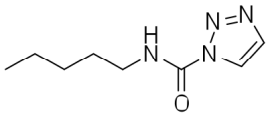
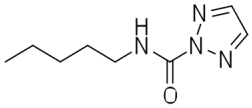
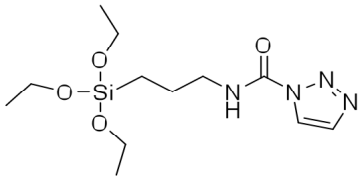
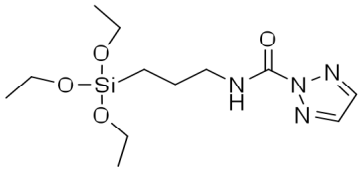
PJM 2-21-1	 Diastereomer 1
PJM 2-21-2	 Diastereomer 2
YA 1-32-1	 Diastereomer 1
YA 1-32-2	 Diastereomer 2
KJF 214	
KJF 1-142	
JC-1	
JC-2	

JC-3	
KJF 1-19-4C	
KJF 1-17C	
PJM 2-67-1	
PJM 2-67-2	
PJM 2-63	
PJM 2-65	
KJF 1-22C	

KJF 1-23C	
KJF 1-24-5C	
PJM 2-77-1	
KJF 1-27-6	
KJF 1-39	
KJF 1-41-7	
PJM 2-85-1	
PJM 2-85-2	

PJM 2-89-1	
PJM 2-89-2	
DKM 1-7-1	
DKM 1-7-2	
KJF 1-46-6	
KJF 1-46-10	
KJF 1-48-5	
KJF 1-49-7	

KJF 1-51-6	
DKM 1-9-1	
DKM 1-9-2	
DKM 1-11-4	
DKM 1-11-1	
DKM 1-17-2	
DKM 1-17-3	
KJF 1-55-7	

KJF 1-51-11	
KJF 1-59-4	
KJF 1-66rc	
KJF 1-65-9C	
DKM 1-19-1	
DKM 1-19-2	
DKM 1-21-1	
DKM 1-21-2	

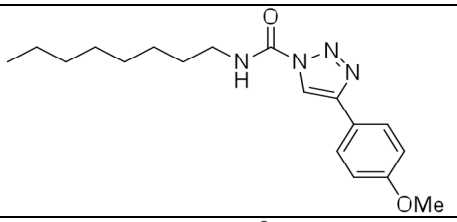
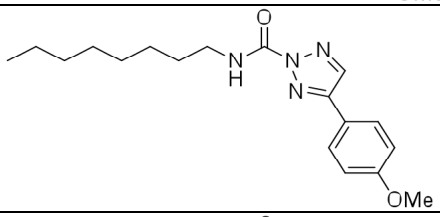
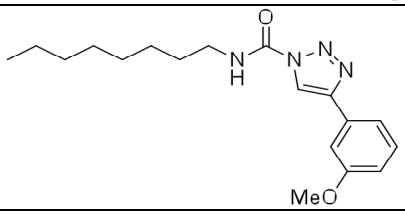
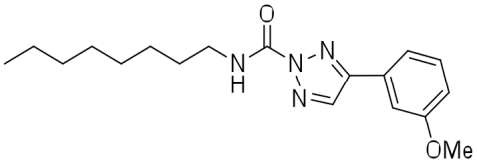
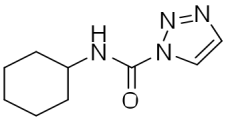
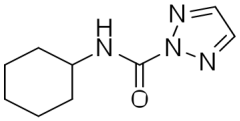
DKM 1-23-1	
DKM 1-23-2	
DKM 1-27-1	
DKM 1-27-2	
DKM 1-29-1	
DKM 1-29-2	

Table 2-1. Compound library included in chemical genetics screen.

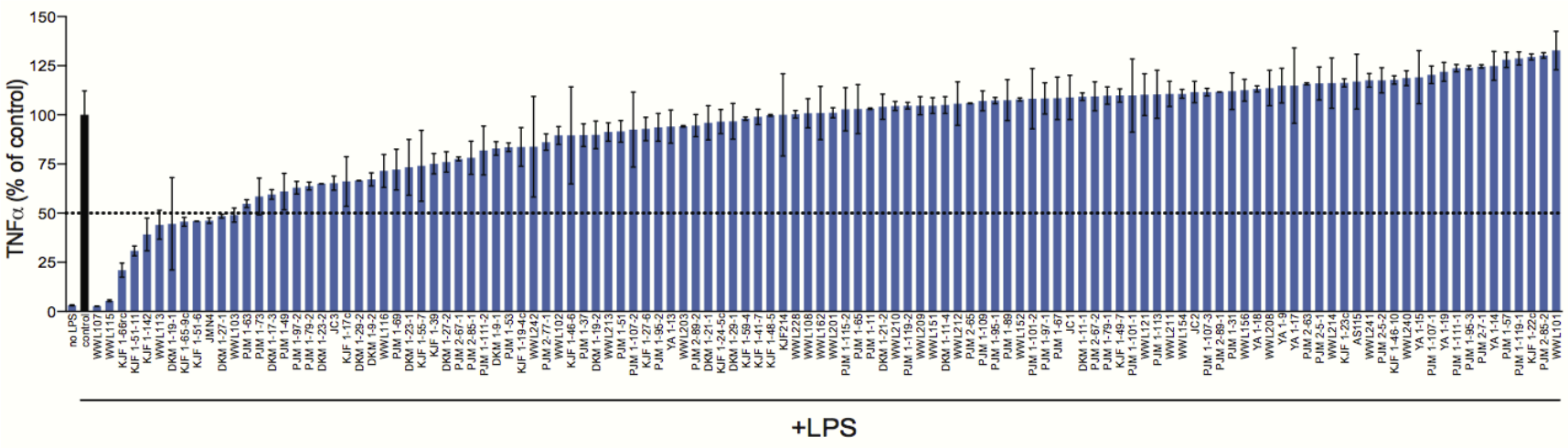


Figure 2-2. Chemical genetic screen for cytokine-lowering effects in THP1 cells. We screened 120 compounds for agents that lower LPS-induced TNFα secretion from THP1 monocytes. THP1 cells were pretreated with each inhibitor (5 μM) in serum-free RPMI for 1 h before stimulating with LPS (2 μg/mL) for 6 h. TNFα levels in media were then assayed by ELISA. Data are displayed as a percent of vehicle-treated, LPS-stimulated controls. Data represent n=2/group.

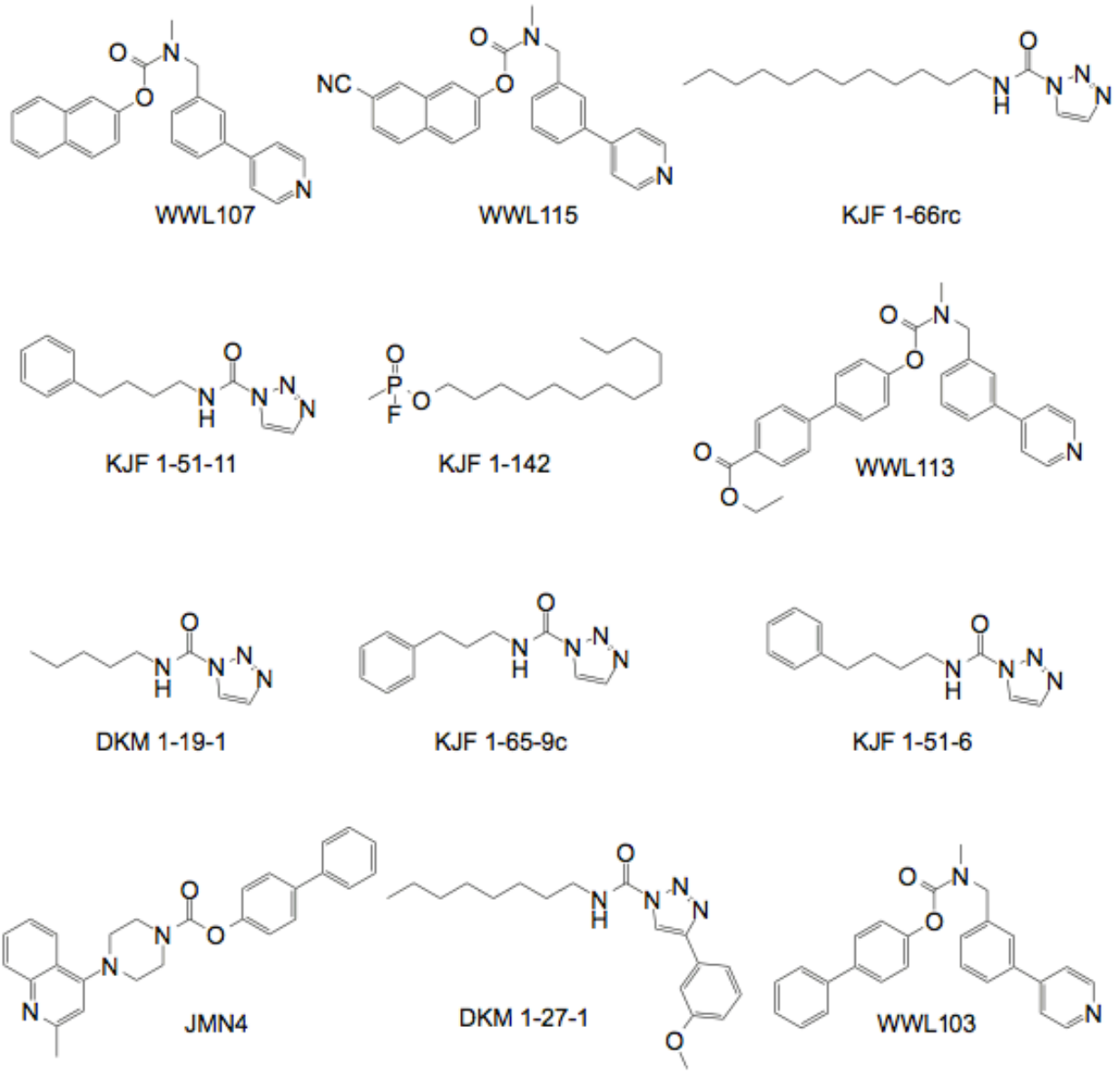


Figure 2-3. Hits from chemical genetics screen. Shown are the structures of the 12 small molecules that decreased LPS-stimulated TNFα secretion by greater than 50%.

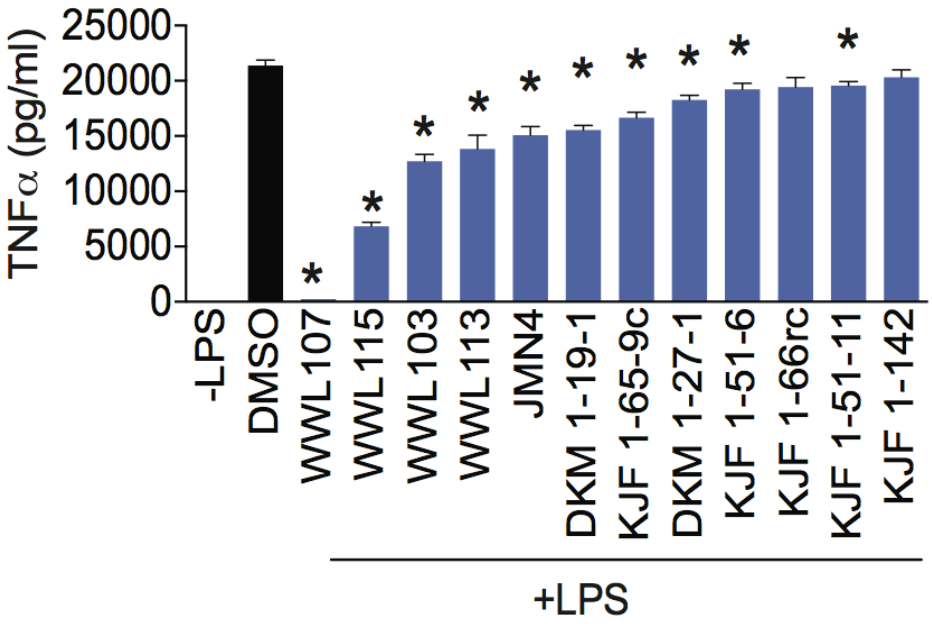


Figure 2-4. Counterscreen for cytokine-lowering effects in BMDMs. We next counterscreened the top 12 compounds to identify agents that also lowered TNFα in primary mouse bone marrow-derived macrophages (BMDMs). BMDMs were preincubated with inhibitor (5 μM) in serum-free DMEM for 1 h before stimulating with LPS (100 ng/mL) for 6 h. The conditioned medium was assayed for TNFα levels by ELISA. Data represent n=3/group. Significance is presented as *$p < 0.05$ comparing inhibitor treated groups to vehicle-treated, LPS-stimulated controls.

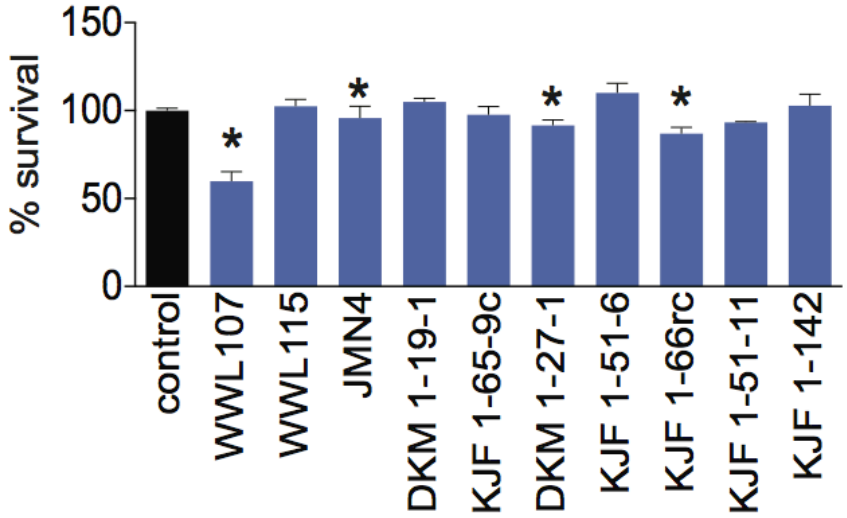


Figure 2-5. Counterscreen for cell death. We also counterscreened the lead compounds for cytotoxic agents by performing a cell survival assay using Hoescht staining. Data are presented as a percent of vehicle-treated cells. Data represent n=3/group. Significance is presented as *$p < 0.05$ comparing inhibitor treated groups to vehicle-treated controls.

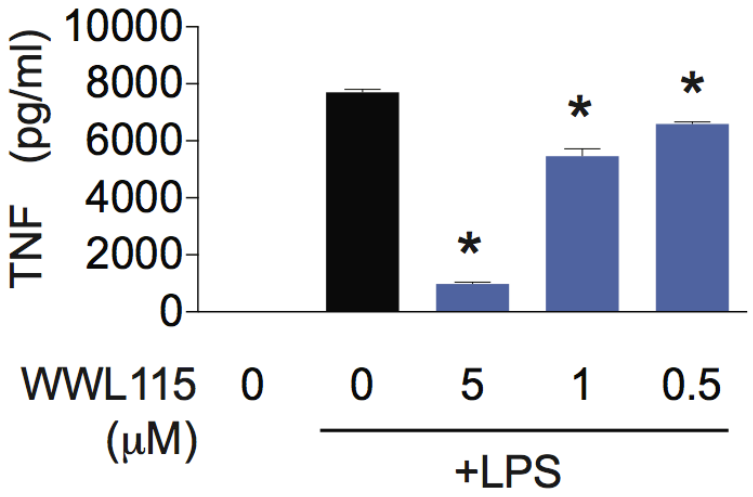


Figure 2-6. Lead compound WWL115. WWL115 is the only compound that significantly lowers LPS- stimulated TNFα by greater than 50% in BMDMs without causing cytotoxicity. We show dose-dependent reductions in LPS-induced TNFα secretion with WWL115 treatment in BMDMs. Data represent n=3/group. Significance is presented as *$p < 0.05$ comparing inhibitor treated groups to vehicle-treated, LPS-stimulated controls.

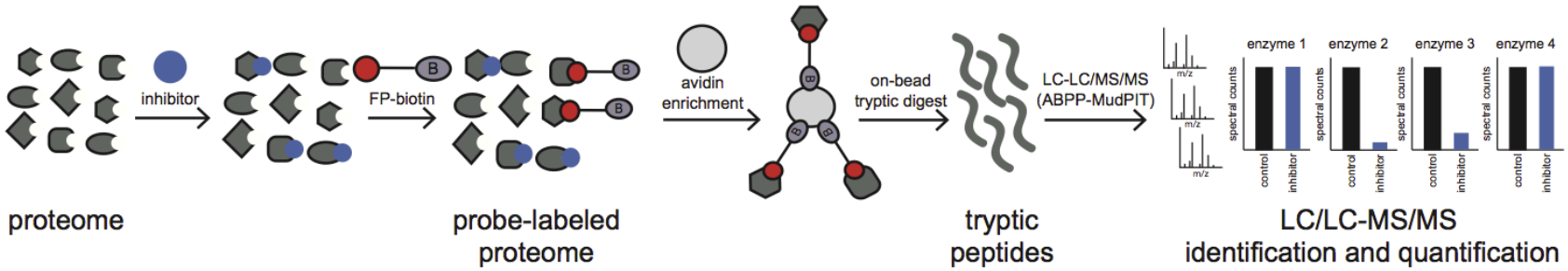


Figure 2-7. Competitive ABPP workflow for WWL115 target discovery. BMDMs were treated in situ with vehicle (DMSO) or WWL115 (5 μM), after which BMDM lysates were labeled with the serine hydrolase activity-based probe FP-biotin followed by avidin enrichment, trypsinization, and analysis of serine hydrolase activities by LC− LC−MS/MS (MudPIT) (ABPP-MudPIT).

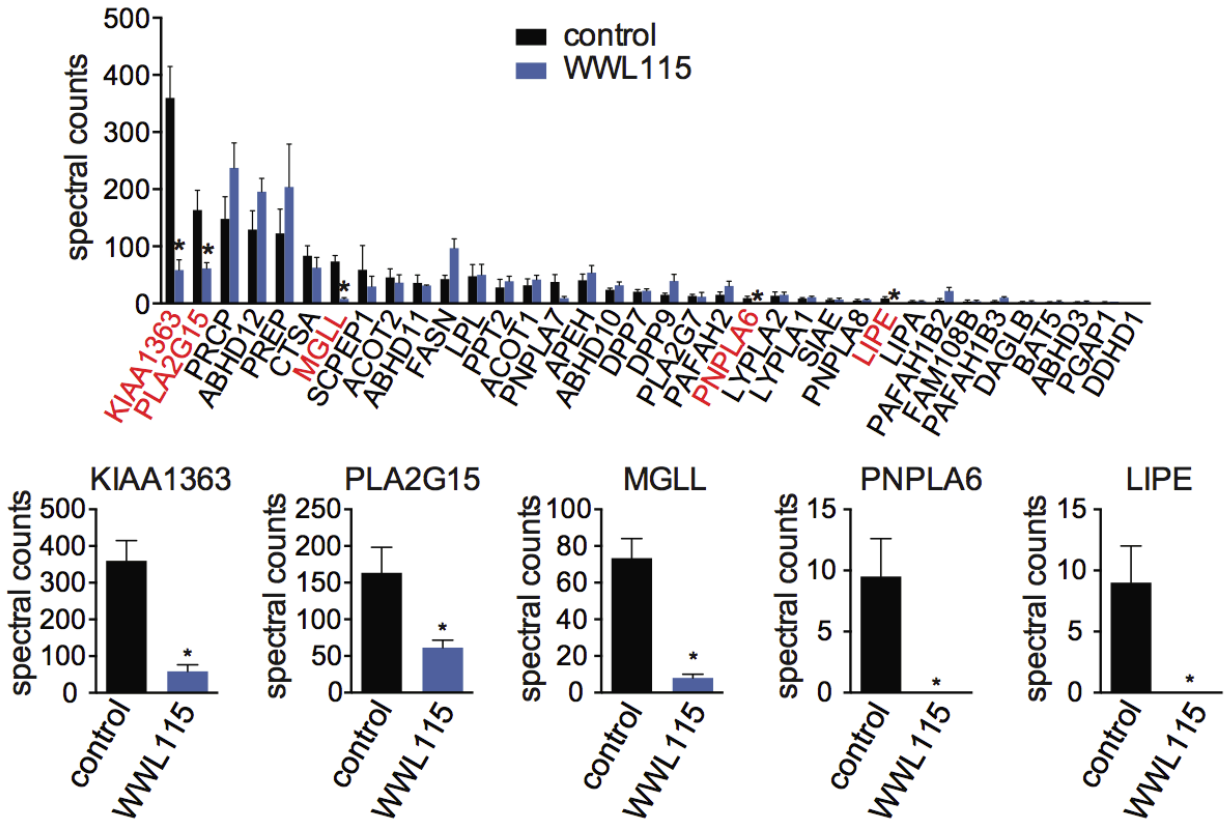


Figure 2-8. WWL115 inhibits 5 serine hydrolases in BMDMs. ABPP-MudPIT profiling of WWL115-treated BMDMs reveals five significantly inhibited serine hydrolases. Data are presented as mean ± SEM; n = 3−4/group. Significance is presented as *p < 0.05 between inhibitor and control-treated groups.

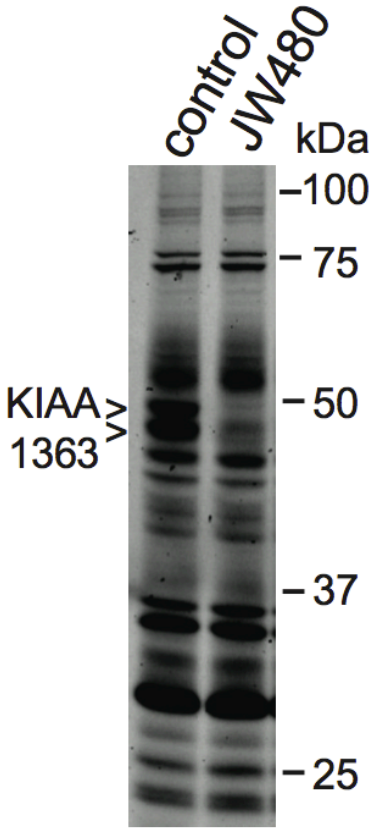


Figure 2-9. Gel-based ABPP analysis of JW480 in BMDMs. Fluorophosphonate-rhodamine labeling of lysate from JW480-treated BMDMs confirms target occupancy and selectivity of JW480. BMDMs were preincubated for 1 h with 5μM JW480. After stimulation with LPS (100 ng/mL) for 6 h, cells were harvested for ABPP analysis.

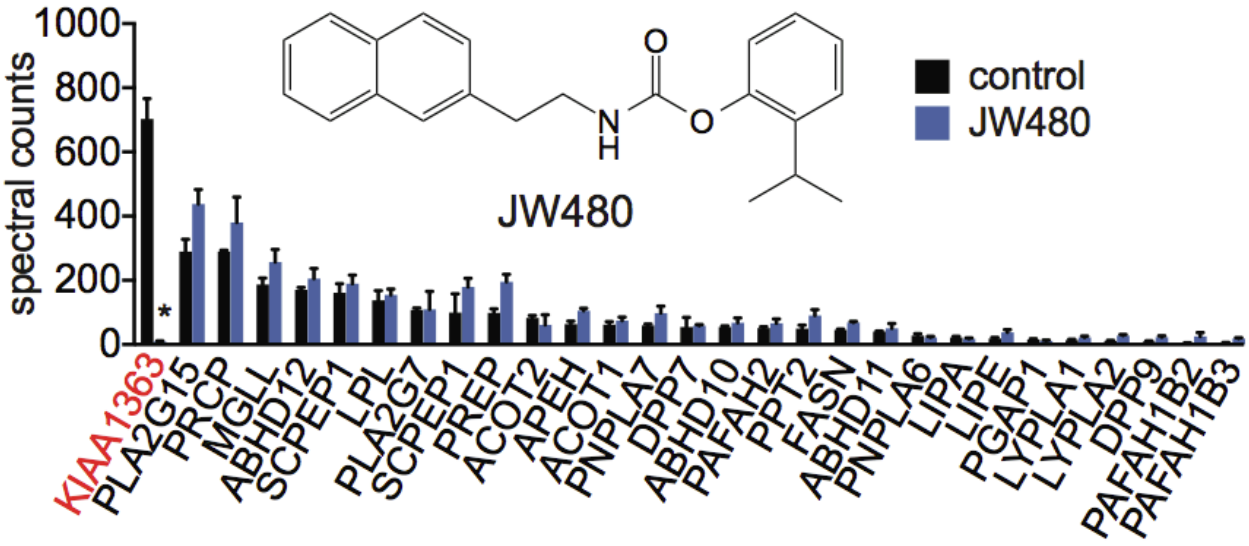


Figure 2-10. ABPP-MudPIT analysis of JW480 in BMDMs. Fluorophosphonate-biotin labeling of lysate from JW480-treated BMDMs confirms target occupancy and selectivity of JW480. BMDMs were preincubated for 1 h with 5μM JW480. After stimulation with LPS (100 ng/mL) for 6 h, cells were harvested for ABPP-MudPIT analysis. Data are presented as mean ± SEM; n = 3−4/group. Significance is presented as *$p < 0.05$, comparing inhibitor-treated groups to vehicle-treated, LPS-stimulated controls.

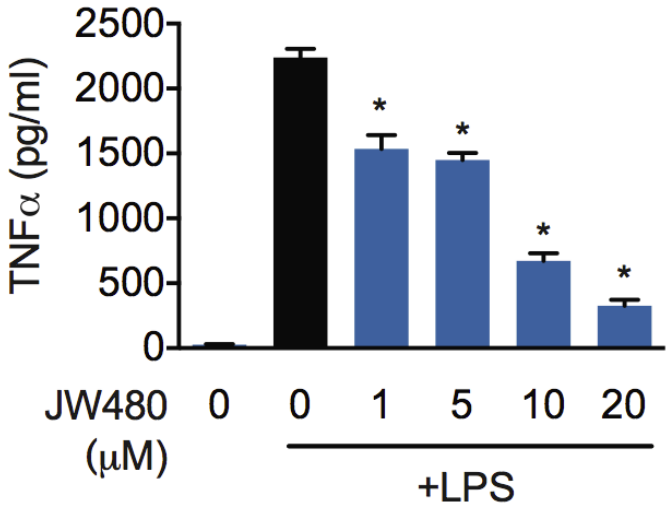


Figure 2-11. JW480 effects on LPS-stimulated TNFα secretion from BMDMs. JW480 lowers LPS-induced TNFα secretion in a dose-dependent manner. BMDMs were preincubated for 1 h with JW480 at the indicated concentrations. After stimulation with LPS (100 ng/mL) for 6 h, the conditioned medium was analyzed for secreted TNFα levels by ELISA. Data are presented as mean ± SEM; n = 3−4/group. Significance is presented as *p < 0.05, comparing inhibitor-treated groups to vehicle-treated, LPS-stimulated controls.

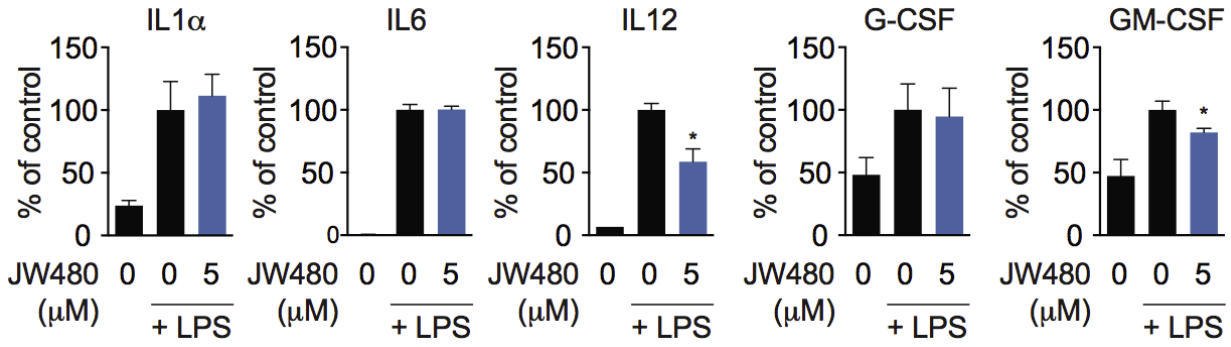


Figure 2-12. JW480 effects on other pro-inflammatory cytokines. KIAA1363 blockade induces selective decreases in LPS-stimulated pro-inflammatory cytokine release from BMDMs. BMDMs were preincubated for 1 h with 5μM JW480. After stimulation with LPS (100 ng/mL) for 6 h, the conditioned medium was analyzed for secreted cytokine levels by ELISA. Data are presented as mean ± SEM; n = 3/group. Significance is presented as *$p < 0.05$, comparing inhibitor-treated groups to vehicle-treated, LPS-stimulated controls.

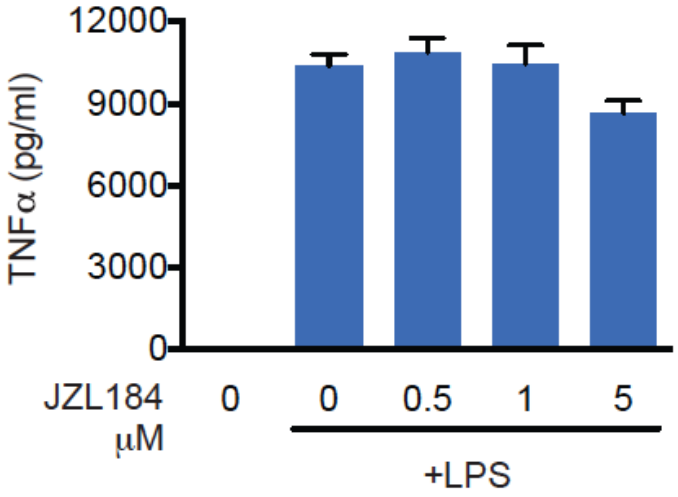


Figure 2-13. MGLL inhibition does not alter LPS-stimulated TNFα secretion. BMDMs were pretreated for 1 h with the selective MGLL inhibitor JZL184 at the indicated concentrations before stimulation with 100ng/mL LPS (6h) in serum-free medium.

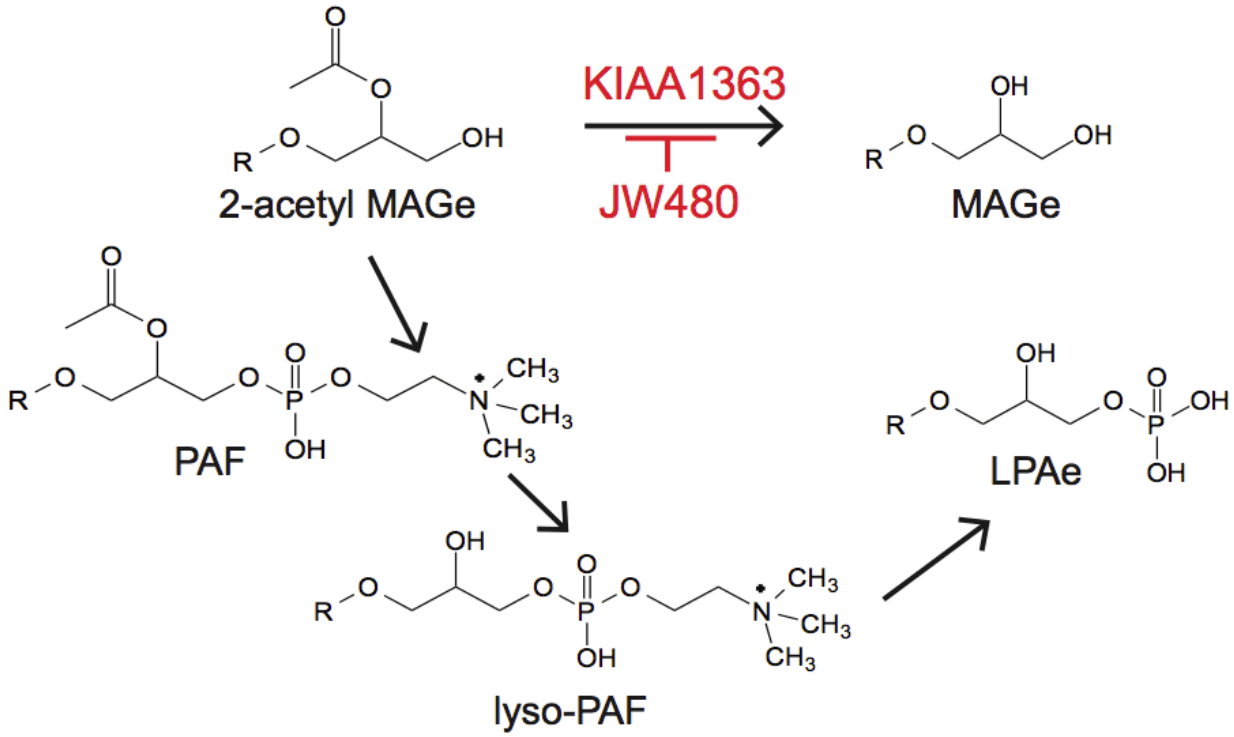


Figure 2-14. KIAA1363-ether lipid pathway. KIAA1363 is thought to control the formation of monoalkylglycerol ether (MAGe) from the hydrolysis of 2-acetyl MAGe, the penultimate precursor in the biosynthesis of platelet-activating factor (PAF).

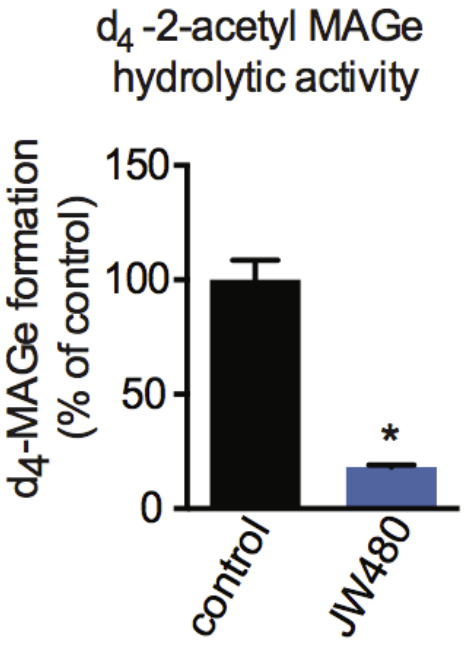


Figure 2-15. KIAA1363 activity assay. KIAA1363 activity, assessed by measuring d4-2-acetyl MAGe hydrolysis, is significantly inhibited in JW480-treated BMDMs (5 μM, 4 h). Data are presented as mean ± SEM; n = 5/group. Significance is presented as *p < 0.05, comparing inhibitor-treated groups to vehicle-treated controls.

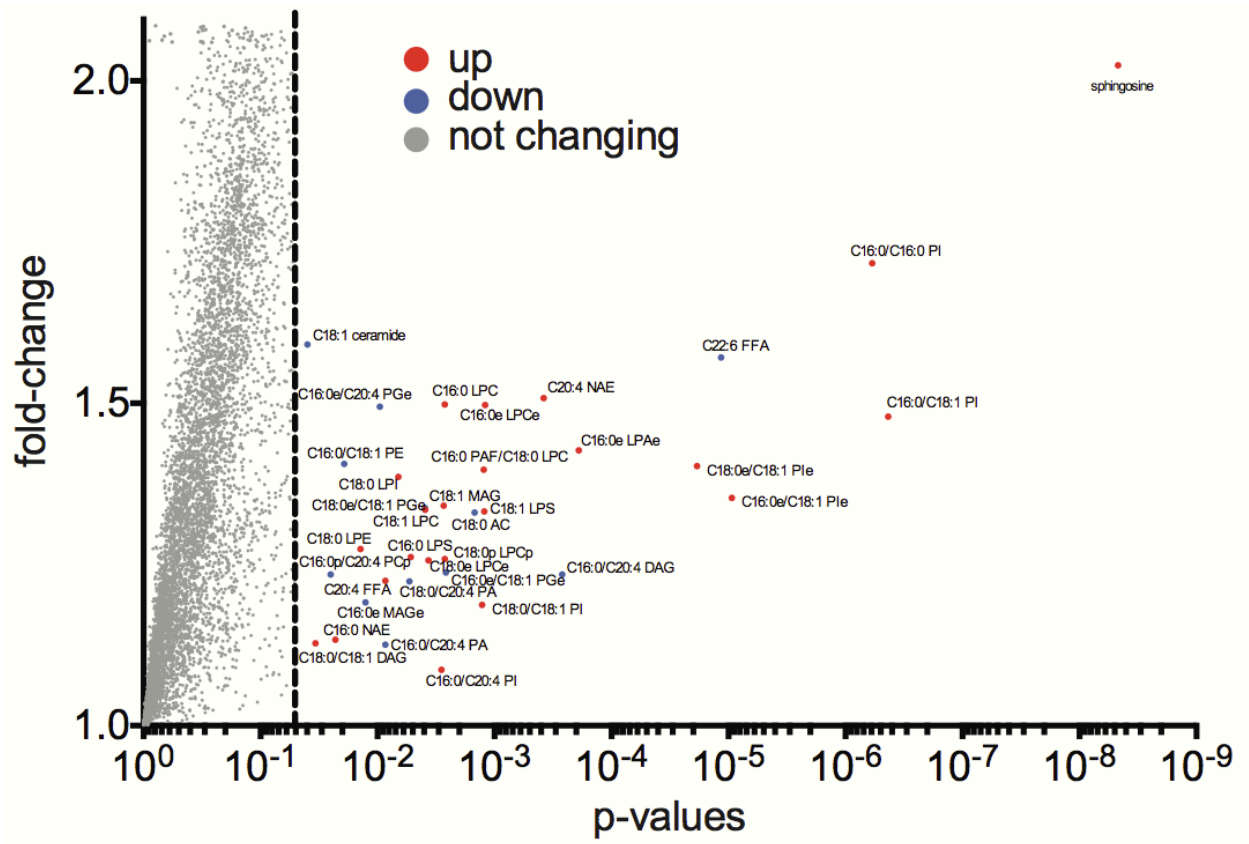


Figure 2-16. Metabolomic signature of KIAA1363 inhibition in BMDMs. Volcano plot shows targeted and untargeted metabolomic analysis of the nonpolar metabolome of JW480-treated BMDMs, revealing 35 lipid species whose levels were significantly altered out of all ions detected.

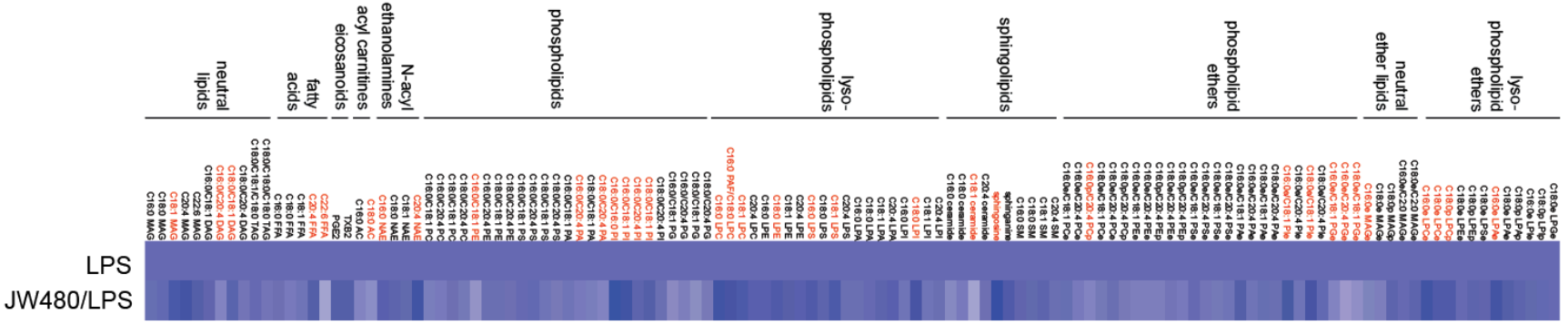


Figure 2-17. Lipidomic profiling of KIAA1363 inhibition in BMDMs. Targeted and untargeted metabolomic analysis of the nonpolar metabolome of JW480-treated BMDMs as shown as a heatmap including all lipidomic data sorted by general lipid class. Significantly changing lipids are highlighted in red.

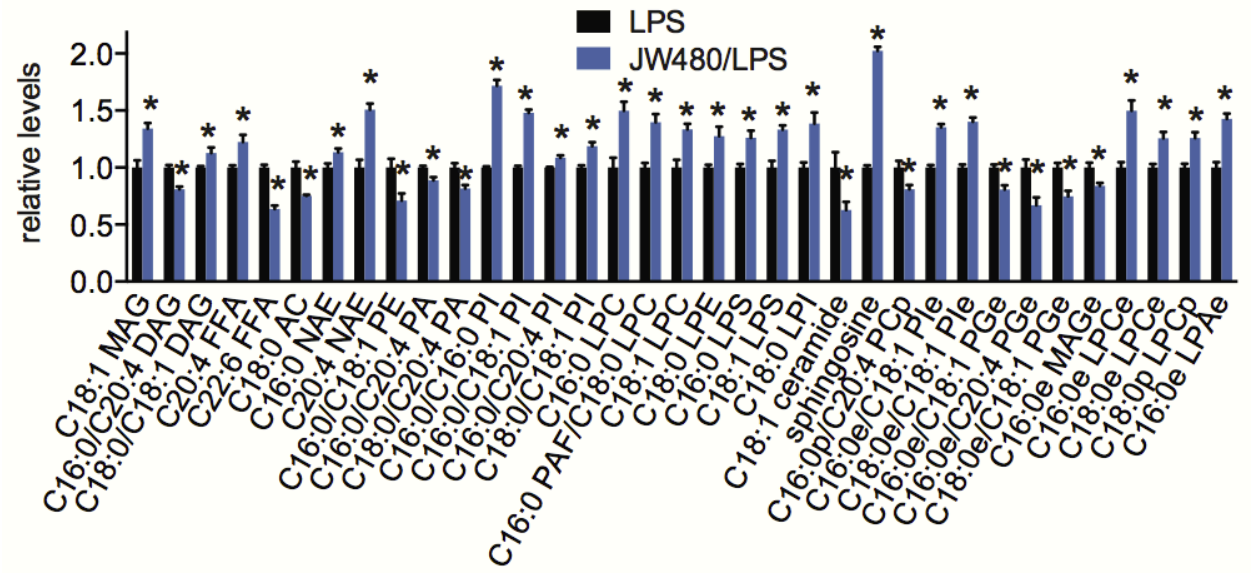


Figure 2-18. Relative levels of significantly altered lipids. JW480-mediated blockade of KIAA1363 changes levels of 35 lipid species. Data are presented as mean ± SEM; n = 5/group. Significance is presented as *p < 0.05, comparing inhibitor-treated groups to vehicle-treated, LPS-stimulated controls.

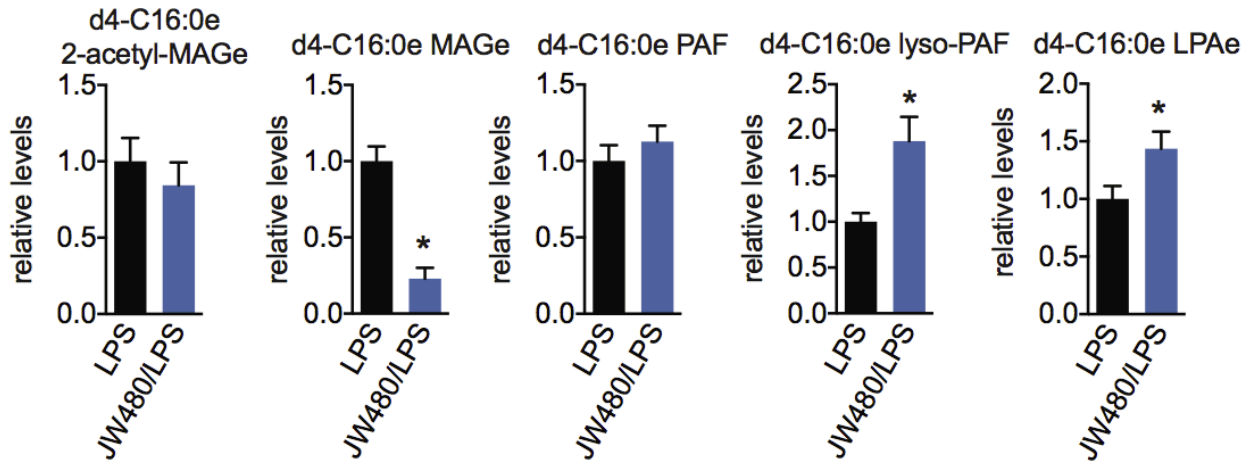


Figure 2-19. D4-2-acetyl MAGe labeling of BMDMs. d4-2-Acetyl MAGe isotopic incorporation into ether lipid metabolites in the KIAA1363 pathway. BMDMs were preincubated with JW480 (5 μM) or vehicle for 1 h before adding LPS (100 ng/mL) and d4-2- acetyl MAGe (10 μM) for 15 min. Isotopic incorporation into ether lipid metabolites was analyzed by LC−MS/MS. Data are presented as mean ± SEM; n = 5/group. Significance is presented as *$p < 0.05$, comparing inhibitor-treated groups to vehicle-treated, LPS-stimulated controls.

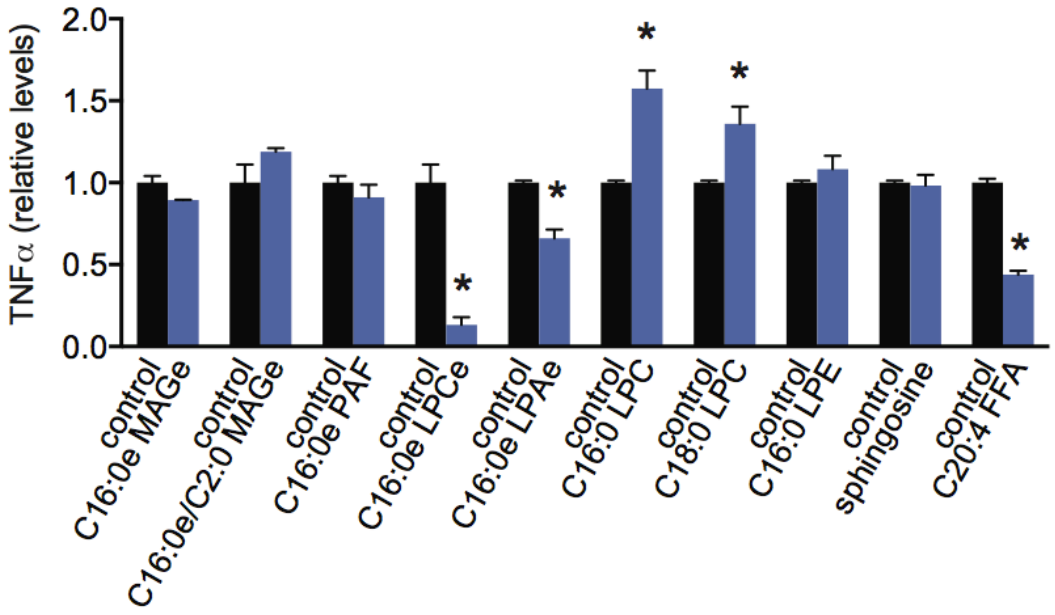


Figure 2-20. Screening KIAA1363-regulated lipids for cytokine-lowering effects. The effect of KIAA1363-regulated lipids on LPS-induced TNFα release from BMDMs. BMDMs were preincubated with each lipid (10 μM) or vehicle for 1 h before adding LPS (100 ng/mL). Conditioned medium was collected and assayed for TNFα by ELISA after 6 hr. Data are presented as mean ± SEM; n = 3−4/group. Significance is presented as *p < 0.05, comparing inhibitor-treated groups to vehicle-treated, LPS-stimulated controls.

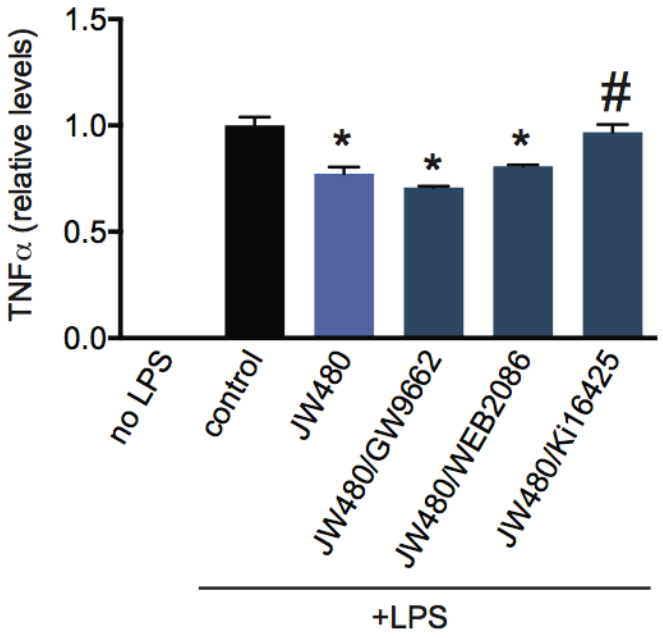


Figure 2-21. Rescue of cytokine-lowering effects with lipid receptor antagonists. Rescue of JW480-mediated TNFα-lowering effects by the LPA receptor antagonist Ki16425 (10 μM) but not by the PPARγ antagonist GW9662 (10 μM) or the PAF receptor antagonist WEB2086 (10 μM). Data are presented as mean ± SEM; n = 3−4/group. Significance is presented as *p < 0.05, comparing inhibitor-treated groups to vehicle-treated, LPS-stimulated controls, or #p < 0.05 compared to JW480-treated controls.

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