Chapter 8

Summary and future prospects

The overarching aim of the research presented in this thesis was to develop and apply chemical tools to study lipid metabolism, transport and signaling.

Chapter 1 provided an overview of the diversity of lipids and highlighted bioorthogonal chemistry as a method for their investigation. Lipids are structurally and functionally diverse biomolecules that are difficult to study, particularly their localization and protein interaction partners. This is due to their small size, lipophilicity, and high rate of metabolism. Most analytical methods for lipids rely on sample homogenization and lipid extraction, which allows for sensitive detection of lipids, but discards spatial information.

Bioorthogonal chemistry has enabled the investigation on lipids in a more versatile manner. A small bioorthogonal ligation handle, such as an alkyne, can be introduced and conjugated to a reporter group through 'click' chemistry such as the copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC).[1] This has allowed the investigation of localization, metabolism, uptake and incorporation of the lipid as post-translational modification. Moreover, to capture noncovalent lipid-protein interactions, a photoactivatable group can be added. Photoactivatable groups can be irradiated to generate a reactive intermediate, which can form a covalent and irreversible bond with a lipid-interacting protein. The combination of a photoactivatable group and bioorthogonal ligation handles has propelled the investigation of

lipid-protein interactions. This approach holds promise for the investigation of polyunsaturated fatty acids (PUFAs) and their metabolites, which have become increasingly appreciated for their role as signaling molecules, especially in the immune system. However, the availability of chemical tools based on these lipids is limited, in part due to their synthetic difficulty.

Chapter 2 provided an overview of lipid-based photoaffinity probes developed in the last decade and their main discoveries. The first implementations of photoactivatable lipid probes contained radioisotopes or bulky fluorophores and were only capable of investigating interactions with a predetermined protein. In recent years, lipid probes have shifted to diazirine- and alkyne-containing structures, partly due to increased synthetic methodologies and commercial availability of compatible click reagents. The scope of projects involving lipid probes has also moved towards large-scale profiling of lipid-interacting proteins and using probes to tackle challenging biological questions. However, challenges in using lipid probes remain their lipophilicity and fast cellular metabolism. Due to this lipophilicity, many probe targets can be non-specific binding partners, which makes it difficult to identify specific interacting proteins. As a resource for future research, a list of promiscuous lipid-binding proteins is compiled in Chapter 2. Looking forward, improved synthetic accessibility—usually followed by commercial availability—of probes and expanding on their functionality will lead to improved understanding of the complex and multifaceted roles of lipids.

Chapter 3 described the synthesis and use of photoaffinity-click (pac)-probes based on the omega-3 fatty acid docosahexaenoic acid (DHA, 22:6 n-3, **1**) and its oxidized metabolite, 17-hydroxy-DHA (17-HDHA) in comparative photoaffinity-based protein profiling (AfBPP). DHA is oxidized through different pathways into biologically active metabolites that lower inflammatory response and signal for the resolution of inflammation.[2] 17-HDHA is an important intermediate in this process. Two photoaffinity probes, pac-DHA and pac-17-HDHA, were synthesized to investigate the specific interaction partners of 17-HDHA in human immune cells. Synthesis of pac-DHA was achieved by generation of the six cis double bonds inherent to DHA through a combination of partial hydrogenation of alkynes and Wittig reactions. To obtain pac-17-HDHA, the hydroxyl was introduced by enzymatic oxidation using soy bean lipoxygenase.

Using the two probes in comparative AfBPP in primary macrophages (Figure 1), prostaglandin reductase 1 (PTGR1) was identified and validated as a specific pac-17-HDHA binding target capable of converting 17-HDHA into 17-oxo-DHA. PTGR1 was shown to act as a metabolic hub in oxidative metabolism of PUFAs, simultaneously inactivating pro-inflammatory lipids and producing anti-inflammatory oxidative metabolites. Moreover, comparative AfBPP was shown to be an effective method to distinguish genuine probe targets.

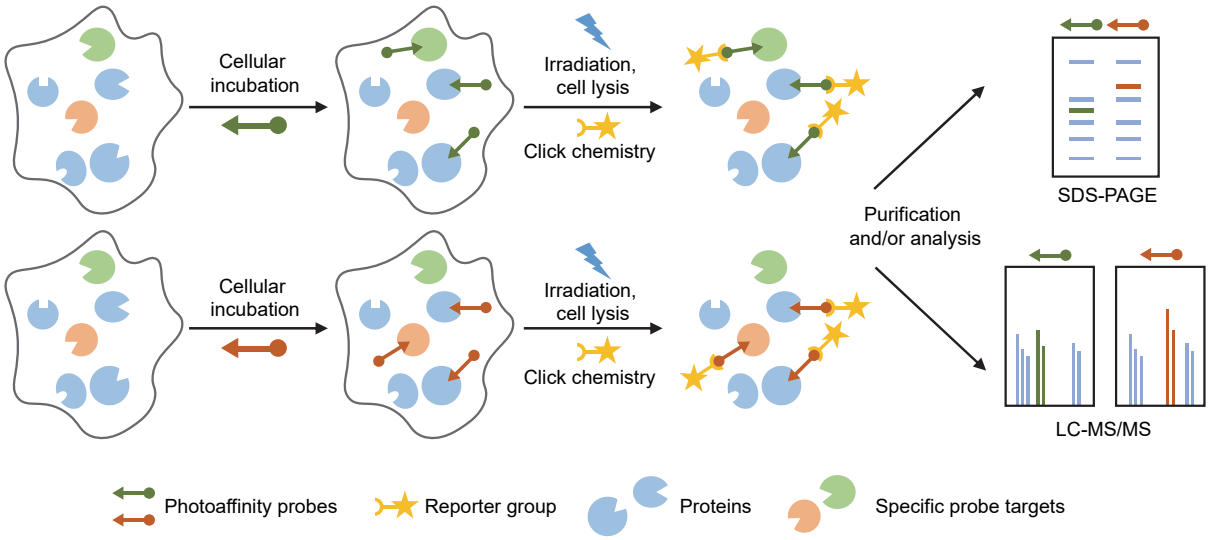


Figure 1 | Schematic representation of comparative AfBPP for lipid photoaffinity probes.

Neutrophils are key players in the acute inflammatory response and perpetuate the response via an autocrine loop that is regulated through the secretion of LTB4.[3] Murine neutrophils produce less 5-HETE and LTB4 after being exposed to 17-HDHA *in vivo*,[4] and previously similar results were found in isolated human neutrophils.[5] However, neutrophils have not been well-studied in the context of chronic inflammation despite their importance in the immune system.[6] The DHA- and 17-HDHA-based probes described in Chapter 3 were used to discover protein interaction partners in primary neutrophils in a similar approach to the M2 macrophages. Comparative AfBPP on neutrophils resulted in 29 UV-enriched targets of pac-17-HDHA (Figure 2, Table S1). The pac-17-HDHA probe was found to UV-enrich the LTB4 receptor and 5-lipoxygenase-activating protein (ALOX5AP), although ALOX5AP was equally enriched by both probes. These protein interaction partners are involved in the metabolism and function of LTB4, and constitute interesting targets for further investigation of the anti-inflammatory properties of DHA and 17-HDHA.

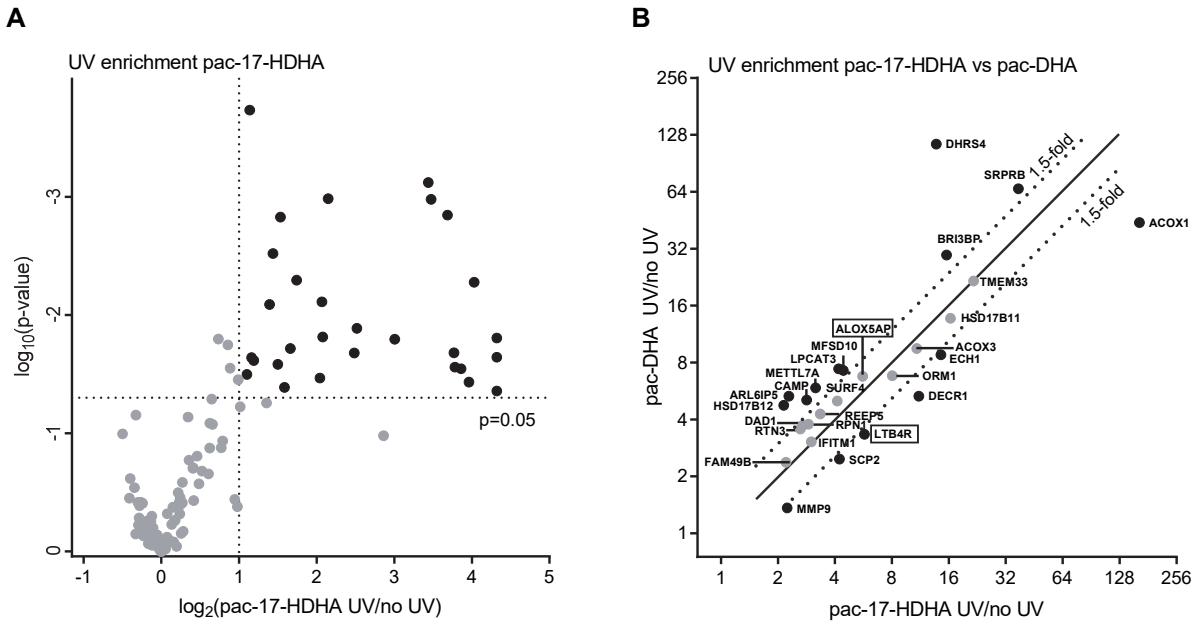


Figure 2 | Comparative AfBPP using pac-DHA and pac-17-HDHA in human neutrophils.

Chapter 4 described the use of a photoaffinity-click probe based on the neuroprotective and anti-inflammatory *N*-docosahexaenoylethanolamine (DHEA). pac-DHEA was synthesized and used to identify its protein binding partners in a microglial cell line. Eleven specific binding partners were identified using the comparative AfBPP approach. Three targets were investigated in further detail. NENF, an excreted neurotrophic factor, reduced inflammatory response, although not in combination with DHEA. Stable overexpression of APMAP and GSTM1 in microglial cells exacerbated LPS-induced inflammation as measured by NO and IL-6 production. These effects were reversed by pretreatment of the cells with DHEA, while DHA was less effective. These results indicate that DHEA confers anti-inflammatory effects through binding to these proteins, although their exact function remains to be established.

Although DHEA has been shown to have direct signaling functions,[7] anti-inflammatory oxidative metabolites have also been reported.[8,9] Oxidative metabolites of PUFAs are mainly detected in cells with high oxidative activity, such as macrophages. Since the binding of pac-DHEA to GSTM1 in microglia could not be recapitulated in an overexpression system, this may suggest that an oxidative metabolite may be responsible for the anti-inflammatory effects of DHEA via GSTM1.

Chapter 5 described an improved, facile method for synthesis of an alkyne-functionalized DHA analog, cDHA (**2**, Figure 3). Synthesis of PUFA derivatives by partial hydrogenation of skipped alkynes suffers from formation of undesired *E*-alkenes, poor reproducibility and overhydrogenated products.[10] In this chapter, an alternative synthetic route for cDHA (**2**) starting from DHA is developed. This clickable lipid was used to visualize DHA metabolism and cellular exchange using thin-layer chromatography and flow cytometry.

This synthetic methodology can be expanded to other PUFA derivatives. For example, conjugation of aldehydes **3** and **4** to isotopically labelled phosphonium salts would afford internal standards **5**, **6** and **7** for mass spectrometry analysis (Figure 3). Moreover, alkyne-functionalized derivatives of arachidonic (AA, 20:4 n-6, **8**) or eicosapentaenoic acid (EPA, 22:5 n-3) would be readily accessible using this synthetic approach, affording cAA (**9**) and cEPA (**10**) respectively.

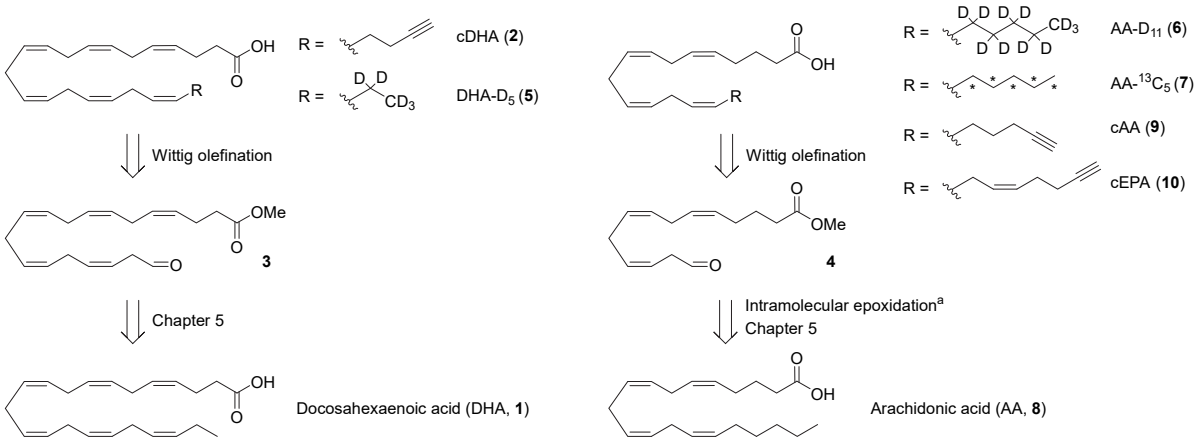


Figure 3 | Synthetic routes towards alkyne- or isotope-modified PUFAs. AA, arachidonic acid, DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; a[11,12]

Another application of cDHA (**2**) is the investigation of protein modification by electrophilic lipids.[13] PUFA oxidation results in the formation of many reactive products, such as aldehydes, α,β-unsaturated ketones and epoxides, which can react with nucleophilic residues of proteins, such as cysteines.[13] Reaction with a catalytic cysteine of an enzyme is often responsible for loss of activity, but a few proteins are actually activated by covalent modification, leading to downstream signaling.[14] Reactive lipids, such as epoxyeicosanoids or 17-oxo-DHA described in Chapter 3, have therapeutic potential in cardiovascular and inflammatory diseases.[15,16] Moreover, electrophilic lipids have a central role in a newly discovered form of cell death, ferroptosis.[17–19] Mapping of lipid electrophile-modified proteins has recently been demonstrated using alkyne-linoleic acid[20] and alkyne-arachidonic acid.[21] This can also be done using cDHA (**2**), which is demonstrated by the visualization of specific protein adducts in N9 microglia and RAW264.7 cells when compared to DHA (**1**) and click-palmitic acid (cPA, **11**) (Figure 4). The cDHA (**2**) reported in this thesis enables the investigation of the proteins modified by electrophilic DHA derivatives.

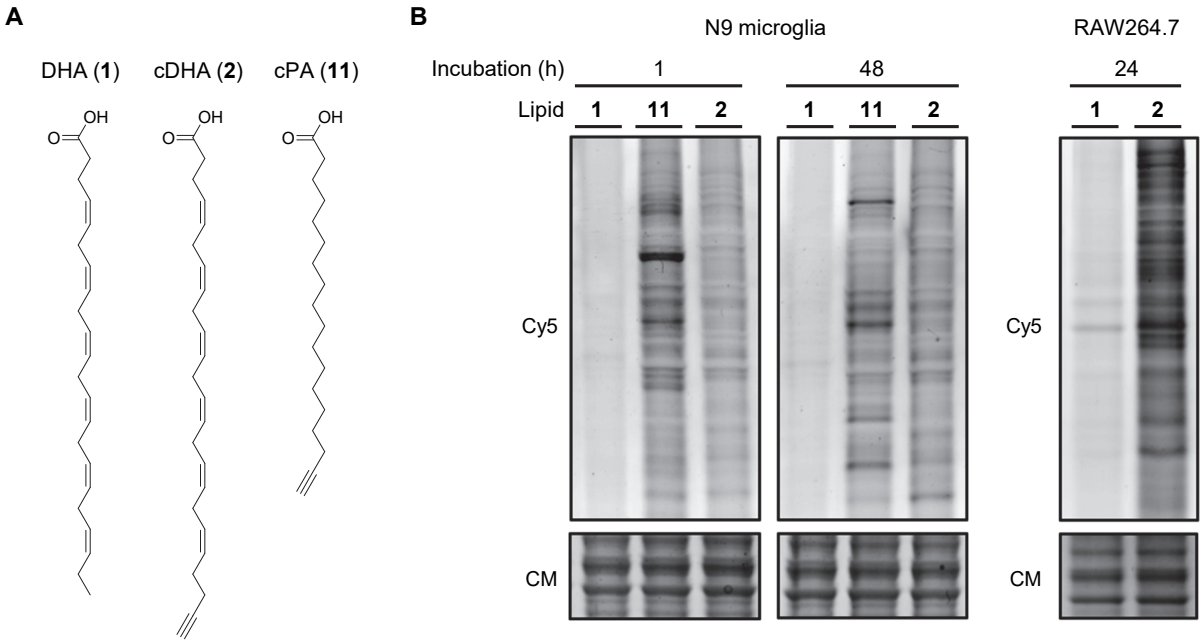


Figure 4 | Mapping of lipid-modified proteins. (A) Structures of DHA (**1**), cDHA (**2**) and cPA (**11**). **(B)** Labeling pattern of proteins derived from N9 microglia and RAW264.7 cells treated with 10 μM indicated lipid for 1, 24 or 48 h. Cells were lysed, reversible conjugates were reduced using $NaBH_4$[13,20] and lipid-bound proteins were conjugated to Cy5-N_3 using CuAAC chemistry and analyzed by SDS-PAGE and in-gel fluorescence scanning. Coomassie served as a protein loading control.

Chapter 6 describes the characterization of an anandamide (AEA) reuptake inhibitor. Anandamide, or *N*-arachidonoylethanolamine, is a lipid signaling molecule in the endocannabinoid system (ECS) and modulates neurotransmitter release via activation of the cannabinoid receptor type 1 (CB1R).[22] Although it is interesting to target the ECS for therapeutic purposes, an AEA transport protein has not been identified.[23–27] Recently, WOBE437 has been reported as a novel, natural product-based AEA reuptake inhibitor.[28] In this thesis, WOBE437 was found to surprisingly increase AEA uptake in Neuro-2a cells, which was accompanied by reduced cellular levels of AEA and related *N*-acylethanolamines (NAEs). A photoaffinity-click probe based on WOBE437 identified SCCPHD, VAT1 and FECH as WOBE437-interacting proteins. However, further genetic studies indicated that SCCPDH and VAT1 were not responsible for the WOBE437-induced reduction in NAE levels. Further exploration of the mechanism of action of WOBE437 should investigate the drop in cellular NAE levels found in this thesis. This observation conflicts with the hypothesis of inhibition of an endocannabinoid membrane transporter, and indicates more complicated processes could be involved.

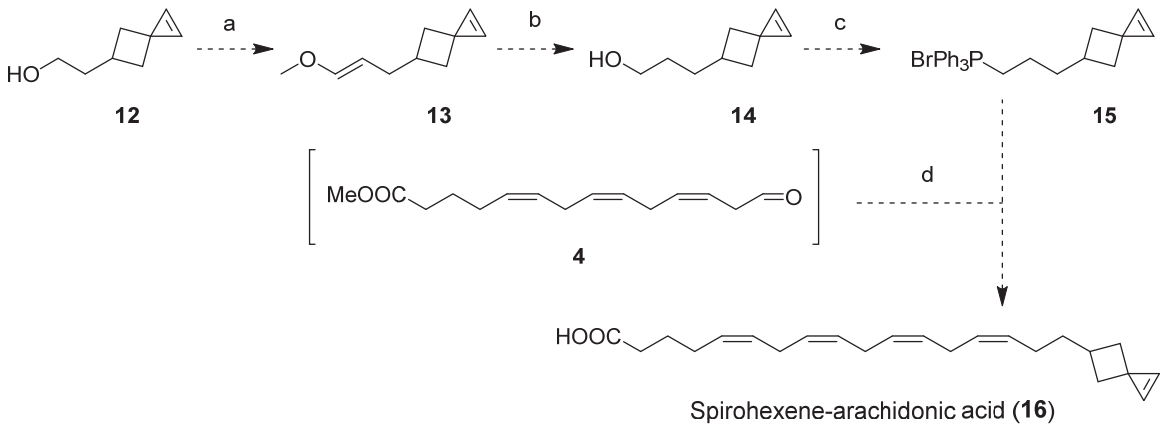
Another path to explore would be the use of chemical biology tools to gain more information on the process of AEA uptake. The current method to measure uptake, using radiolabeled AEA, does not take into account metabolism or excretion, measuring only a single endpoint. Moreover, the use of radiolabeled AEA is costly and requires special equipment. A more versatile tool would be alkyne-tagged AEA, which could be used to measure uptake and

exchange[30] as well as metabolism[31,32] and localization.[33] This approach would result in a better understanding of the process of AEA uptake. A facile synthetic route would be the selective epoxidation of the terminal alkene of arachidonic acid[11,12] followed by the steps outlined in Chapter 5 (Figure 3).

Notwithstanding the versatility of alkyne-tagged lipids, the required copper catalyst excludes their use in living cells. This problem is addressed in **Chapter 7**, which describes the synthesis of cyclopropene-modified arachidonic acid and use of 1,2-substituted cyclopropene lipids for live-cell imaging. Cyclopropenes act as dieneophiles in an inverse-electron demand Diels–Alder (IEDDA) reaction with tetrazine.[34,35] A cyclopropene lipid found in nature, sterculic acid, was tested in U2OS cells using a tetrazine-quenched BODIPY fluorophore. This resulted in successful IEDDA reaction in the cells as indicated by an intense fluorescent signal with a good signal-to-noise ratio, which was due to the fluorogenic property of the tetrazine-quenched BODIPY. Motivated by these results, cyclopropene-tagged arachidonic acid was synthesized through partial hydrogenation of three skipped alkynes and addition of the cyclopropene through nucleophilic substitution.

Although it was possible to synthesize and isolate cyclopropene-tagged arachidonic acid, it proved to be unstable. This instability is likely due to ene chemistry which would explain the observed tendency for degradation under solvent- and oxygen-free conditions.[36,37] Future use of cyclopropene-tagged arachidonic acid and its derivatives should take these properties in consideration and minimize handling steps.

Another possibility would be to use 3,3-substituted cyclopropenes, as they are more stable yet more reactive towards tetrazines in IEDDA reactions (Scheme 1).[38] Elongation[39] of reported spirohexene **12**[38] followed by Wittig reaction to aldehyde **4** would afford spirohexene-arachidonic acid **16** with all four alkenes intact, although it remains to be seen if this modification is tolerated for the investigation of arachidonic acid and derivatives in live cells.



Scheme 1 | Synthetic route towards spirohexene-tagged arachidonic acid 16. Reagents and conditions: (a) DMP, DCM, then added to $MeOCH_2PPh_3Cl$, KO*t*Bu, THF; (b) aq. HCl, then $NaHCO_3$, $NaBH_4$; (c) CBr_4, PPh_3, DCM, then PPh_3, ACN; (d) KO*t*Bu, THF, then aq. LiOH.

Challenges and opportunities for lipid probes

Lipids are a diverse class of biomolecules that serve a multitude of roles in the cell, from energy storage and membrane architecture to serving as signaling molecules in numerous cellular processes. The cellular distribution of lipids and their role in signaling events is regulated by a complex network of transport proteins and metabolic enzymes. Although technical advances in analytical chemistry have allowed for the detection and quantification of many lipid metabolites, unraveling the network of proteins and cellular processes involved is still a difficult task. This is especially true for polyunsaturated fatty acids and their metabolites, many of which have been found to have immunomodulatory signaling functions through unknown targets, while being processed through interweaving metabolic pathways. For concurrent investigation of localization, metabolism, intercellular exchange and signaling functions, innovative approaches are required.

Chemical biology offers a solution to this problem, as multiple aspects of lipid biology can be studied using the same chemical tool. Alkyne-modified lipids, for example, are versatile tools to study lipid localization and transport using fluorescence microscopy[33] and flow cytometry,[30] metabolism by LCMS[40] or thin-layer chromatography,[40] as well as incorporation as post-translational modification[41] and reaction with proteins by reactive oxidative metabolites.[20,21] Since protein modification by these reactive species is an important aspect in ferroptosis, chemical tools will be valuable to study this newly discovered form of cell death.[14,18] These tools would benefit from increased synthetic and commercial availability, along with approachable procedures to employ them. This will enable their use in labs with different areas of expertise–as has happened with tools to profile serine hydrolases–to tackle questions in lipid biology in a multidisciplinary fashion.[42,43]

Photoaffinity probes have proven to be powerful tools to study lipid-protein interactions, which is highlighted by the diversity of reported probes discussed in Chapter 2. However, lipid photoaffinity probes have a 'blind spot' for high-affinity interactions with low-abundant proteins, such as G-protein coupled receptors.[44] Although appropriate control experiments can distinguish specific interactions, abundant lipid-interacting proteins will quickly saturate the analytical capacity of gel- or mass spectrometry-based approaches. Technological advances in mass spectrometry analysis will mitigate this problem, as identification coverage of probe-bound proteins will increase. Moreover, optimization of experimental conditions can be guided by prior biological knowledge to improve analysis of relevant probe targets. For example, when investigating cytosolic, the proteome can be prefractionated by centrifugation and removal of membrane proteins.[45]

The next step for lipid probes is the study of signaling events in living cells, but more challenges need to be overcome. As the CuAAC reaction requires a cytotoxic metal catalyst, other bioorthogonal chemistry is required. However, selectivity and reaction speed are crucial in order to visualize dynamic processes.[46] Inverse-electron demand Diels-Alder (IEDDA) is a

bioorthogonal reaction that possesses sufficient selectivity and a high reaction speed, and is possible with strained cyclopropenes as the dienophile.[47] The use of cyclopropenes as a bioorthogonal handle shows potential for the investigation of signaling lipids, as it is a minimally intrusive modification. The use of IEDDA for real-time imaging of lipid metabolism has been demonstrated using a method developed by Dr. Baskin and coworkers, termed IMPACT.[48,49] This thesis provides evidence that 1,2-substituted cyclopropenes are capable of visualizing lipids in live cells and are deserving of further examination.

Another challenge is the metabolic fate of exogenous lipids, which may not mimic the subcellular distribution of lipids synthesized by the cellular machinery.[50] A promising approach is the addition of a caging group, which can be released upon irradiation.[51–53] This allows for unbiased distribution and spatiotemporally controlled release of a signaling lipid,[52] and this approach has been applied to photoactivatable lipids to afford trifunctional probes.[54] As these probes are extremely versatile tools, extension of this approach to other lipids will be of great value for the investigation of lipids as signaling molecules.

Conclusion

In this thesis, a number of chemical tools are reported which enable the study of different aspects of lipid biology. The focus was the study of polyunsaturated fatty acids, which are underrepresented in the field of chemical biology due to the synthetic challenge posed by their derivatives. The photoaffinity probes reported in Chapters 3 and 4 as well as the comparative AfBPP approach were used to discover new lipid-protein interactions and to provide more background for the anti-inflammatory properties of DHA and its metabolites. The synthetic method developed in Chapter 5 allows for improved accessibility of DHA derivatives and demonstrated the versatility of clickable lipids. The photoaffinity probe reported in Chapter 6 allowed for target discovery of an inhibitor of anandamide reuptake, although outstanding questions remain about this process. The first use of 1,2-substituted cyclopropenes for live-cell imaging of lipids is reported in Chapter 7, as well as the synthesis of cyclopropene-tagged arachidonic acid for future investigation of anandamide in real-time.

Overall, the use of chemical tools is a versatile approach for the investigation of lipid biology and the increasing repertoire of tools and techniques in chemical biology will lead to a greater understanding of lipids as signaling molecules.

Experimental procedures

General

Lipids were purchased from Cayman Chemicals and stored as 10 mM ethanolic stocks under nitrogen at -80 °C. Cy5-N_3 and cPA (**11**) were prepared as previously reported in literature.[55,56] All cell culture disposables were from Sarstedt. All other reagents were purchased from Sigma Aldrich or Cayman Chemicals unless otherwise specified.

Cell culture

RAW264.7 cells were cultured at 37 °C under 7% CO_2 in DMEM (Sigma Aldrich, D1145) containing phenol red, stable glutamine, 10% (v/v) New Born Calf Serum (Thermo Fisher) and penicillin and streptomycin (200 µg/mL each, Duchefa). Cells were passaged twice a week at 80-90% confluence by scraping and resuspension in fresh medium. N9 microglia were cultured at 37 °C under 5% CO_2 in RPMI 1640 (Sigma) containing phenol red, stable glutamine, 10% (v/v) sterile-filtered Fetal Calf Serum (Thermo Fisher) and penicillin and streptomycin (200 µg/mL each, Duchefa). Cells were passaged every two days by washing with PBS and trypsinization, subsequently quenching the trypsin with medium and removal of the trypsin by spinning down the cell suspension (200 *g*, 5 min). The resulting pellet was resuspended in culture medium by vortexing and subsequently seeded on new plates. Cells were tested regularly for mycoplasma contamination. Cultures were discarded after 2-3 months of use.

SDS-PAGE analysis of protein lipidation

For gel-based profiling, cells were plated on 6-well plates and treated with 10 µM of indicated lipid for the indicated time. Then, cells were washed with PBS (1 mL) and harvested by pipetting and scraping into ice-cold PBS (1 mL), then pelleted by centrifugation (1,000 *g*, 10 min, 4 °C). The supernatant was removed and the cells were lysed by resuspension in lysis buffer (250 mM sucrose, 20 mM HEPES pH 7.5, 1 mM $MgCl_2$) and sonication in a bath sonicator (10 s, 0 °C). Protein concentration was measured by Qubit™ assay (Invitrogen) and the samples were adjusted to 1.5 mg/mL and a volume of 100 µL, after which reversible adducts were reduced[13,20] using $NaBH_4$ (5 mM, 45 min, 4 °C) which was quenched with acetone (1 µL, 15 min, 4 °C). Then, the samples were treated with 10.95 µL click mix (5.5 µL aq. 25 mM $CuSO_4$, 3.25 µL aq. 250 mM NaAsc, 1.1 µL 25 mM THPTA in DMSO, 1.1 µL 0.9 mM Cy5-N_3 in DMSO) at rt for 1 h. Then, samples were quenched with 4X Laemmli buffer, boiled (5 min, 95 °C) and resolved by SDS-PAGE (10% acrylamide gel, ±80 min, 180 V) along with protein marker (PageRuler™ Plus, Thermo Fisher). In-gel fluorescence was measured in the Cy3- and Cy5-channel (Chemidoc™ MP, Bio-Rad) and the gels were subsequently stained with Coomassie and imaged as a loading control.

AfBPP analysis of neutrophils

Neutrophils, harvested as described in Chapter 3, were isolated in DPBS with $MgCl_2$ and $CaCl_2$ (D8662, Merck) and divided over two 50 mL falcon tubes, centrifuged (800 *g*, 5 min, no brakes) and resuspended in DPBS supplemented with 0.1% (w/v) delipidated BSA (15 mL) with pac-DHA or pac-17-HDHA (10 µM from 10 mM ethanolic stock) by careful vortexing. Next, neutrophils were incubated at 37 °C for 25 min. The cells were pelleted by centrifugation (800 *g*, 5 min, no brakes), the supernatant was aspirated and the cells were resuspended in ice-cold PBS (6.3 mL) by careful vortexing. The cells were distributed over 12-well plates (1 mL portions) and were irradiated at using a Caprobox™ (10 min, 4 °C, 350 nm, "UV") or exposed to ambient light (10 min, 4 °C, "No UV"). The cells were then transferred to 1.5 mL tubes, spun down (10 min, 1,000 *g*) and the supernatant was aspirated. The cells were then lysed with lysis buffer (250 µL, 250 mM sucrose, 2X protease inhibitor cocktail (Roche), 20 mM HEPES pH 7.5, 1 mM $MgCl_2$). This was sonicated (Branson Sonifier probe sonicator, 10 x 1 s pulses, 10% amplitude). Protein concentration was measured by Qubit™ assay (Invitrogen) and the samples were adjusted to 0.6 mg/mL and a volume of 400 µL. This lysate is subjected to proteomic analysis as described in Chapter 3 under 'Mass spectrometric analysis of tryptic peptides, identification and quantification'.

Supplementary data

Table S1 | Proteins significantly UV-enriched by pac-17-HDHA in neutrophils.

Gene name	Unique peptides	Description	UV/no UV pac-17-HDHA	p-value	UV/ no UV pac-DHA	Enrichment pac-17-HDHA/ pac-DHA
ACOX1	7	Peroxisomal acyl-coenzyme A oxidase 1	163.14	0.0437	43.95	3.71
SRPRB	7	Signal recognition particle receptor subunit beta	37.36	0.0156	66.43	0.56
TMEM33	4	Transmembrane protein 33	21.62	0.0227	21.60	1.00
HSD17B11	6	Estradiol 17-beta-dehydrogenase 11	16.32	0.0053	13.71	1.19
BRI3BP	2	BRI3-binding protein	15.57	0.0367	29.58	0.53
ECH1	5	Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial	14.53	0.0283	8.79	1.65
DHRS4	2	Dehydrogenase/reductase SDR family member 4	13.75	0.0275	114.23	0.12
DECR1	2	2,4-dienoyl-CoA reductase, mitochondrial	11.12	0.0010	5.31	2.09
ACOX3	4	Peroxisomal acyl-coenzyme A oxidase 3	10.83	0.0008	9.48	1.14
ORM1	4	Alpha-1-acid glycoprotein 1	8.04	0.0160	6.82	1.18
LTB4R	4	Leukotriene B4 receptor 1	5.73	0.0129	3.33	1.72
ALOX5AP	6	Arachidonate 5-lipoxygenase-activating protein	5.60	0.0208	6.76	0.83
MFSD10	3	Major facilitator superfamily domain-containing protein 10	4.44	0.0010	7.27	0.61
SCP2	6	Non-specific lipid-transfer protein	4.23	0.0153	2.47	1.71
LPCAT3	2	Lysophospholipid acyltransferase 5	4.19	0.0077	7.40	0.57
SURF4	4	Surfeit locus protein 4	4.12	0.0340	5.00	0.82
REEP5	3	Receptor expression-enhancing protein 5	3.35	0.0051	4.28	0.78
METTL7A	2	Methyltransferase-like protein 7A	3.16	0.0191	5.87	0.54
IFITM1	2	Interferon-induced transmembrane protein 1	3.00	0.0408	3.04	0.99
RPN1	7	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1	2.90	0.0015	3.78	0.77
CAMP	7	Cathelicidin antimicrobial peptide	2.83	0.0260	5.06	0.56
DAD1	2	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit DAD1	2.71	0.0030	3.72	0.73
RTN3	3	Reticulon-3	2.63	0.0081	3.56	0.74
ARL6IP5	2	PRA1 family protein 3	2.28	0.0241	5.30	0.43
MMP9	9	Matrix metalloproteinase-9	2.24	0.0229	1.36	1.65
FAM49B	2	Protein FAM49B	2.20	0.0002	2.37	0.93
HSD17B12	5	Very-long-chain 3-oxoacyl-CoA reductase	2.15	0.0317	4.76	0.45

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