Visualization of vitamin A metabolism

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Chapter 2

Opportunities for Lipid-based Probes in the Field of Immunology

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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 blocks of membranes and energy storage to cell signaling. The discovery of the inhibitory effect of aspirin on prostaglandin biosynthesis showed that lipids can modulate the immune system and that the enzymes involved in their metabolism constitute potential drug targets.[1] Since this initial discovery, many connections have been made between the immune system and signaling lipids in the field of endocannabinoids, resolvins, steroid hormones and vitamins A and D.[2–5] 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 signaling lipids and their protein interaction landscapes as well as their biological function. Since its inception the field of lipodomics, which is the analysis of lipids and their interacting partners within a biological system, has made great strides forward.[6] Standardization of protocols, increased availability of deuterated lipids and the high mass accuracy and resolution of modern mass spectrometers have made it possible to identify and quantify many lipids in complex biological samples.[7]

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 and generally accepted definition of a lipid, which in their view comprises a small hydrophobic or amphipathic molecule that is formed at least partially by the condensation of ketoacyl thioesters and/or isoprene units.[8] Based on these two building blocks eight major lipid classes are defined within this classification: glycerophospholipids, glycerolipids, fatty acyls, sphingolipids, sterol lipids, prenol lipids, saccharolipids and polyketides (**Fig. 2.1**).[9]

The variability in chemical and physical properties 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.[10] 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) 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 or azide tags.[11,12] Using bioorthogonal ligation chemistry these functionalized lipids can be visualized and identified.[13] This method has been used for the visualization of lipids in membranes, modification of proteins by lipids and to study lipid metabolism.[14–16]

Although the introduction of a ligation handle 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 bifunctional 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).[17] Both methods allow for the visualization and identification of protein-lipid interactions.[18]

In this chapter, the recent developments concerning activity-based protein probes based on lipids are summarized. The probes are grouped based on the enzyme class they target. As well, some opportunities for future research are presented.

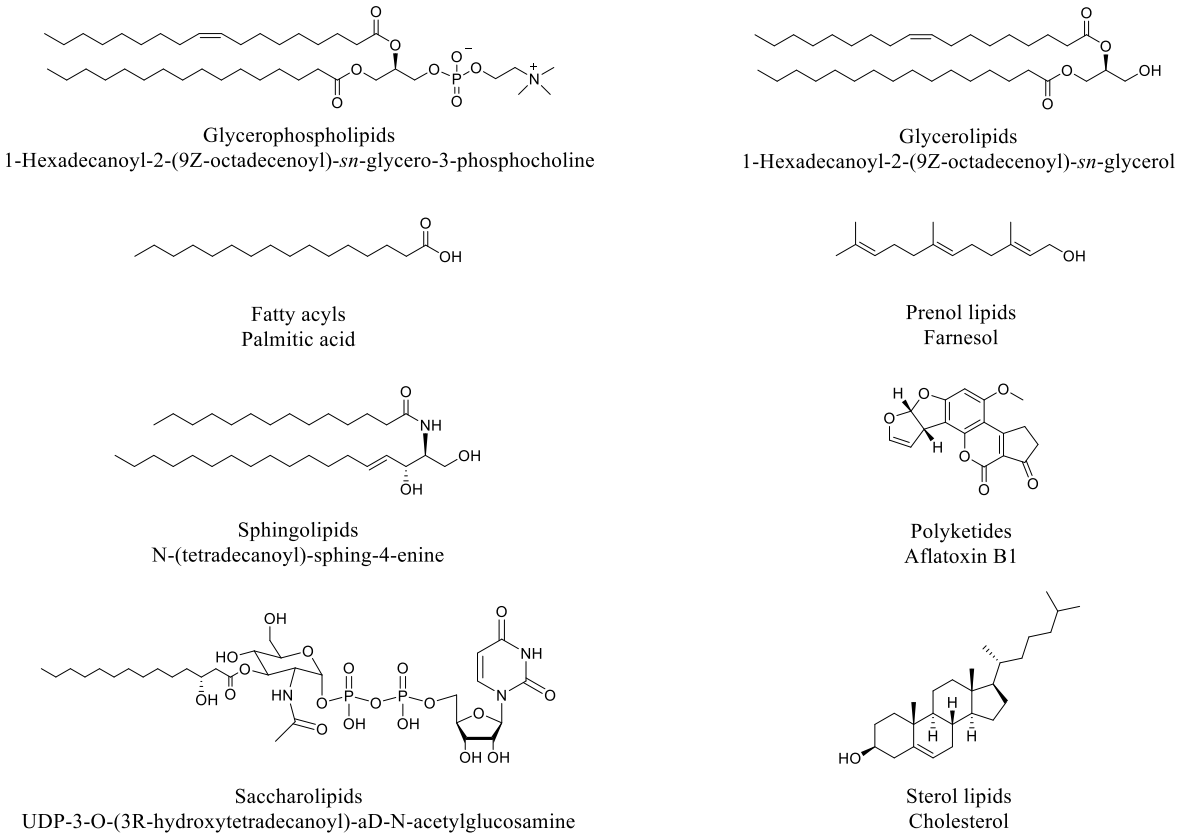

Fig. 2.1 | Representative structures of the eight lipid categories as defined by the LIPID MAPS consortium.[8,9]

Lipid-based 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 classified in one-step and two-step probes based on the presence of a reporter tag during protein labeling or ligation of the tag after the labeling event using click chemistry, respectively. The chemical structure of the probe combined with the warhead determines the affinity, reactivity 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 foundational work in the field of ABPP has been done in the field of serine hydrolases. This class of enzymes consists of more than 200 enzymes in humans of which about half are serine proteases and the other half metabolic enzymes with an active site serine.[19] The group of metabolic serine hydrolases can be divided into (thio)esterases, lipases, and peptidases and thus can hydrolyze a wide variety of substrates.[20] Lipases hydrolyze triglycerides feeding liberated fatty acids back into the β-oxidative pathway. Next to their role in the energy household of cells, these 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 first ABP developed for serine hydrolases is fluorophosphonate (FP)-biotin, which is a one-step probe with a FP-warhead.[21] Together with the FP-TAMRA probe **1**, they are routinely used for the mapping of activity profiles of serine hydrolases in different animals or tissues as well as for the determination of the selectivity profiles of inhibitors.[22–26]

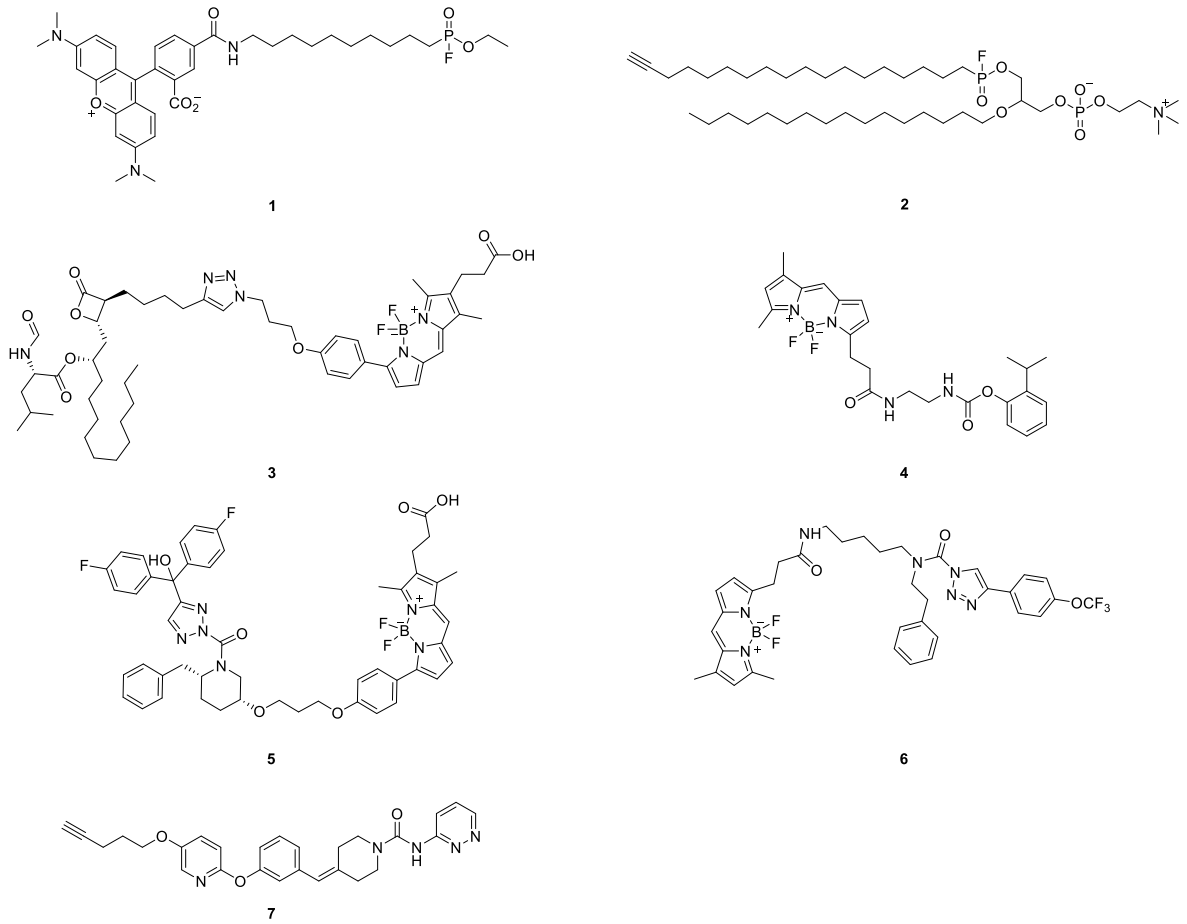

Fig. 2.2 | Structures of some activity-based probes for serine hydrolases. Broadspectrum serine hydrolase FP probes **1-2**, THL based probe MB064 **3**, KIAA1363 selective probe JW576 **4**, DAGL-α and DAGL-β selective probes DH379 **5** and HT-01 **6**, FAAH selective probe PF-04457845yne **7**.

By tweaking the scaffold of traditional FP probes, subclass-selective probe **2** for serine phospholipases was made.[27] This demonstrates that the scaffold and reactivity of the warhead are key for affinity and selectivity of a probe (**Fig. 2.2**). The FP-probes enable measurement of the activity of a wide range of serine hydrolases, but do not cover the entire family. Several complementary ABPs have therefore been developed over the past decade.

For example, MB064 (**3**) is based on the scaffold of tetrahydrolipstatin (THL), a promiscuous lipase inhibitor that also labelled diacylglycerol lipase alpha (DAGL-α), which is not targeted by FP-TAMRA (**1**).[28] Compared to FP-TAMRA (**1**), MB064 (**3**) covered a smaller subset of serine hydrolases, but labelled enzymes not targeted by FP-TAMRA (**1**).[22] Another example is JW576 (**4**), an ABP selective for KIAA1363, which can also be used as a biomarker in imaging (**Fig. 2.2**).[29]

Another group of selective serine hydrolase probes are the triazole urea probes represented by DH379 (**5**) and HT-01 (**6**).[30,31] These probes show selectivity for DAGL-α and DAGL-β. HT-01 has been used to study the regulatory role of DAGL-β in the inflammatory response of macrophages.[31] The last example in this group is the fatty-acid amide hydrolase 1 (FAAH)-selective probe PF-04457845yne (**7**) (**Fig. 2.2**).[32] The amidase FAAH hydrolyses a variety of endocannabinoid lipid amides, thereby inactivating these lipid messengers. Probe **7** was synthesized to study the off-targets of the covalent FAAH inhibitor PF-04457845 directly. This ABP proved to be selective for FAAH in mouse brain and liver tissue.

More recently, two probes (**8** and **9**) for lipid modifying lysosomal cysteine hydrolases, have been published (**Fig. 2.3**).[33,34] This group of hydrolases is 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 catalytic cysteine. However, they use different warheads for this purpose. The carmofur-based probe **8** uses a urea group and ARN14686 (**9**) uses a β-lactam. Probe **8** labeled acid ceramidase (AC) and ARN14686 (**9**) was shown to label both AC and *N*-acylethanolamide acid amidase (NAAA). Changing the ligation tag of **9** led to the development of norbornene-ABP **10** and an BODIPY-ABP **11** based on a *N,O*-substituted threonine-β-lactam.[35] Both probes react with NAAA and could be used to label NAAA directly (**Fig. 2.3**).

Another interesting group of ABPs are the probes **12** and **13** for β-glucosidases, such as glucosylceramidase (GBA) that hydrolyze glucosylceramide (**Fig. 2.3**). ABP **12** was shown to be selective for GBA1, whereas ABP **13** acted as a broadspectrum probe for GBA1, GBA2, GBA3 and lactase/phlorizin hydrolase (LPH).[36,37] These probes are currently used to study Gaucher disease in which deficiency of GBA leads to accumulation of its substrate.

ABPP has also made its entry into the field of the lipid vitamins. RPE65 or retinoid isomerohydrolase is essential in the visual cycle and converts all-*trans*-retinyl esters to 11-*cis*-retinol.[38] 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.[39]

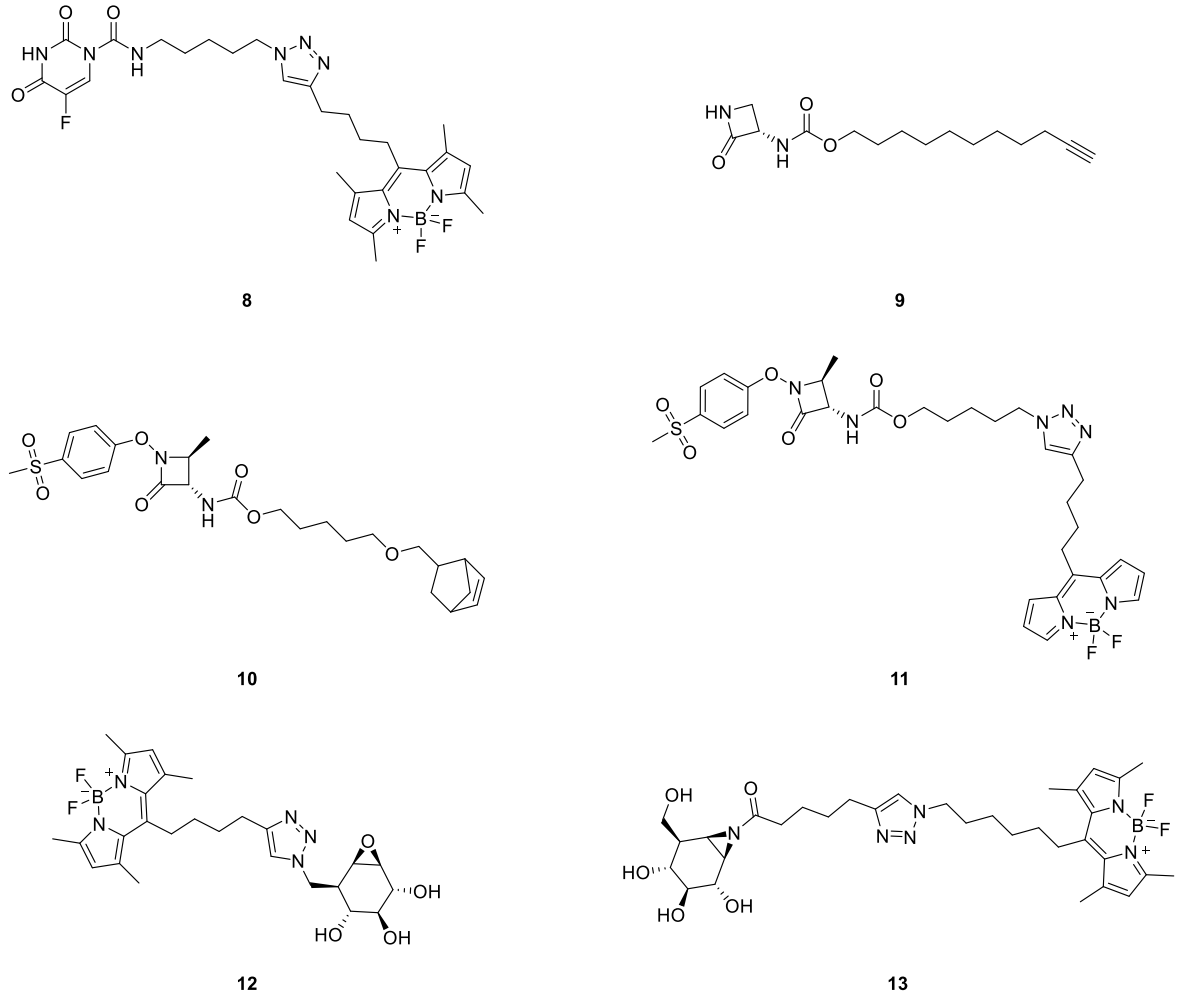

Fig. 2.3 | Structures of activity-based probes for cysteine hydrolases 8-11 and β-glucosidases 12-13.

The one-step probe, all-*trans*-retinyl chloroacetate (RCA) (**14**), mimicking retinyl acetate was synthesized in 2002 and contains a chloroacetate warhead and a cleavable biotin linker (**Fig. 2.4**).[40] This ABP **14** was shown to label RPE65 and lecithin retinol acyltransferase (LRAT) in retinal pigment epithelial membrane.[41]

Another lipid soluble vitamin, vitamin D_3, was used as a scaffold for probe **15** (**Fig. 2.4**). Probe **15** was shown to bind the vitamin D-binding protein (DBP) in a similar fashion as the natural vitamin D_3 ligand, positioning the bromoacetate electrophile for S_N2 displacement of the bromide by a neighboring tyrosine phenol.[42] The resulting covalent complex could be visualized via the carbon-14 (^{14}C) of probe **15** using phosphor imaging. After separation of the proteins using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) the gel is placed on a photostimulable phosphor plate.

Exposure to the ionizing radiation from the ^{14}C radioisotope excites electrons in the phosphor plates and traps them in this excited state. This latent image can be released using a scanning laser, which lets the excited electrons return to their base state, while emitting photons. The luminescence is captured and used to visualize the gel image. 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).[43]

Lipoxygenases oxygenate polyunsaturated fatty acids (PUFAs) and are essential metabolic enzymes in the formation of resolvins. N144 (**16**) is a recently developed ABP for 15-lipoxygenase-1 (15-LOX-1).[44] 15-LOX-1 oxidizes its natural substrate linoleic acid through a radical mechanism. N144 (**16**) mimics linoleic acid and interacts via its bis(alkyne)core with the active site of the enzyme (**Fig. 2.4**). At the active site a hydrogen is abstracted from the bis(alkyne)core resulting in a radical, which can insert itself into one of the neighboring amino acid bonds. As a result the enzyme is irreversibly inhibited and covalently labelled by N144 (**16**). Probe **16** possesses a terminal alkene, which can be utilized as a bioorthogonal handle in an oxidative Heck reaction with biotinylated phenylboronic acid to ligate a biotin reporter tag.[45]

Arylfluorosulfate probe **17** has recently been shown to covalently label intracellular lipid binding proteins (**Fig. 2.4**). Probe **17** and similar arylfluorosulfate 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).[46] These arylfluorosulfates enable the visualization of these lipid trafficking proteins and their ligands without the need of probes with a photoaffinity group.

Opportunities

Activity-based probes are relatively underrepresented in the category of lipid-based chemical probes.[47] The development of these lipid-based ABPs provides opportunities in the field of chemical biology. Even more so in chemical immunology, as lipid signaling molecules are increasingly recognized as important regulators of the immune response.

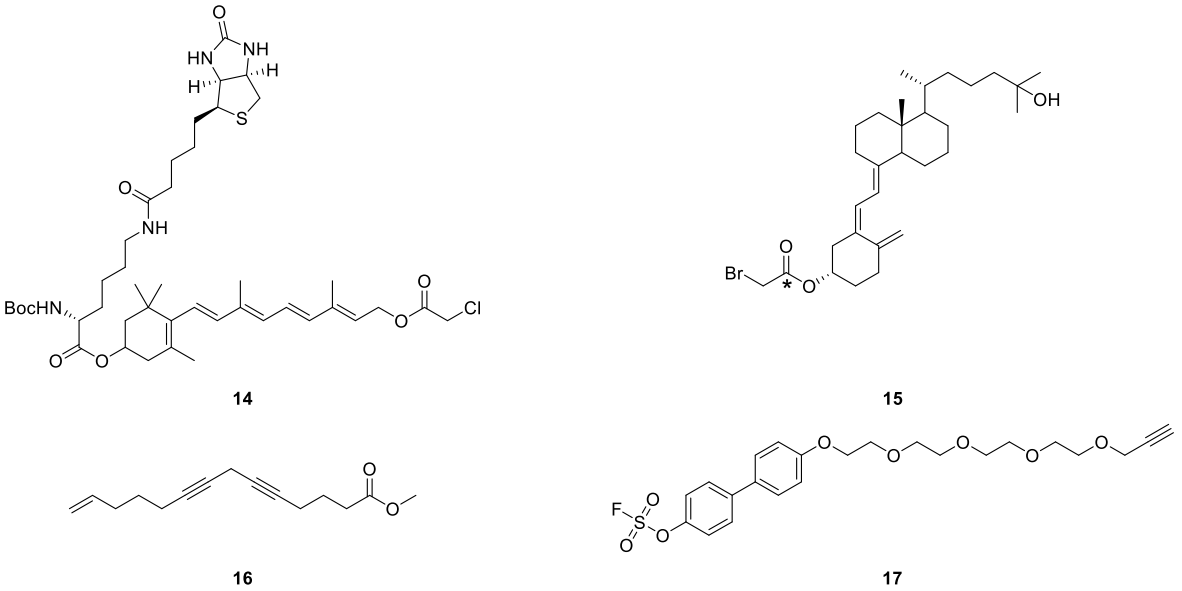

Fig. 2.4 | Structures of activity-based probes based on retinyl acetate 14, vitamin D 15 (* indicates ^{14}C), linoleic acid 16 and arylfluorosulfonate probe 17.

Endocannabinoids

The serine hydrolase ABPs discussed previously 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.[48]

Since the discovery of the CB_2 receptor, determining its expression level in different immune cell types is done by either quantifying its mRNA levels or by Western blotting.[49] Both of these methods however have drawbacks. While mRNA levels do not always correlate with protein levels, they also provide no information on the activity of the protein. 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.[50] 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 be used as an alternative to antibodies.[51]

With endocannabinoids being implicated in several immune diseases, the field of immunology presents a clear opportunity for lipid-based chemical probes.[52–55] These ABPs would enable the visualization of levels of active enzyme in immune cells as has been shown with the HT-01 probe **6** for DAGL-β in macrophages.[31] 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 compare healthy and diseased tissues.

Steroid hormones

The steroid hormones can be divided into two categories: sex steroids and corticosteroids. They are 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 been implicated to also act via a different, faster signaling mechanism within the plasmamembrane.[56] 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.[57,58] Women display a more robust immune response, but are more susceptible to autoimmune diseases possibly due to the modulatory effects of estrogen.[59,60] Most research in this field has focused on administering steroid hormones, removing hormone producing organs or protein knockouts.[61,62] It is therefore envisioned that steroid hormone-based probes have potential in the field of immunology. For example, no steroid hormone-based activity-based probes have been published so far. These probes would enable investigation of steroid hormone modifying enzymes in a biological system.

Lipid soluble vitamins

Vitamin A and D regulate gene expression by binding to their nuclear receptors the retinoic acid receptor (RAR) and vitamin D receptor (VDR), respectively.[63] 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.[5] A radioactive photoaffinity probe and a radioactive affinity-based probe based on vitamin D have been synthesized and applied to characterize the binding pocket of the vitamin D binding protein.[42,64]

These probes could be replaced by new ABPs with a diazirine as a less bulky photoactive group and addition of a click-handle to enable proteomics studies. A vitamin D-based ABP would enable the study of enzymes involved in the metabolism of vitamin D, which could be used to study its involvement in immunological processes.

In the case of vitamin A and its metabolites, direct photoaffinity labeling with radioactive retinoic acid was enabled by its inherent photoreactive characteristics.[65,66] A radioactive photoaffinity probe based on retinoic acid has been synthesized, but A*f*BPP has not been performed due to lack of a ligation handle for application in chemical proteomics studies.[67] The ABP for retinyl ester processing enzymes has not yet been tested outside of the retinal pigment epithelial membrane.[41] The application of this ABP in the field of immunology would be interesting as vitamin A is involved in the immunohomeostasis of the gut.[68] As these retinyl ester processing enzymes are involved in the metabolism of vitamin A, comparing their activity levels in intestinal epithelial cells in different disease models could indicate whether vitamin A is processed for storage in the liver or used to regulate the local immune response.[69–71] Furthermore, ABPs based on retinol, retinal and the immunologically active metabolite, retinoic acid (RA), would be valuable additions to this field as RA is involved in the differentiation and gut-homing of lymphocytes.[68,72] Probes based on these lipids would therefore enable research into the factors regulating their metabolism.

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.[73,74] Next to its antioxidant activity, it has also been implicated as a potential orphan nuclear receptor ligand.[63,75] The synthesis of a vitamin E derivative containing a diazirine and an alkyne click-handle would therefore be a potential 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 E biology.

Conclusion

Chemoproteomics using lipid-based probes is an invaluable strategy to study the biology of lipid messengers. A*f*BPP using photoaffinity probes can provide insight into the target interaction landscape of lipid messengers with previously unknown proteins, such as transporters and receptors, while ABPP using activity-based probes could identify the enzymes that control the metabolism of these important messengers in health and disease. 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 current set of lipid-based probes, however, does 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 are inherently instabile. Although chemists are still working on the total synthesis of these low abundant biologically active lipids and their probes, there is still a need for further elucidation of important cellular processes performed by lipids, especially in the field of immunology.[76,77]

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