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Opportunities for Lipid-Based Probes in the Field of Immunology

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Contents

Abstract Lipids perform a wide range of functions inside the cell, ranging from structural building block of membranes and energy storage to cell signaling. The mode of action of many signaling lipids has remained elusive due to their low abundance, high lipophilicity, and inherent instability. Various chemical biology approaches, such as photoaffinity or activity-based protein profiling methods, have been employed to shed light on the biological role of lipids and the lipid–protein interaction profile. In this review, we will summarize the recent developments in the field of chemical probes to study lipid biology, especially in immunology, and indicate potential avenues for future research.

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1 Introduction

Lipids are defined as hydrophobic biomolecules that dissolve in organic solvents, but not in water. They perform a wide range of functions inside the cell, ranging from structural building block of membranes and energy storage to cell signaling. The discovery of the link between aspirin and prostaglandin synthesis showed that lipids can modulate the immune system and that the enzymes involved in their metabolism constitute interesting drug targets (Vane 1971). Since then, many associations have been made between the immune system and signaling lipids in the field of endocannabinoids, resolvins, steroid hormones, and vitamins A and D (Mora et al. 2008; Pandey et al. 2009; Marshall-Gradisnik et al. 2009; Serhan and Petasis 2011). However, due to the low abundance, high lipophilicity, and inherent instability of many lipid signaling molecules, their lipid–protein interaction profile and mode of action have remained largely elusive.

In recent years, several technical advances in mass spectrometry and innovative chemical biology strategies have been developed to shed light on these lipids and their protein interaction landscapes to study their biological function. Since its inception, the field of lipidomics, which is the analysis of lipids and their interacting partners within a biological system, has made great strides forward (Wenk 2005). Standardization of protocols, increased availability of deuterated lipids, and the high mass accuracy and resolution of modern mass spectrometers have made it possible to measure many lipids in complex biological samples (Wenk 2010). In an effort to systematically classify the rapidly expanding database of characterized lipids, the Lipid Metabolites and Pathways Strategy (LIPID MAPS) consortium has come up with a more concise definition of a lipid: a small hydrophobic or amphipathic molecule that is formed at least partially by the condensation of ketoacyl thioesters and/or isoprene units (Fahy et al. 2011). Based on these two building blocks eight major lipid classes are defined: glycerophospholipids, glycerolipids, fatty acyls, sphingolipids, sterol lipids, prenol lipids, saccharolipids, and polyketides (Fig. 1) (Fahy et al. 2009).

The variability in chemical characteristics between the various lipid groups makes it a challenge to measure all lipid species in one experiment. Different sample preparation methods and measuring techniques are required to quantify different classes of lipids (Yang and Han 2016). Adding to the challenge is the wide range of alterations that lipids can undergo to form still non-identified lipid derivatives. By combining the fields of chemistry and biology, some of these challenges can be overcome.

The field of chemical biology has developed two main approaches to study lipids: a) novel chemically modified lipids to study their biological role and b) chemical tools to visualize and modulate the proteins involved in lipid metabolism. To track lipids in a biological system, they can be functionalized with alkyne tags (Gaebler et al. 2013; Robichaud et al. 2016). Using ligation chemistry, these functionalized lipids can be visualized and identified (Hein and Fokin 2010). This method has been used for the visualization of lipids in membranes, modification of

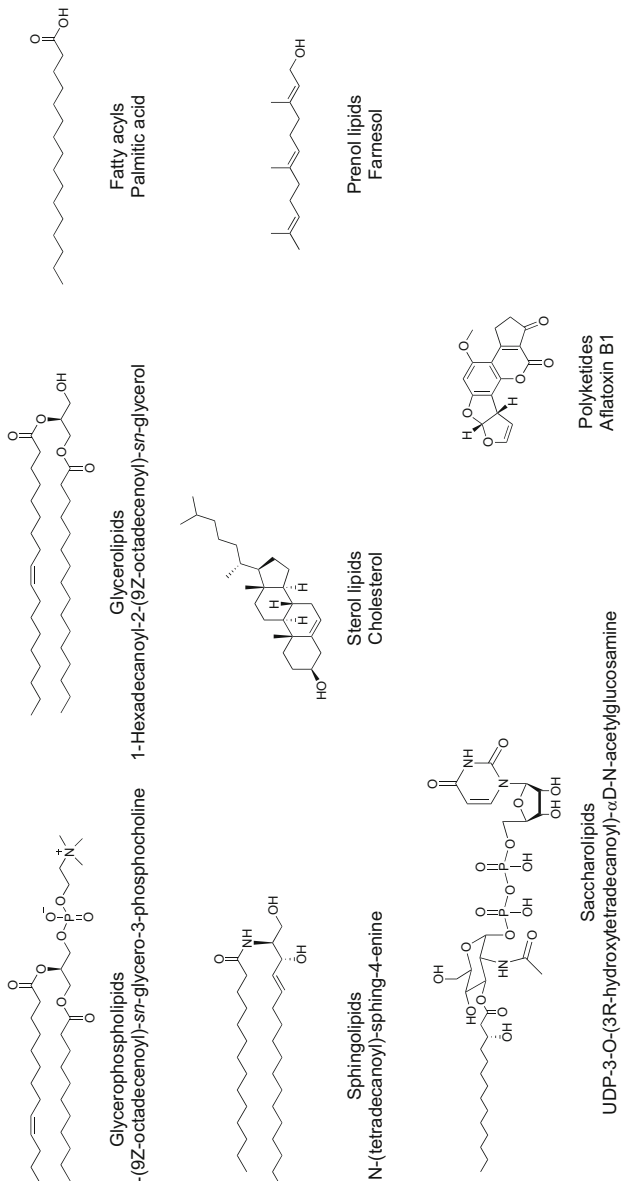

Fig. 1 Representative structures of the eight lipid categories according to LIPID MAPS (Fahy et al. 2009, 2011)

proteins by lipids, and their metabolism (Hofmann et al. 2014; Tate et al. 2015; Gaebler et al. 2016). Although the alkyne functionalization enables affinity purification, this will only reveal protein interaction partners that are covalently bound to the lipid.

To visualize lipid-interacting partners, such as binding proteins or metabolizing proteins, photoaffinity or activity-based labeling can be used. These methods require bifunctionalized lipids and probes. These molecules contain a photoreactive group or an electrophilic warhead and an alkyne or azide, which are employed in affinity-based protein profiling (A*f*BPP) and activity-based protein profiling (ABPP) (Cravatt et al. 2008). Both methods enabled the visualization and identification of the protein–lipid interactions (Wright and Sieber 2016).

In this review, we will summarize the recent developments concerning lipid-based probes. The chemical tools are divided into two classes: affinity- and activity-based probes. The affinity-based probes are discussed using the LIPID MAPS classification system, while the activity-based probes are grouped based on their enzyme class. Finally, we present some opportunities for future research.

2 Lipid Photoaffinity Probes

Classical techniques to study protein–lipid interactions include microscale thermophoresis, isothermal titration calorimetry, and surface plasmon resonance. Recently, new techniques have emerged to study these types of interactions in a native environment, such as live-cell imaging with fluorescent proteins or affinity purification lipidomics. Most of these methods, however, rely on a predetermined protein-of-interest and its modification. Compared to these techniques, photoaffinity lipids offer new possibilities regarding throughput and biocompatibility.

Photoactivatable lipids are synthetic derivatives that retain the overall structure and interactions of the parent lipid, but contain a photoactivatable moiety that may form a covalent bond with its interacting partner. This covalent bond essentially 'freezes' the interaction and allows for affinity purification. Of interest, this interaction can be captured in complex native environments (Haberkant et al. 2013). A number of photoreactive moieties are available, with differing synthetic accessibility, reactivity, efficiency, and structural impact on a probe. Three photoreactive groups are routinely used: diazirines, benzophenones, and aryl azides (Fig. 2). Their properties and reactivity have been previously discussed in excellent reviews (Bush et al. 2013; Sakurai et al. 2014; Kleiner et al. 2017).

The choice of photoactivatable group depends on its application. However, a shift from benzophenones to diazirines has been observed in the last decade. Increased synthetic efforts, the desire for a smaller modification, and the application in a relative water-free inner membrane have contributed to this trend. In this chapter, lipid photoaffinity probes for fatty acyls, glycero(phospho)lipids, sphingolipids, and sterols will be discussed.

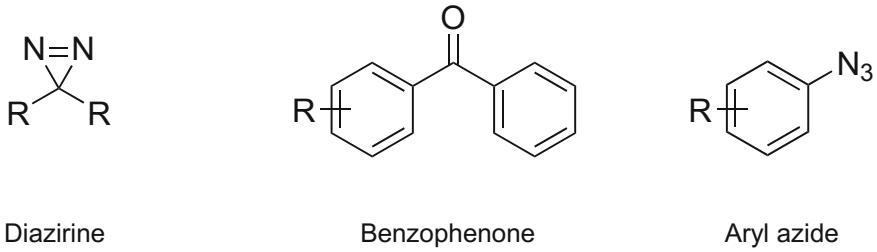

Fig. 2 Structures of three commonly used photoreactive groups

2.1 Glycero(Phospho)Lipids

One of the most well-studied lipid classes is the glycero(phospho)lipids. Their amphipathic nature is essential for the formation of lipid bilayers, and they are universally present in cellular membranes. Glycerolipids are composed of a glycerol backbone that has been mono-, di-, or trisubstituted with a fatty acyl. In most glycerophospholipids, the *sn*-3-position is esterified with a phosphate group, which in turn can be substituted with different head groups. The most common substitutions are a choline, ethanolamine, glycerol, serine, or inositol, giving rise to phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylserine (PS), and phosphatidylinositol (PI), respectively. The length and degree of unsaturation of the fatty acyl groups determine the physical properties of the lipid, and its substitution pattern on the phosphate group dictates its protein interactions partners on the membrane surface (Yeagle 2016). Different types of lipids and their compartmentalization between cellular structures do not only determine the physical properties of the biomembrane, but also regulate protein activity, localization, or signaling events via lipid–protein interactions. Such interactions can be discovered via application of photoaffinity lipid probes.

Phosphatidylserine (PS) lipids are primarily found on intracellular membrane surfaces. When this asymmetry is disturbed during apoptosis, PS presentation is a trigger for phagocytosis. Several PS analogues **1**–**5** containing a benzophenone and alkyne click-handle were synthesized and shown to label PS-interacting proteins in PS-lipid-mediated engulfment, such as prothrombin-1 (Fig. 3) (Bandyopadhyay and Bong 2011).

To study membrane-associated proteins that are under-represented by traditional proteomics, lipid probes based on phosphatidylcholine were made (Gubbens et al. 2009). The goal was to detect proteins interacting with the phospholipid head groups in inner mitochondrial membranes. To this end, the photoreactive group was installed on the solvent-exposed head group of the lipid. The aryl azide probe **6** and benzophenone probe **7** were used to isolate proteins from *Saccharomyces cerevisiae* mitochondria (Fig. 3). The identified targets consisted of known membrane interacting proteins, like Gut2p and Cox2p, but also new proteins. Of note, the authors

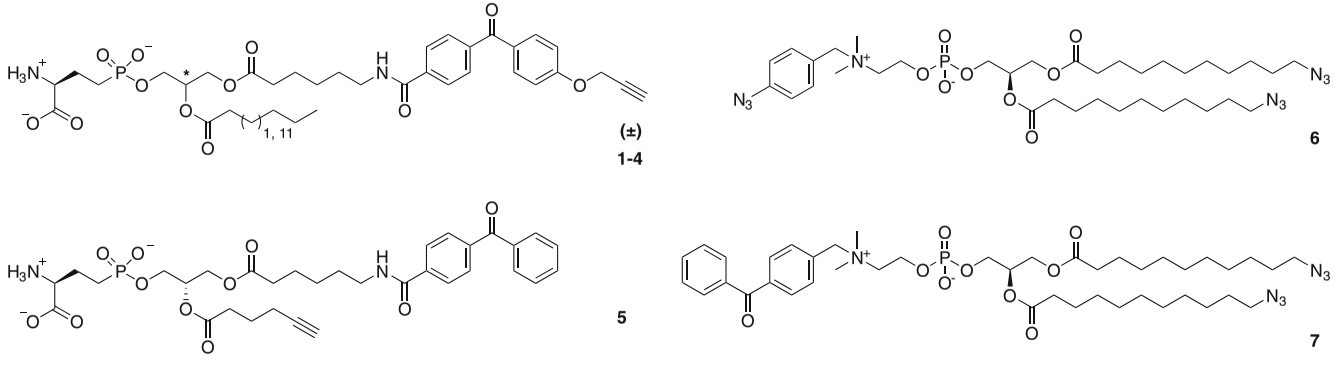

Fig. 3 Structures of photoaffinity probes based on phosphatidylserine **1**–**5** and phosphatidylcholine **6**–**7**

concluded that aryl azide **7** is the preferred probe due to high background labeling and labeling of highly abundant proteins with benzophenone **6** (Gubbens et al. 2009).

Phosphatidylinositol (PI) polyphosphates are a family of signaling lipids that act as ligands for protein-membrane association. To discover the proteins that associate with these lipids, two chemical probes based on the head group of phosphatidylinositol 3,4,5-triphosphate have been synthesized (Rowland et al. 2011). Probes **8** and **9** were tested on the purified pleckstrin homology domain of protein kinase B, a known phosphatidylinositol phosphate binder (Fig. 4). The shorter **8** gave better signal, which can be attributed to its shorter linker length which positioned the benzophenone closer to the protein. Probe **8** was applied to MDA-MB-435 cell extracts, and 265 proteins were identified as phosphatidylinositol 3,4,5-triphosphate-binding proteins (Rowland et al. 2011).

To study the membrane-binding domain of PON1, a high-density lipoprotein (HDL)-associated protein, phospholipid probe **10** was made with the photoactivatable group on the head of the lipid (Fig. 4). After covalent photocrosslinking, digestion and analysis by MS several residues localized closely together were found on the surface of PON1, indicating the HDL-binding domain (Gu et al. 2016).

To study the ability of cardiolipin to form a complex with the mitochondrial protein cytochrome *c*, several photoaffinity probes **11**–**13** were synthesized (Fig. 4). These probes induced similar or higher cytochrome *c* peroxidase activity compared to endogenous tetraoleoyl cardiolipin, indicating that the central hydroxyl group is not necessary for activation. No follow-up study applying the labeling functionality has appeared (Abe et al. 2015).

Several photoactivatable analogs of phosphatidylcholines are commercially available. However, it is argued that lipids supplied to a living cell are differently distributed than lipids synthesized within the cell, which distorts the interaction profile reported by the probe (Peng et al. 2014). Moreover, palmitoleic and myristoleic acid probes that contain both a diazirine and an alkyne can be incorporated in various glycerolipids and used as post-translational modifications (PTMs), thereby further complicating the interpretation of the results of a photoaffinity experiment. The solution was to incubate cells with an alkyne-tagged choline **14** and diazirine-containing fatty acid **15** (Fig. 5). In this manner, the photoaffinity experiment only captured the proteins that interacted with the in situ synthesized phosphatidylcholine. Different combinations of alkyne- and diazirine-containing components of PC were tested, but the combination of **14** and **15** gave the best results (Wang et al. 2017). This strategy yielded a PC-protein interaction map in living cells. Two hundred and eleven high-confidence PC-interacting proteins, mostly present in the cytosol, ER, mitochondria, and nucleus were identified. The authors concluded that the double incorporation strategy offered significant improvements for global mapping of genuine protein–lipid interactions and indicated that the strategy is expandable to different lipid classes, such as phosphatidylinositols (Wang et al. 2017).

Dilinolenoyl monogalactosyldiacylglycerol (MGDG) is an anti-inflammatory signaling lipid (Ulivi et al. 2011). Three photoactivatable probes **16**–**18** were synthesized to elucidate its mode of action (Fig. 5). The linolenoyl groups were

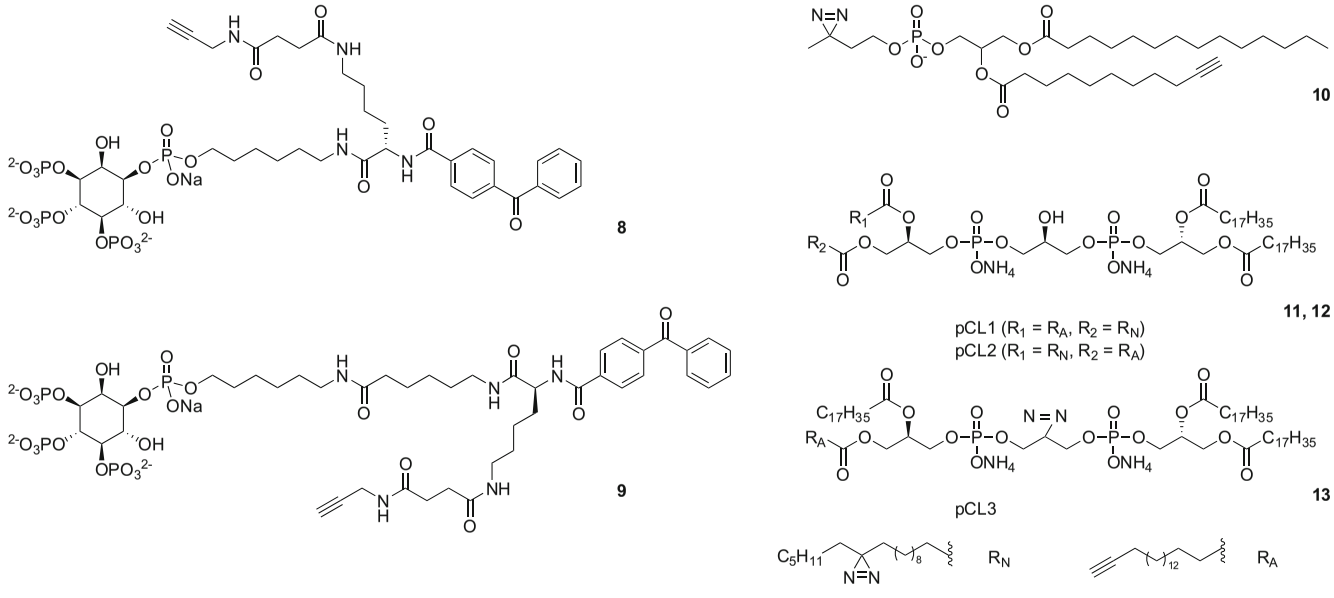

Fig. 4 Structures of photoaffinity probes based on phosphatidylinositol 3,4,5-triphosphate **8–9**, phospholipid **10** and cardiolipin **11–13**

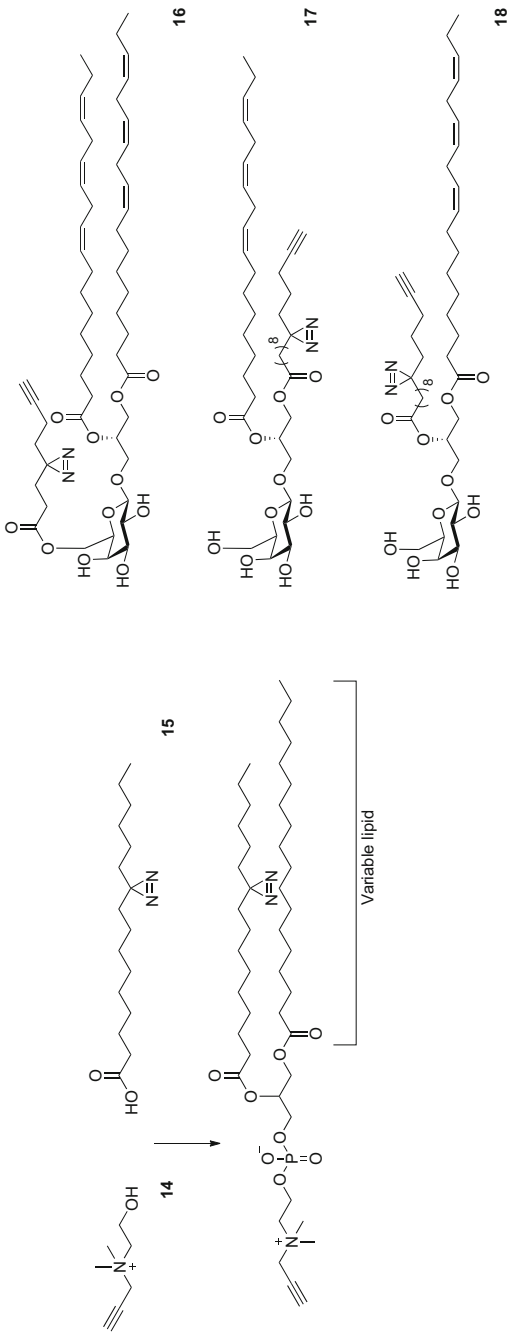

Fig. 5 Structures of photoaffinity probes based on phosphatidylcholine **14+15** and dilinolenoyl MGDG **16–18**

replaced with a similar photoaffinity-click fatty acid (pacFA), or a minimalist linker was tagged to the sugar moiety (Li et al. 2013). **16** Did not show any activity in an inflammatory assay, but **17** and **18** had comparable activity to MGDG. This indicated that an unmodified galactosyl moiety is essential for MGDG activity. Moreover, when incubated, UV-exposed and washed, **17** lost all anti-inflammatory activity, whereas **18** was still active. This implied that **18** was capable of both binding and labeling an unknown target. With structure **16** as negative control, TLR4 was identified as a probe target of **18** in human chondrocytes. Subsequent orthogonal experiments demonstrated MGDG acted as TLR-4 antagonist (Liu et al. 2016).

Recently, caged photoaffinity probes including **19–21** were developed to study biologically active signaling lipids with temporal, spatial, and subcellular resolution (Figs. 6, 7 and 8). These caged probes contain a fluorescent coumarin group, which is released upon irradiation with a certain wavelength light that does not activate the diazirine moiety. Microscopy experiments indicated that all lipids were indiscriminately localized to internal membranes and the cytoplasm. Uncaging, photocrosslinking, and ligation with a different fluorophore showed that each lipid localized to distinct cellular components. Controlled release of DAG via uncaging was also shown to be feasible. Elevated diacylglycerol (DAG) levels are known to trigger translocation of C1-domain-containing proteins to the plasma membrane (Nadler et al. 2013). Immediate translocation of C1-linked green fluorescent protein (GFP) was observed when **19** was uncaged in HeLa cells (Höglinger et al. 2017). DAG turnover was subsequently quantified on a population-wide and single-cell level. The authors suggested that standard biochemical experiments to measure DAG metabolism were inherently flawed, since they only measure combined lipid transport and metabolism. Moreover, striking differences were found between DAG turnover on a cell-to-cell level, indicating that heterogeneity might be an underrated complication of lipid signaling (Höglinger et al. 2017). In addition, a pull-down experiment with probe **19–21** was performed with HeLa cell proteome affording 130 **19-**specific binding proteins. As the probe contains arachidonic acid, the hits were compared to the targets of arachidonic acid-based probes **22** and **23** (Fig. 7). Remarkably, only 17 proteins of the 130 hits were overlapping, showing that probe **19** has mostly DAG-specific interactions. Thus, the two activatable functionalities allowed to investigate different aspects of the lipids with the same probe (Höglinger et al. 2017).

2.2 Fatty Acids

Fatty acids are constituents of other lipid classes, such as glycerol(phospho)lipid, glycolipids, and ceramides, but also have their own signaling roles. In addition, they can be incorporated by proteins as a post-translational modification embedding the proteins into the lipid membrane. The length of fatty acid varies, just as its degree of unsaturation. An unsaturated lipid has one or more double bonds, usually in a cis-configuration and with one methylene group in between. Furthermore, oxidative enzymes, such as lipoxygenases, cyclooxygenases, and cytochrome

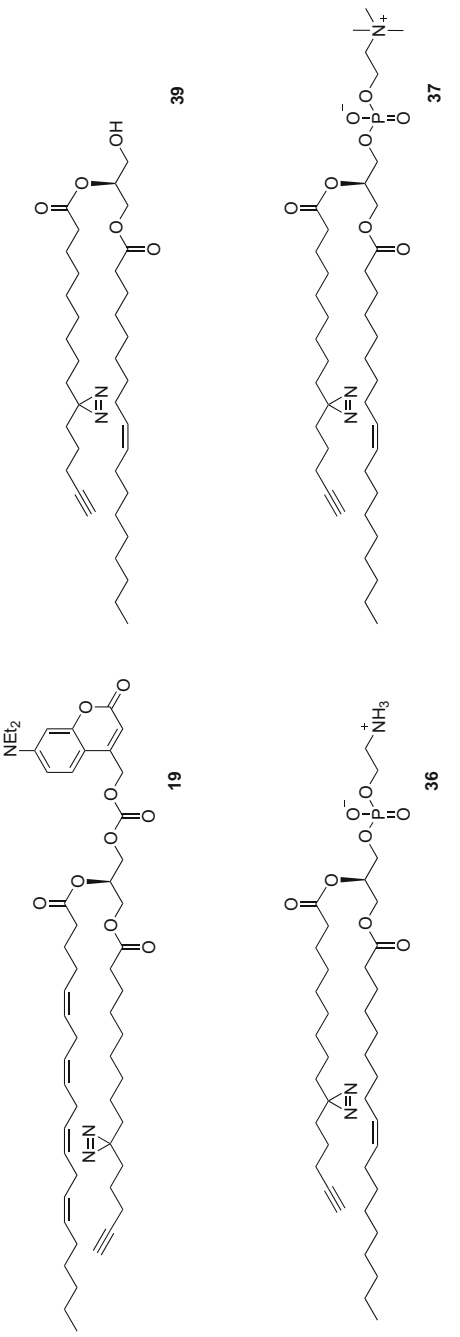

Fig. 6 Structures of photoaffinity probes based on diacylglycerol **19** and **39**, phosphatidylethanolamine **36,** and phosphatidylcholine **37**

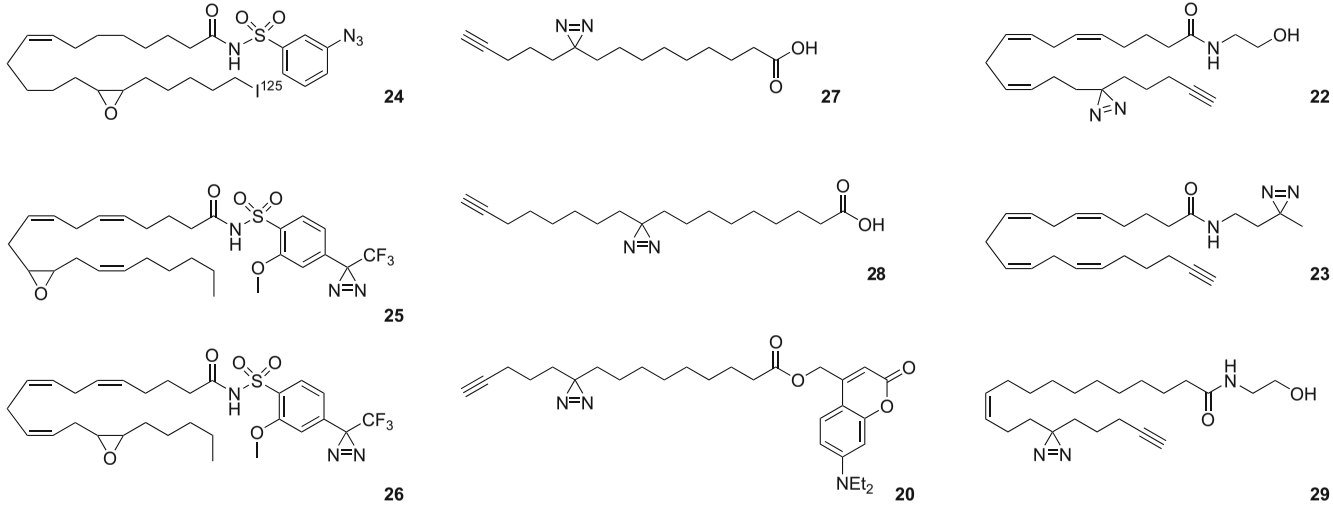

Fig. 7 Structures of photoaffinity probes based on 14,15-epoxyeicosatrienoic acid **24–26**, bifunctional lipid probes **27–28**, caged lipid probe **20,** and anandamide-based probes **22**, **23,** and **29**

P450 s, metabolize polyunsaturated fatty acids (i.e., arachidonic acid) into bioactive signaling molecules. For example, endothelium-derived epoxyeicosatrienoic acids (EETs) are lipid signaling molecules with various biological activities. In search of a high-affinity G protein-coupled EET receptor for 14,15-epoxyeicosatrienoic acid, epoxide-containing lipid **24** equipped with a photoactivatable aryl azide, and radioactive iodide as reporter group was synthesized (Fig. 7). The probe showed EET agonist activity and labeled a 47-kDa band which could be outcompeted with several EET agonists and antagonists (Chen et al. 2011).

The structurally related probes **25** and **26** have been used to study the binding mode of EETs in the soluble epoxide hydrolase (sEH) enzyme (Fig. 7). The stereoselectivity of the epoxide dictated the binding mode, but this has not been confirmed by testing the individual enantiomers of the probes (Lee et al. 2017).

Bifunctional probe **27** was synthesized to study in vivo protein–lipid interactions (Fig. 7) (Haberkant et al. 2013). A palmitic acid mimic was incorporated into different lipid classes and proteins. Proteomic analysis resulted in the identification of 214 lipid-interacting proteins. Ligation to a fluorophore visualized the probe-bound proteins in nematode larvae. Moreover, the probe was metabolically incorporated in 185 additional proteins, thereby highlighting the versatility of bifunctional probes in studying protein–lipid interactions (Haberkant et al. 2013).

Others also exploited this type of metabolic incorporation. For example, probe **28** was incorporated in S-palmitoylated membrane proteins (Fig. 7). The photoaffinity group allowed the capture protein–protein interactions of IFITM3, a protein with antiviral properties. This method was validated by studying VAPA—a known interaction partner of IFITM3—after which a pull-down experiment afforded 12 novel interaction partners of IFITM3 (Peng and Hang 2015).

The endocannabinoid anandamide is a signaling lipid involved in neurotransmission. To map the anandamide-binding proteins, photoaffinity probes **22** and **23** and two control probes based on oleoylethanolamide and palmitoylethanolamide were synthesized (Fig. 7). More than thousand interacting proteins were identified, including NUCB1, NENF, and VAT1. These probes were subsequently employed to discover ligands for the lipid-binding pockets of said targets using competitive fluorescence polarization assays (Niphakis et al. 2015). In addition, lipid probes **22**, **23,** and **29** were utilized to determine the selectivity profile of small molecules occupying lipid-binding pockets (Fig. 7). This strategy was referred to as lipid–protein interaction profiling (LiPIP) (Lum et al. 2017).

2.3 Sphingolipids

Sphingolipids are lipids that have sphingosine (or a derivative) as scaffold. Similar to glycerolipids, most of the sphingolipids exist in the form of a phosphate ester and a fatty acyl amide bond. Sphingomyelin, one of the most common sphingolipids, serves mainly a structural purpose, but its derivatives are increasingly recognized as important signaling molecules.

The last decade has witnessed a continuous progression in the development of chemical probes to identify sphingolipid-binding proteins. For example, Haberkant et al. synthesized probes **30** and **31**, which were rapidly incorporated into sphingolipids (Fig. 8). Caveolin-1 and nicastrin were identified as sphingolipid-interacting proteins (Haberkant et al. 2008). In 2010, photoactivatable sphingosine **32** was made and was co-incubated together with radioactive [^{3}H]choline in fibroblasts from healthy subjects or patients with Niemann-Pick A disease (Fig. 8) (Aureli et al. 2010). The storage of sphingomyelin was found to be disregulated in fibroblasts of the patients. In 2015, the photoaffinity-click sphingosine (pacSph) **33** was developed, which led to the identification of 186 pacSph-enriched proteins (Haberkant et al. 2016). As a control, fatty acid probe **27** was used. Although pacSph **27** and **33** have a different structure and are metabolized via separate pathways, substantial overlap between the interacting proteins was found (Figs. 7 and 8). It was suggested that different photoaffinity-click lipids might not be suitable as control compounds. Four potential issues should be taken into consideration: (i) Both lipid probes could be incorporated into phosphatidyl cholines (PCs), (ii) proteins may have two or more lipid-binding sites, (iii) a single lipid-binding site may be able to recognize multiple lipids, and (iv) alterations made on the lipids may alter their physiochemical properties (Haberkant et al. 2016). Nevertheless, pacSph **27** probe seems to be a more versatile tool to discover new sphingosine-binding proteins compared to **30** and **31**. Finally, functional uncaging of pacSph **21** afforded 64 pacSph-specific binding proteins, of which 14 were also found using uncaged pacSph (Höglinger et al. 2017).

Ceramide, a sphingosine containing a fatty acyl amide, is a signaling molecule with pro-apoptotic activity. In search of ceramide-binding proteins, a number of photoaffinity probes with pacFA as basis have been synthesized. The targets of ceramide probe (pacCer) **34** were compared to the interaction partners of glucosylceramide (pacGlcCer) **35** in cytosolic fractions of various cell lines. CERT, a protein with a StAR-related lipid-transfer domain was chosen as model protein to study structure–activity relationships of the lipid-binding pocket using probes **34**–**39** (Figs. 6 and 9) (Bockelmann et al. 2018).

2.4 Sterols

Sterols are lipids with rigid, fused rings with one or more hydroxyl groups, which gives them amphiphilic properties. Cholesterol is the most abundant sterol found in mammalian cells. Cholesterol alters the fluidity of the membrane and is a constituent of lipid rafts, which are liquid-ordered regions of the plasma membrane high in cholesterol and glycosphingolipids, sequestering specific proteins (Pike 2003). The lipid environment alters the biological properties of the embedded proteins, and the noncovalent interaction of sterols to proteins has had a surge of interest in recent years.

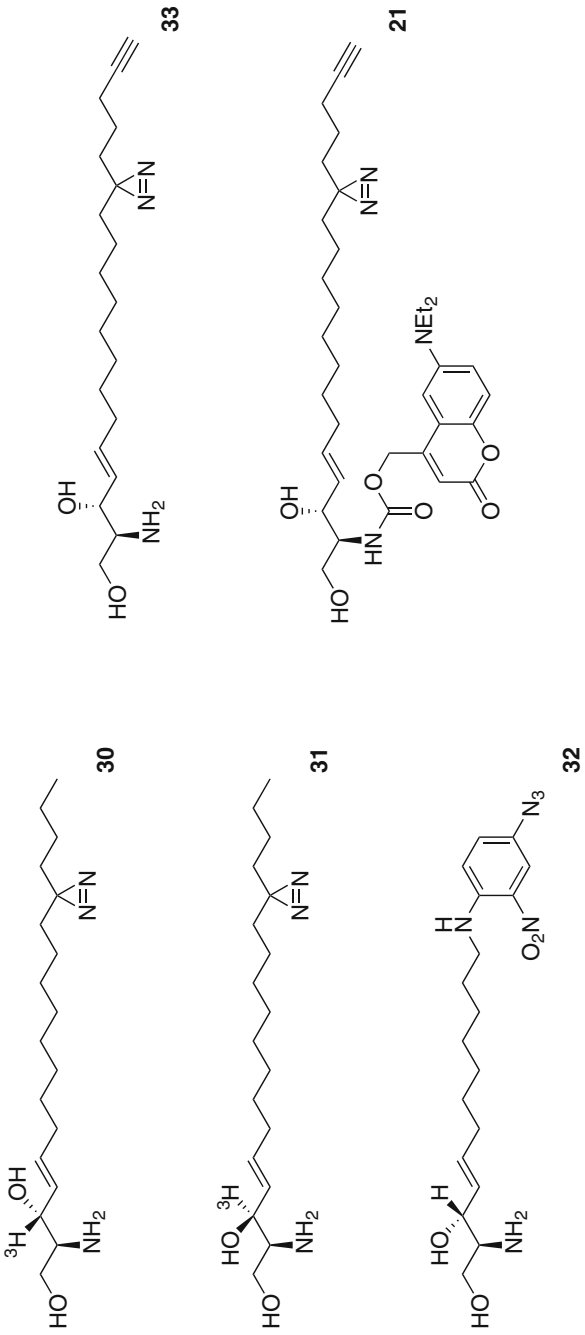

Fig. 8 Structures of photoaffinity probes based on sphingosine **30**, **31**, **32**, **33**, and **21**

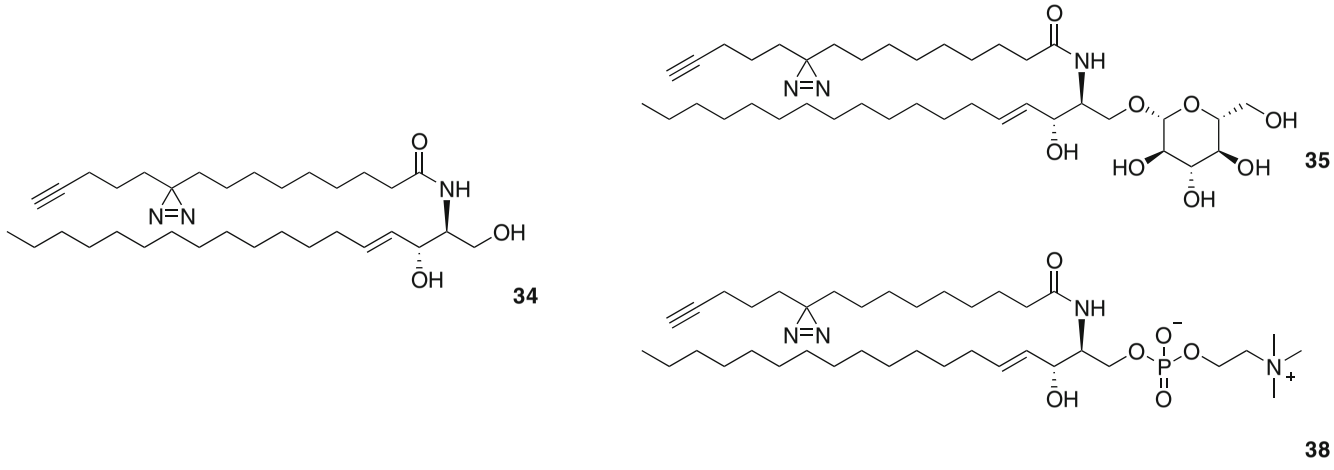

Fig. 9 Structures of photoaffinity based probes based on ceramide **34**, glucosylceramide **35,** and sphingomyelin **38**

Various types of cholesterol probes have been synthesized. Structures **40–42** are diastereomers and showed similar labeling patterns on gel (Fig. 10) (Hulce et al. 2013). In a pull-down experiment with trans-sterol probe **40** about 850 proteins were enriched using no UV and no probe as controls. Nearly 700 proteins showed a preference over a palmitoylethanolamide-based probe.

To map cholesterol binding sites in VDAC1, **43** and **44** were synthesized (Fig. 10). Purified recombinantly expressed mouse VDAC1 was used in a top-down and bottom-up proteomics analysis to map the binding pocket. The binding site was found to include Thr83 and Glu73 (Budelier et al. 2017).

To study the transfer of cholesterol between NPC1 and NPC2, the cholesterol derivative **45** has been synthesized to stabilize the protein transition state during the handoff (Fig. 10). Supported by previously reported cross-linked bile acids and modeling studies, the probe was supposed to stabilize protein dimer complexes. No follow-up studies have been reported to date (Byrd et al. 2015).

Bile acids are sterols that aid in dietary lipid digestion, but also act as signaling molecules that regulate lipid and glucose metabolism (Zhou and Hylemon 2014). Three probes based on the general structure of bile acids have been synthesized with the diazirine and alkyne positioned on different parts of the scaffold (Zhuang et al. 2017). Probes **46–48** incubated at 50 μM and competition with a twofold excess of competitor afforded 331 proteins that were labeled by all three structures, which provided evidence to assign them as bile acid interacting proteins (Fig. 11). Six known and unknown bile acid binding proteins were validated by overexpression, labeling with or without a competitor and immunoblotting to show probe- and UV-specific enrichment of these proteins. In view of their structural similarity to cholesterol probes **40–42,** the bile acid binding proteins were compared to the cholesterol targets. 146 proteins were shared by both lipid classes.

The development of betulinic acid-based probes has been reported, including photoactivatable probes **49** and **50** (Fig. 11) (Guo et al. 2017). Probe **49** was armed with a 2-aryl-5-carboxytetrazole, a recently developed photoactivatable linker with high crosslinking efficiency (Herner et al. 2016). A pull-down experiment performed with both probes afforded 150 proteins, which were subsequently triaged using control experiments. This afforded 9 and 13 unique proteins for structure **49** and **50**, respectively. The lack of overlap between the two probes was rationalized by the difference in location and reactivity of the photoactivatable group. Of note, most of the **50**-bound hits coincided with the results of Zhuang et al. 2017, indicating the reliability of these hits (Guo et al. 2017).

Two probes based on the sterol oleanolic acid have been synthesized (Zhang et al. 2012). To test functional similarity to the parent structure, they were tested in a RMPGa inhibition assay, where **51** showed a twofold and **52** a fivefold reduction of potency compared to oleanolic acid (Fig. 11). Probe **51** labeled two bands UV-dependently in soluble proteomes prepared from HepG2 cells which could be outcompeted with oleanolic acid (Zhang et al. 2012).

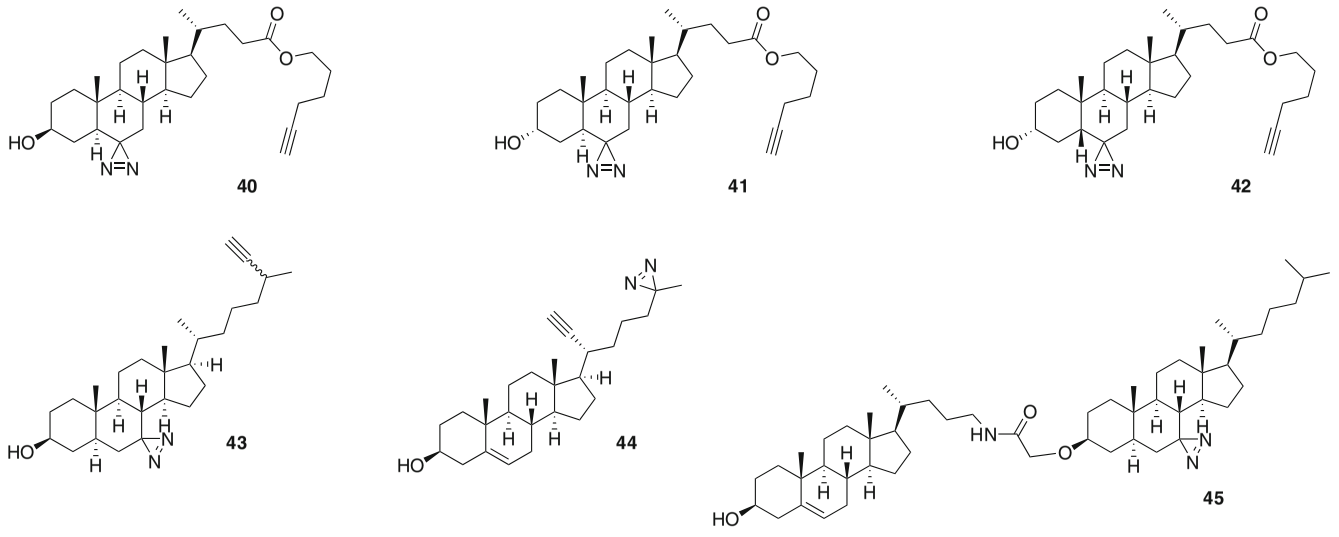

Fig. 10 Structures of photoaffinity probes based on cholesterol **40–45**

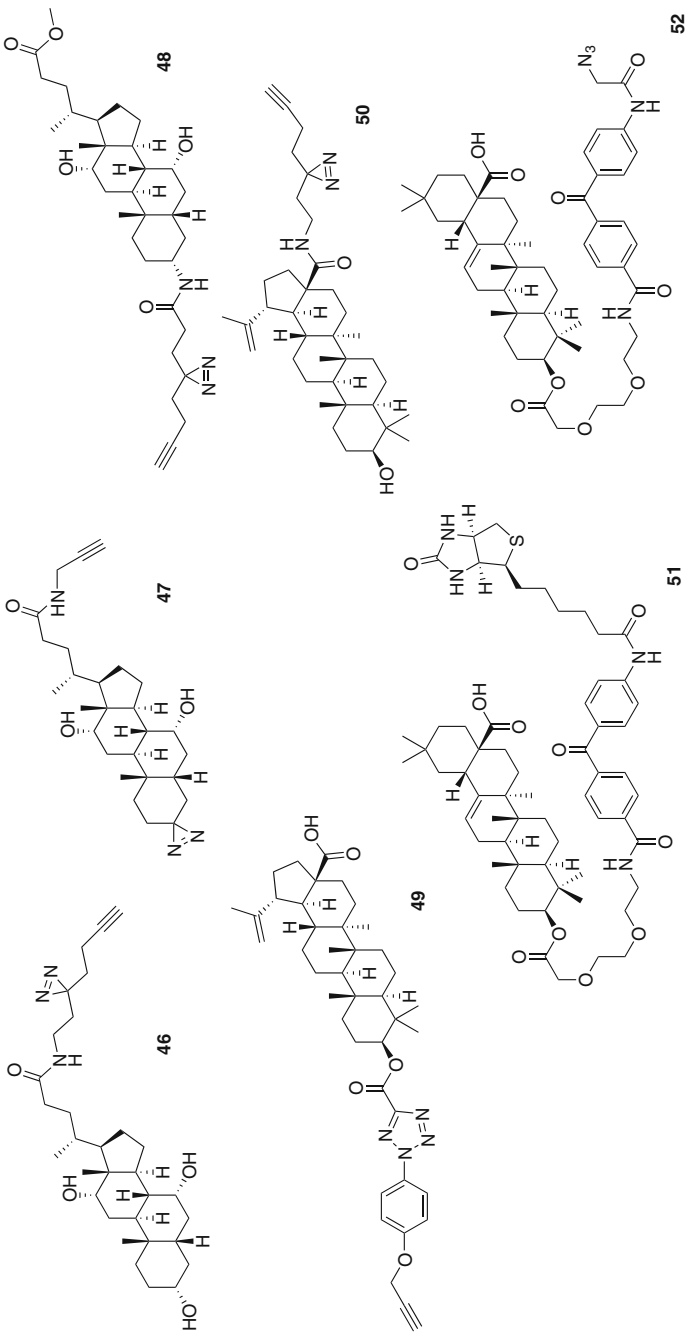

Fig. 11 Structures of photoaffinity probes based on bile acid **46–48**, on betulinic acid **49–50,** and on oleanolic acid **51–52**

2.5 *Promiscuous Lipid-Binding Proteins*

Chemical proteomic datasets contain an enormous amount of data, making it a challenge to distinguish real specific interacting partners from background proteins for a chemical probe. This necessitates the careful design of the experiment with negative controls. Due to a bias toward highly abundant proteins and potential co-purification of other proteins with probe targets, negative controls do not always cover the whole spectrum of background proteins. To combat this problem, twelve laboratories have combined the data of >300 negative control experiments to establish a database of common background proteins. This contaminant repository for affinity purification (CRAPome) is a useful tool to identify common background proteins (Mellacheruvu et al. 2013). In case of affinity-based protein profiling, one also needs to keep in mind the specific background labeling proteins associated with each individual photoreactive group (Kleiner et al. 2017). The CRAPome database can be used for analyzing (photoaffinity) proteomic datasets. However, it is confined to the background of the purification method (CRAPome) or photoreactive group (Kleiner et al. 2017). When conducting chemical proteomics with lipid probes, one also has to account for the nonspecific interactions due to the lipophilic character of the probes. A different set of background proteins could therefore be envisioned based on hydrophobic interactions with lipid probes.

To compile a database of promiscuous lipid-binding proteins, we have combined, compared, and ranked all the enriched target proteins of probes **8**, **14**, **15**, **19**, **21**, **22**, **23**, **27**, **29**, **33**, **40,** and **47**. For each probe, the criteria of the authors for assigning probe targets were used. Where possible, the proteins identified in multiple cell lines were used. This resulted in a list of 1367 distinct proteins of which 176 targets were found in ≥ 4 experiments (from a total of 11 experiments) (Fig. 12a). Of note, only 13 of these proteins were identified as background proteins

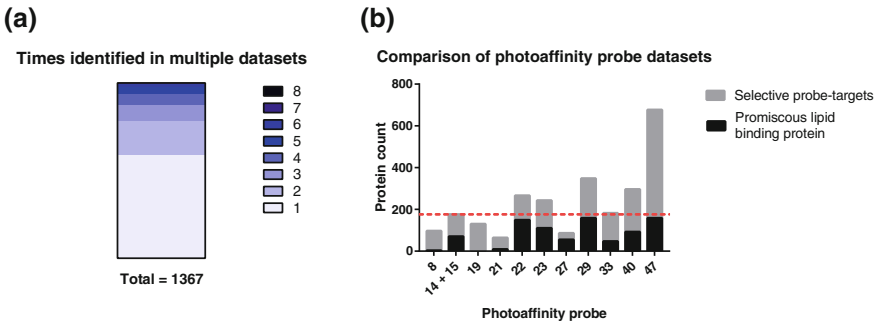

Fig. 12 **a** Overview of how many times a protein was targeted by different probes. In total, 1367 proteins were identified of which 176 (13%) were found in at least 4 of the 11 experiments. **b** Overview of the number of probe targets and promiscuous lipid-binding proteins identified by each probe. The red line is set at 176 and visualizes the maximum amount of promiscuous lipid-binding proteins

by the CRAPome (using >20% of total entries as cutoff criteria) (Mellacheruvu et al. 2013). An overview of the 176 most promiscuous lipid-binding proteins can be found in the supplementary. It was found that (caged) photoaffinity probes **8**, **19,** and **21** were highly selective (Fig. 12b) with the smallest number of common targets. The other probes interacted with targets also detected by different probes to some extent (Fig. 12b). Without diligent controls, such as competition with the endogenous ligand, caution would therefore be advised before assigning these targets as probe-specific proteins.

3 Lipid Activity-Based Probes

Activity-based protein profiling (ABPP) uses a chemical probe to covalently label and identify an enzyme or class of enzymes in a biological sample. Activity-based probes (ABPs) consist of a reactive group or warhead and a reporter tag. ABPs can further be divided into one-step and two-step probes based on the presence of a reporter tag during labeling or ligation of the tag after the labeling event using click chemistry, respectively. The scaffold of the probe combined with the reactive group determines the affinity and selectivity of the ABP.

The advantage of using ABPP over transcriptomics or whole-cell proteomics is the ability to quantify the amount of active proteins, whereas alternative methods do not discriminate between active or inactive protein forms. If a protein is inactive due to a post-translational modification (PTM) or blocked by a non-allosteric inhibitor, it will not react with the ABP, because the reactive group interacts with the active site of the enzyme.

Most of the groundwork for ABPP has been done in the field of serine hydrolases. This class of enzymes consists of more than 200 enzymes of which half are serine proteases and other half metabolic enzymes with an active site serine (Long and Cravatt 2011). The group of metabolic serine hydrolases can be divided into (thio)esterases, lipases, peptidases and thus can hydrolyze a wide variety of bonds (Simon and Cravatt 2010). Lipases hydrolyze triglycerides feeding liberated fatty acids back into the β-oxidative pathway. Next to their role in the energy household of cells, lipases also play a role in cellular signaling as some of their products, such as 2-Arachidonoylglycerol (2-AG) and anandamide are endogenous ligands of the cannabinoid receptors. The amidase FAAH hydrolyzes a variety of endocannabinoid lipid amides, thereby inactivating these lipid messengers. It is because of their wide variety in role and function that this protein class has gotten so much attention in the last years.

The first ABP for this class of enzymes was fluorophosphonate (FP)-biotin which is a one-step probe with a FP-warhead (Liu et al. 1999). Together with the FP-TAMRA probe **53**, they are routinely used in this field for the mapping of activity profiles of serine hydrolases in different animals or tissues or for the determination of selectivity profiles of inhibitors (Baggelaar et al. 2017; Zweerink et al. 2017; Van Esbroeck et al. 2017; Lentz et al. 2018; Rooden et al. 2018).

By tweaking the scaffold of traditional FP probes, subclass-selective probe **54** for serine phospholipases was made (Tully and Cravatt 2010). This demonstrates that the scaffold and reactivity of the warhead are key for affinity and selectivity of a probe (Fig. 13).

The FP probes enabled measurement of the activity of a wide range of serine hydrolases, but do not completely cover the entire family. Several complementary ABPs have therefore been developed. For example, MB064 (**55**) is based on the scaffold of tetrahydrolipstatin (THL), a promiscuous lipase inhibitor that also labeled DAGL-α, which is not targeted by FP-TAMRA (Baggelaar et al. 2013). Compared to FP-TAMRA (**53**), MB064 (**55**) showed a more limited labeling pattern, thereby complementing the serine hydrolase toolbox (Baggelaar et al. 2017). Another example is JW576 (**56**), an ABP selective for KIAA1363, which can also be used as an imaging biomarker (Fig. 13) (Chang et al. 2012).

Another group of selective serine hydrolase probes is the triazole urea probes DH379 (**57**) and HT-01 (**58**) (Hsu et al. 2012; Ogasawara et al. 2016). These probes show selectivity for DAGL-α and DAGL-β. HT-01 has been used in the field of immunology to study the regulatory role of DAGL-β in the inflammatory response of macrophages (Hsu et al. 2012). The last example in this group is the FAAH-selective probe PF-04457845yne (**59**) (Fig. 13) (Ahn et al. 2011). This probe was synthesized to study the off-targets of covalent FAAH inhibitor PF-04457845 directly. This ABP proved to be selective for FAAH in mouse brain and liver tissue, thus illustrating how ABPP can be used to profile the off-targets of covalent drug candidates.

More recently, two probes (**60** and **61**) for a related class of enzymes, the lysosomal cysteine hydrolases, have been published (Fig. 14) (Ouairy et al. 2015; Romeo et al. 2015). This group of hydrolases is also involved in the hydrolysis of lipids, but performs its activity via an active site cysteine instead of a serine. Both probes are activity-based and react with the active site cysteine. However, they use different warheads for this purpose. The carmofur-based probe **60** uses a 5-fluorouracil group and ARN14686 (**61**) uses a β-lactam. Probe **60** labeled acid ceramidase (AC) and ARN14686 (**61**) was shown to label both AC and N-acylethanolamine acid amidase (NAAA). Changing the ligation tag of **61** led to the development of norbornene-ABP **62** and an BODIPY-ABP **63** based on a N, O-substituted threonine-β-lactam (Petracca et al. 2017). Both probes react with NAAA and could be used to label NAAA directly (Fig. 14).

Another interesting group of ABPs is the probes **64** and **65** for beta-glucosidases, such as GBA that hydrolyze glucosylceramide (Fig. 14). ABP **64** was shown to be selective for GBA1 (Witte et al. 2010), whereas ABP **65** acted as a broad-spectrum probe for GBA1, GBA2, GBA3, and LPH (Kallemeijn et al. 2012). These probes are currently used to study Gaucher disease in which deficiency of GBA1 leads to accumulation of its substrate.

RPE65 or retinoid isomerohydrolase is essential in the visual cycle and converts all-*trans*-retinyl esters to 11-*cis*-retinol (Cai et al. 2009). LRAT transfers acyl groups from lecithin to all-*trans*-retinol yielding retinyl esters, which provides the substrate for RPE65 and is therefore also part of the visual cycle (Jahng et al. 2003b). ABPP has also made its entry into the field of the lipid vitamins.

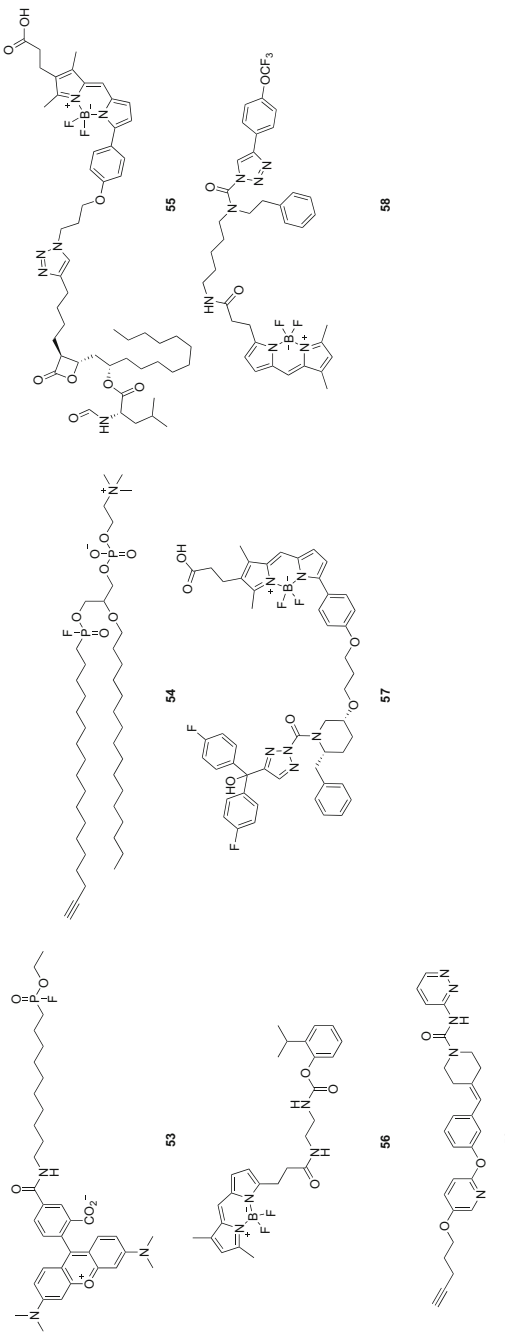

Fig. 13 Structures of activity-based probes for serine hydrolases. Broad-spectrum serine hydrolase FP probes **53**–**54**, THL-based probe MB064 **55**, KIAA1363 selective probe JW576 **56**, DAGL-α and DAGL-β selective probes DH379 **57** and HT-01 **58**, FAAH-selective probe PF-04457845yne **59**

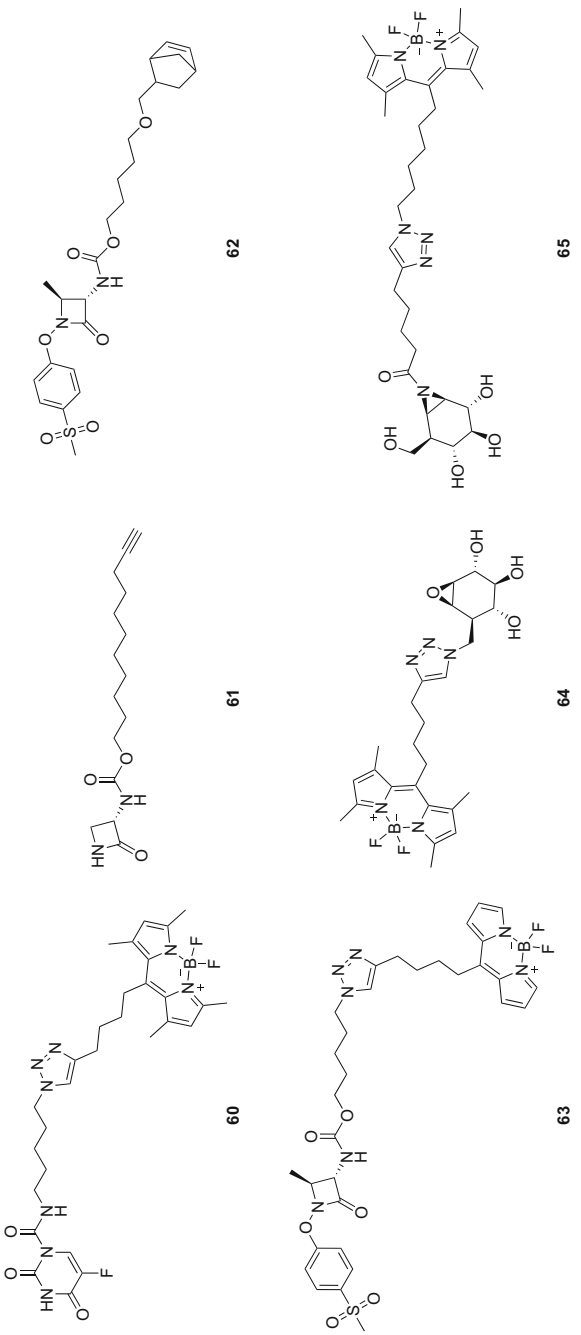

Fig. 14 Structures of activity-based probes for cysteine hydrolases **60**–**63** and beta-glucosidases **64**–**65**

An one-step probe all-*trans*-retinyl chloroacetate (RCA) (**66**) mimicking retinyl acetate was synthesized in 2002 containing a chloroacetate warhead and a cleavable biotin linker (Fig. 15) (Nesnas et al. 2002). This ABP **66** was shown to label RPE65 and lecithin retinol acyltransferase (LRAT) in retinal pigment epithelial membrane (Jahng et al. 2003a). Although this ABP **66** has proven to be a great tool for visualizing interactions of retinoids with enzymes involved in the visual cycle, it has so far found no application outside of the field.

Another lipid soluble vitamin, vitamin D_3, has a chemical ^{14}C labeled probe **67** visualized by phosphorimaging (Fig. 15). Though not activity-based, this probe **67** was able to covalently label the vitamin D-binding protein (DBP) by reacting with a bromoacetate to the tyrosine of the binding protein (Swamy et al. 2000a). A similar affinity probe based on the 1,25-dihydroxyvitamin D_3 equipped with a bromoacetate was shown to label the vitamin D nuclear receptor (VDR) (Swamy et al. 2000b).

Lipoxygenases oxygenate polyunsaturated fatty acids (PUFAs) and are essential metabolic enzymes in the formation of resolvins. N144 (**68**) is a recently developed ABP for 15-lipoxygenase-1 (15-LOX-1) (Eleftheriadis et al. 2016). N144 (**68**) mimics linoleic acid using a bis(alkyne)core to interact with the active site of the enzyme via its radical mechanism (Fig. 15). It also possesses a terminal alkene, which can be utilized as a handle in an oxidative Heck reaction with biotinylated phenylboronic acid to ligate a biotin reporter tag (Ourailidou et al. 2014).

Although not activity-based, arylfluorosulfate probes **69** have recently been shown to covalently label intracellular lipid-binding proteins (Fig. 15) (Chen et al. 2016). These probes have been shown to react with a reactive tyrosine inside the binding pocket of cellular retinoic acid binding proteins (CRABPs) and fatty acid binding proteins (FABPs). These arylfluorosulfates enable the visualization of these lipid trafficking proteins and their ligands without the need of probes with a photoaffinity group.

4 Opportunities

So far the majority of lipid chemical probes consist of photoaffinity probes. A general trend can be observed when surveying the published structures for lipid A*f*BPP from the last seven to eight years. Before that, many photoaffinity lipids consisted of benzophenone-modified lipids, lacking a reporter group. When they did contain a reporter group, it was usually a fluorophore or radioactive isotope and was exclusively used for the study on purified proteins or cell lysates (Xia and Peng 2013). These probes could be used to locate binding sites on known binders or show binding proteins on a gel. However, identifying novel proteins with these reporters (or lack thereof) is challenging.

In recent years, the radioactive reporters, bulky fluorophores and linker-attached biotin groups are being replaced with an alkyne, ameliorating the challenging synthesis of probes based on biomolecules. Besides its minimal impact on the

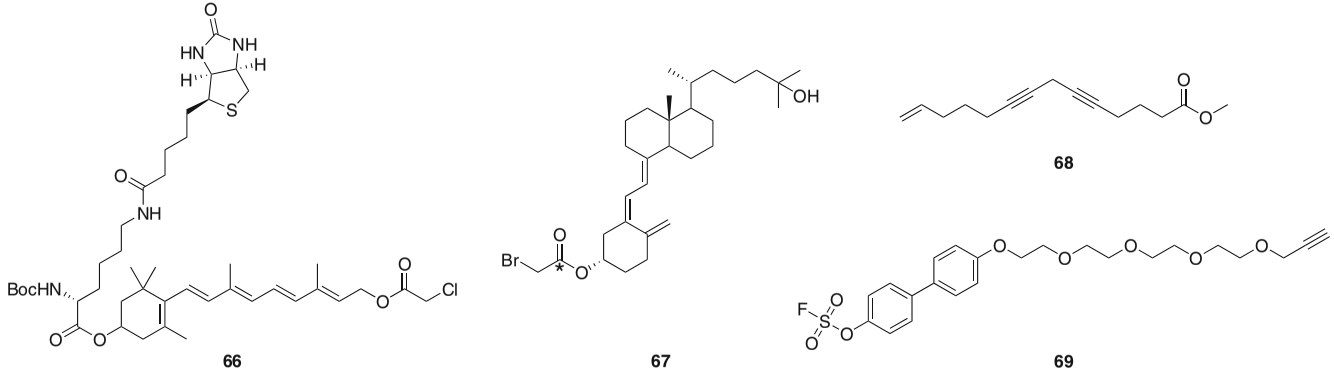

Fig. 15 Structures of activity-based probes based on retinyl acetate **66**, vitamin D **67** (*indicates ^{14}C), linoleic acid **68,** and arylfluorosulfonate probe **69**

structure, the alkyne group also offers increased flexibility in the experimental setup. With an increasing amount of click chemistry possibilities and a large library of commercially available azides, the same alkyne photoaffinity probe can be used for detailed binding studies and global A*f*BPP.

An interesting combination of a global and detailed study is described for lipid probes **22** and **23**, in which a probe target and its binding site were discovered in living cells without any protein purification or overexpression, using the isoTOP-ABPP platform and a isotopically labeled cleavable TEV linker. This double enrichment method, first probe-bound protein enrichment followed by probe-bound peptide enrichment, allows the discovery of probe targets and binding sites in a complex mixture (Weerapana et al. 2007; Niphakis et al. 2015). Techniques like isoTOP-ABPP becoming more feasible will be a major driving force in the applicability of lipid probes.

Protein–protein interactions can be mediated through lipid PTMs. Introduction of both an alkyne and diazirine seems to moderately affect the capability of a lipid to be recognized by its interacting proteins and incorporated as a PTM. This strategy has been used by several groups to discover lipidated proteins and their interaction partners (Peng and Hang 2015; Wright et al. 2015). As lipidation is a common PTM, this approach should be applicable to lipids other than simple fatty acids, most notably the class of prenyl lipids.

Challenges for lipid probes include their metabolism and high lipophilicity. Membrane lipid composition is tightly regulated, and any imbalance is quickly rectified, complicating the identification of the exact lipid species by the tagged protein (Haberkant et al. 2016). Making metabolically inactive, 'caged' derivatives are suggested to ameliorate this problem, allowing the lipid probe to distribute before releasing the bioactive lipid in a controlled manner (Höglinger et al. 2017). Moreover, the metabolism can also be exploited, as demonstrated by Aureli et al. and Wang et al. by incubating the cells with a reporter group and photoactivatable group installed on different parts of a bioactive molecule. Only the assembled combination of the two parts will function as a probe (Aureli et al. 2010; Wang et al. 2017). Of note, different probes based on the same lipid have overlapping, but distinct labeling profiles. To increase the reliability of a photoaffinity project, multiple active and preferably even inactive variants should, therefore, be made (Arrowsmith et al. 2015). Moreover, it suggested that probes should be outcompeted by their parent lipid to increase the amount of specific binders in a probe target dataset. However, a-specific lipophilic interactions often prevail and cannot be outcompeted (Hulce et al. 2013; Zhuang et al. 2017; Bockelmann et al. 2018).

A-specific binding is also an inherent issue with photoactivatable groups, which lead Sieber and colleagues to make an inventory of common off-targets bound by aryl azides, benzophenones, and most thoroughly diazirines. Next to the CRAPome, this database should be consulted when performing photoaffinity pull-down experiments (Mellacheruvu et al. 2013; Kleiner et al. 2017). Moreover, the data in Chapter 2.5 from analysis performed on the targets of over ten different lipid photoaffinity probes can also be used to recognize promiscuous lipid binders.

As noted above, activity-based lipid probes are relatively under-represented, thereby providing great opportunities in the field of chemical biology. Particularly in the field of immunology, where lipid signaling molecules are increasingly recognized as important regulators of the immune response, there is ample room for novel activity-based lipid probes.

4.1 Endocannabinoids

The serine hydrolase ABPs discussed in Sect. 3 have been widely used to study the endocannabinoid system (ECS) and the effect of its lipid messengers on the central nervous system (CNS). The main receptors for these lipids are the cannabinoid receptor type 1 and type 2 (CB_1 and CB_2 receptor respectively). While CB_1 is mainly expressed within the central nervous system, CB_2 is mainly expressed in immune cells and tissue (Cabral and Griffin-Thomas 2009). Since the discovery of the CB_2 receptor, a lot of effort has gone into determining its expression level in different immune cell types (Turcotte et al. 2016). This work is done either by looking at the mRNA levels of the receptor or by western blotting with specific antibodies. Both of these methods, however, have drawbacks. While mRNA levels do intuitively correlate with protein levels, it gives no information about the actual activity of the receptor. Protein degradation, PTMs, and protein–protein interactions are important factors in regulating the amount of active enzyme. The presence of protein mRNA does therefore not predictably correlate with the amount and activity of an enzyme. While correlating with the amount of protein, antibodies on the other hand can have cross-reactivity problems (Weller 2016). Without the necessary quality controls and with different groups using different antibodies, it is a difficult task to determine which results are significant and reproducible. Recently, it has been shown that photoaffinity probes can also be used as an alternative to antibodies (Soethoudt et al. 2018).

With endocannabinoids being implicated in more and more immune diseases, the field of immunology is a clear application area for existing and future lipid chemical probes (Basu and Dittel 2011; Pacher and Mechoulam 2011; Chiurchiù et al. 2015, 2018). These ABPs would enable the visualization of levels of active enzyme in immune cells as has been shown with the HT-01 probe **58** for DAGL-β in macrophages (Hsu et al. 2012). Coupled with cell sorting techniques, the effect of different kinds of stimulation on the ECS can be analyzed. This technique could also be used to make a comparison between tissue/cells in healthy and disease states.

4.2 Steroid Hormones

The steroid hormones can be divided into two categories: sex steroids and corticosteroids. They are all derived from cholesterol and are fat-soluble.

These hormones act as lipid messengers and interact with nuclear steroid receptors influencing gene expression. More recently, they have also been implicated to act via different, faster pathways localized in the cytoplasm (Norman et al. 2004). Due to the sexual dimorphism in the immune system, sex steroids have long been implicated to affect the immune system. Testosterone seems to suppress the immune system leading to a lowered immune response to infections and vaccines in men (Furman 2015; Trigunaite and Dimo 2015). Females seem to have a more robust immune response, but are more susceptible to autoimmune diseases possible due to the modulatory effects of estrogen (Cunningham and Gilkeson 2011; Waldmann et al. 2016). Most research in this field has focused on administering steroid hormones, removing hormone-producing organs or protein knockouts (Homo-Delarche et al. 1991; Lai et al. 2012). We therefore envision potential for steroid hormones based lipid probes. For example, no photoaffinity probes based on the scaffold of its steroid hormone derivatives and no activity-based probes have been published so far. These probes would enable investigation of steroid hormone interactions while keeping the biological system in its natural state. They could also reveal previously unknown hormone–protein interactions.

4.3 Lipid Soluble Vitamins

The lipid soluble vitamins, vitamin A and D, exercise their effect via the same mechanism as the steroid hormones by binding to their nuclear receptors, the retinoic acid receptor (RAR) and vitamin D receptor (VDR), respectively (Carlberg 1999). Vitamin D, synthesized in the skin under influence of light, is a secosteroid and therefore also structurally resembles the steroid hormones. Both lipid vitamins have been implicated to affect the immune system (Mora et al. 2008). A radioactive photoaffinity probe and a radioactive affinity-based probe for Vitamin D have been synthesized and applied (Ray et al. 1991; Swamy et al. 2000a). These probes may be replaced by novel ABPs with a diazirine as a less bulky photoactive group and addition of a click-handle to enable proteomics studies. An activity-based probe to study the metabolism of the active metabolite of vitamin D throughout the immune system would be a new addition to this toolbox.

In the case of vitamin A, direct photoaffinity labeling with radioactive retinoic acid was enabled by its inherent photoreactive characteristics (Bernstein et al. 1995; Chen and Radominska-Pandya 2000). A radioactive photoaffinity probe based on retinoic acid has been synthesized, but A*f*BPs has not been performed due to lack of a ligation handles for application in chemical proteomics studies (Shimazawa et al. 1991). The activity-based probe for retinyl ester processing enzymes has not yet been tested outside of the retinal pigment epithelial membrane (Jahng et al. 2003a). The application of this ABP in the field of immunology would be interesting. Furthermore, activity-based probes based on retinol, retinal and the immunologically active metabolite, retinoic acid, would be valuable additions to this field.

The other lipid soluble vitamins, such as vitamin E and K, have not been studied using ABPs. Vitamin E has been associated with T-cell differentiation and decreased cellular immunity in aging (Moriguchi 1998; Moriguchi and Kaneyasu 2003). Next to its antioxidant activity, it has also been implicated as a potential orphan nuclear receptor ligand (Soontjens et al. 1996; Carlberg 1999). The synthesis of a vitamin E derivative containing a diazirine and an alkyne click-handle would therefore be an important chemical tool to unravel its protein interactions and mechanism of action. Such a photoaffinity probe could help to find the proposed nuclear receptor or discover other cellular pathways involved in Vitamin K biology.

5 Conclusions

Proteomics using chemical probes is an invaluable strategy to study the biology of lipid messengers. A*f*BPP using photoaffinity probes provides insight into the target interaction landscape of lipid messengers with previously unknown proteins, such as transporters and receptors, while ABPP using activity-based probes identifies the enzymes that control the metabolism of these important messengers in health and disease. We believe that the combination of A*f*BPP and ABPP is a powerful approach to obtain a global and detailed view of the biological processes mediated by lipid signaling molecules. The chemical probes, however, do not cover many lipid classes yet. A potential reason could be that most long-chain, polyunsaturated lipids and their metabolites are challenging to synthesize and have an inherent instability. Although chemists are still working on the total synthesis of these low abundant biologically active lipids and their probes (Ogawa et al. 2017; Rodriguez and Spur 2017), there is still a need for further elucidation of important cellular processes performed by lipids, especially in the field of immunology. So we call on chemists, biologists, and immunologists to combine their expertise to tackle these topics and become chemical immunologists.

Supplementary

List of proteins identified as promiscuous lipid-binding proteins. The count shows the amount of experiments in which the protein was identified as a probe target. Proteins colored red are the proteins flagged by the CRAPome database.

Accession	Protein	Count
Q96A33	CCD47_HUMAN	8
P43307	SSRA_HUMAN	8
Q9BV23	ABHD6_HUMAN	7
P51648	AL3A2_HUMAN	7
P51572	BAP31_HUMAN	7
Q16850	CP51A_HUMAN	7
P50402	EMD_HUMAN	7
Q9NRY5	F1142_HUMAN	7
O75844	FACE1_HUMAN	7
Q96AG4	LRC59_HUMAN	7
Q86UE4	LYRIC_HUMAN	7
O00264	PGRC1_HUMAN	7
Q9Y512	SAM50_HUMAN	7
Q9UBV2	SE1L1_HUMAN	7
Q99623	PHB2_HUMAN	7
P53365	ARFP2_HUMAN	6
Q9HD20	AT131_HUMAN	6
Q96G23	CERS2_HUMAN	6
Q9NZ45	CISD1_HUMAN	6
Q07065	CKAP4_HUMAN	6
O96005	CLPT1_HUMAN	6
Q53GQ0	DHB12_HUMAN	6
Q15392	DHC24_HUMAN	6
P40939	ECHA_HUMAN	6
Q8TCT9	HM13_HUMAN	6
P30519	HMOX2_HUMAN	6
P42166	LAP2A_HUMAN	6
Q9Y6C9	MTCH2_HUMAN	6
Q9Y6Q9	NCOA3_HUMAN	6
Q9NX40	OCAD1_HUMAN	6
Q9UHG3	PCYOX_HUMAN	6
O15173	PGRC2_HUMAN	6
P35232	PHB_HUMAN	6
P51571	SSRD_HUMAN	6
Q9Y4P3	TBL2_HUMAN	6
P21796	VDAC1_HUMAN	6
Q8N0U8	VKORL_HUMAN	6
P16615	AT2A2_HUMAN	6
P27824	CALX_HUMAN	6
P04843	RPN1_HUMAN	6
P28288	ABCD3_HUMAN	5
Q9BRR6	ADPGK_HUMAN	5
P24539	AT5F1_HUMAN	5
Q8WY22	BRI3B_HUMAN	5
P07339	CATD_HUMAN	5
O75746	CMC1_HUMAN	5
P23786	CPT2_HUMAN	5
Q8WVC6	DCAKD_HUMAN	5
Q9BUN8	DERL1_HUMAN	5
Q96J17	DHRS1_HUMAN	5
Q9Y394	DHRS7_HUMAN	5
Q9UBX3	DIC_HUMAN	5
O75477	ERLN1_HUMAN	5
O00461	GOLI4_HUMAN	5
Q92643	GPI8_HUMAN	5
Q3SXM5	HSDL1_HUMAN	5
P07099	HYEP_HUMAN	5
Q06136	KDSR_HUMAN	5
Q86SY8	KTAS1_HUMAN	5
O95202	LETM1_HUMAN	5
Q9NZJ7	MTCH1_HUMAN	5
Q969V3	NCLN_HUMAN	5
Q15738	NSDHL_HUMAN	5
Q02818	NUCB1_HUMAN	5
Q9Y5Y5	PEX16_HUMAN	5
Q96S52	PIGS_HUMAN	5
P50897	PPT1_HUMAN	5
P61026	RAB10_HUMAN	5
P04844	RPN2_HUMAN	5
O95197	RTN3_HUMAN	5
Q9NQC3	RTN4_HUMAN	5
Q8NBX0	SCPDL_HUMAN	5
Q8IWL2	SFTA1_HUMAN	5
O95470	SGPL1_HUMAN	5
O15260	SURF4_HUMAN	5
O14925	TIM23_HUMAN	5
O43615	TIM44_HUMAN	5
Q9NX00	TM160_HUMAN	5
Q6NUQ4	TM214_HUMAN	5
Q15363	TMED2_HUMAN	5
P49755	TMEDA_HUMAN	5
P57088	TMM33_HUMAN	5
Q9BTV4	TMM43_HUMAN	5
Q9H3N1	TMX1_HUMAN	5
Q9Y320	TMX2_HUMAN	5
Q9NS69	TOM22_HUMAN	5
O14656	TOR1A_HUMAN	5
P45880	VDAC2_HUMAN	5
O95831	AIFM1_HUMAN	5
Q3ZCQ8	TIM50_HUMAN	5
Q9NRG9	AAAS_HUMAN	4
Q8WTS1	ABHD5_HUMAN	4
O95870	ABHGA_HUMAN	4
O95573	ACSL3_HUMAN	4
Q8NHH9	ATLA2_HUMAN	4
Q6DD88	ATLA3_HUMAN	4
Q9UHQ4	BAP29_HUMAN	4
P35613	BASI_HUMAN	4
P11021	BIP_HUMAN	4
Q9UBD9	CLCF1_HUMAN	4
CP20A_HUMAN	CP20A_HUMAN	4
O15121	DEGS1_HUMAN	4
Q96KC8	DNJC1_HUMAN	4
Q8N766	EMC1_HUMAN	4
P0DO92	ENOL_HUMAN	4
P24390	ERD21_HUMAN	4
Q14534	ERG1_HUMAN	4
Q9BSJ8	ESYT1_HUMAN	4
A0FGR8	ESYT2_HUMAN	4
Q96A26	F162A_HUMAN	4
Q8WVX9	FACR1_HUMAN	4
P37268	FDFT_HUMAN	4
Q9P035	HACD3_HUMAN	4
Q9NRV9	HEBP1_HUMAN	4
P22830	HEMH_HUMAN	4
O60725	ICMT_HUMAN	4
Q8TCB0	IFI44_HUMAN	4
Q70UQ0	IKIP_HUMAN	4
Q8N5M9	JAGN1_HUMAN	4
Q9BZL6	KPCD2_HUMAN	4
Q14739	LBR_HUMAN	4
Q02978	M2OM_HUMAN	4
Q6P1A2	MBOA5_HUMAN	4
Q4ZIN3	MBRL_HUMAN	4
O43772	MCAT_HUMAN	4
Q9H2D1	MFTC_HUMAN	4
Q16891	MIC60_HUMAN	4
Q10713	MPPA_HUMAN	4
Q9BTX1	NDC1_HUMAN	4
Q9Y639	NPTN_HUMAN	4
P80303	NUCB2_HUMAN	4
Q8NFH5	NUP53_HUMAN	4
O60313	OPA1_HUMAN	4
Q9H7Z7	PGES2_HUMAN	4
Q9H490	PIGU_HUMAN	4
Q8TEM1	PO210_HUMAN	4
P18031	PTN1_HUMAN	4
P62820	RAB1A_HUMAN	4
Q9H0U4	RAB1B_HUMAN	4
P62491	RB11A_HUMAN	4
Q8TC12	RDH11_HUMAN	4
Q9NTJ5	SAC1_HUMAN	4
Q9NR31	SAR1A_HUMAN	4
Q9Y6B6	SAR1B_HUMAN	4
P67812	SC11A_HUMAN	4
O43819	SCO2_HUMAN	4
Q8WTV0	SCRB1_HUMAN	4
Q14108	SCRB2_HUMAN	4
Q9UGP8	SEC63_HUMAN	4
Q15005	SPCS2_HUMAN	4
Q9NQZ5	STAR7_HUMAN	4
Q9UJZ1	STML2_HUMAN	4
P46977	STT3A_HUMAN	4
Q8TCJ2	STT3B_HUMAN	4
Q9UH99	SUN2_HUMAN	4
Q9NZ01	TECR_HUMAN	4
Q8WUY1	THEM6_HUMAN	4
O60830	TI17B_HUMAN	4
Q9HC07	TM165_HUMAN	4
Q13445	TMED1_HUMAN	4
Q9Y3B3	TMED7_HUMAN	4
Q96008	TOM40_HUMAN	4
Q9H4I3	TRABD_HUMAN	4
P53007	TXTP_HUMAN	4
Q95292	VAPB_HUMAN	4
Q96GC9	VMP1_HUMAN	4
Q93050	VPP1_HUMAN	4
Q5BJH7	YIF1B_HUMAN	4
Q8TAD4	ZNT5_HUMAN	4
P05141	ADT2_HUMAN	4
P25705	ATPA_HUMAN	4
P06576	ATPB_HUMAN	4
P14625	ENPL_HUMAN	4
P20700	LMNB1_HUMAN	4
P07237	PDIA1_HUMAN	4
P08670	VIME_HUMAN	4

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