Review

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Research Advances Through Activity-Based Lipid Hydrolase Profiling

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Abstract: Activity-based proteomic profiling (ABPP) enables the functional study of enzymes by employing small molecule probes that bind covalently to the active site of an enzyme. Activity-based probes can penetrate cells and tissues and thereby allow enzymes to be targeted/labelled in their native state. Probes can be designed to target individual enzymes or whole enzyme groups, which makes ABPP a versatile protein profiling technique. In this review, we give an overview of research advances through ABPP in the context of lipid hydrolase research. We report of lipid hydrolases that were discovered and characterized through ABPP, and aim to give an overview of commonly used probes as well as inhibitors that were discovered and characterized by competitive ABPP. Lastly, this review aims to raise caveats and current limitations of this protein profiling technique.

Keywords: ABPP · functional proteomics · lipid hydrolase · serine hydrolase

1. Introduction

Enzymes are macromolecules that act as a catalyst to enable essential biochemical reactions. Without them the complex interplay between the molecules that make up our cells and enable life would not be possible.[1] The spectrum of reactions that can be carried out by enzymes is vast, ranging from one-step reactions to more complex processes. While some enzymes can act alone, others require cofactors like ions or coenzymes.[1] The activity of some enzymes can further be influenced through post-translational modifications, such as phosphorylation or acetylation, or through interaction with other macromolecules such as co-activators or inhibitors, as well as their subcellular localization.[2] This gives rise to a discrepancy between protein abundance and enzymatic activity. Likewise, numerous studies have found that there is only weak correlation between mRNA abundance and protein abundance reflecting divergent stabilities of corresponding mRNA and proteins.[3] While studying gene expression (qPCR, transcriptomics) or protein abundance (western blot, proteomics) can be informative, a major caveat of these approaches is the poor correlation to the enzymatic proteome, i.e., the enzymatic activities of proteins.

In an effort to enable the study of protein functionality as opposed to abundance, activity-based proteomic profiling (ABPP) has emerged as a robust tool to study enzymatic activities in their native environments.[4] By employing small molecule probes that are directed towards enzymatic active sites, and subsequent identification and quantitation by mass spectrometry, this tool permits the investigation of whole enzyme classes.[5] It also enables the identification of novel enzymes based on active site similarity, as well as the identification and characterization of enzyme inhibitors.[6] Another asset of this technique is its applicability to native biological systems.[7] The employed activity-based probes (ABP) can be fine-tuned to one or a few enzymes, but more promiscuous probe designs enable investigation of many enzymes at once. A prominent example of probes that target a whole enzyme group are the fluorophosphonate probes published by the Cravatt group for the first time in 1999.[5] Phosphonate probes target serine hydrolases by mimicking the transition state of their native substrates. The predominant majority of enzymes breaking down lipids (lipid hydrolases/lipases) – many of which are also post-translationally regulated – are members of the serine hydrolase family. Therefore, the use of phosphonate probes and ABPP in general

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is well suited for the study of lipid hydrolases and lipid metabolism.

Lipids play fundamental roles in mammalian cells. Virtually every cell in mammals is surrounded by a lipid bilayer, and intracellular lipids are used as building blocks for bio-membranes, as an energy source or as signalling molecules.[8] Accordingly, impairments in lipid metabolism can cause severe problems resulting in different diseases ranging from cardiovascular diseases to diabetes to some forms of cancer. In recent years, the study of intracellular fatty acid mobilization has gained interest and activity-based proteomic profiling has enabled the identification of novel lipases and lipid hydrolase inhibitors. In this review we aim to give an overview of recent advances in the investigation of lipid hydrolases with a focus on ABPP methods.

2. Activity-Based Lipid Hydrolase Profiling

2.1 Lipid Hydrolases and Their Mechanism of Action

Lipid hydrolases belong to the large enzymatic family of carboxylic ester hydrolases (EC 3.1.1) which includes following subclasses: carboxylesterases (EC 3.1.1.1), arylesterase (3.1.1.2), triacylglycerol lipases (EC 3.1.1.3), phospholipases A2 (EC 3.1.1.4), lysophospholipases (EC 3.1.1.5), acetylesterases (EC 3.1.1.6), acetylcholinesterases (EC 3.1.1.7) and cholinesterases (EC 3.1.1.8).[9] In general, lipid hydrolases are a versatile group of enzymes, characterized by their ability to cleave hydrophobic esters (such as triglycerides), thereby releasing free fatty acids.[10] Although the term "lipases" is often referred to the enzymatic subclass of neutral lipid hydrolases (EC 3.1.1.3), enzymes capable of hydrolysing lipids can be found also in other subclasses of the carboxylic ester hydrolase superfamily.[11]

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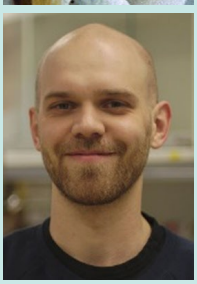

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Ruth Birner-Gruenberger is Full Professor at TU Wien and Associate Professor at Medical University of Graz. She has developed and applied ABPP of lipid hydrolases for 20 years and employs proteomics and metabolomics to study the regulation of lipid and energy homeostasis in health and lipid-associated diseases.

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Given their role in hydrolysis of mainly water-insoluble molecules, lipases often act on the border of the two phases i.e., the water-lipid interphase. Though this unique property enables the emulsification of otherwise insoluble molecules, it also renders them particularly challenging enzymes for direct activity studies, as their kinetics is affected by both physical and chemical properties of the interphase.[12]

In the context of substrate specificity of a lipase, the following information is commonly summarized to characterize the selectivity of a given lipase:

(1) the type of lipid that is hydrolysed (e.g., tri- (TG), di- (DG), mono-acylglycerols (MG), cholesteryl esters (CE), or retinyl esters (RE));
(2) the position and/or orientation of the fatty acid in the molecule or region (e.g., *sn* position or R/S orientation);
(3) the selection of groups of similar fatty acids (e.g., longer or shorter chain fatty acids).[13]

Inside of a cell, the process of hydrolysis of complex lipids is often mediated by an enzymatic cascade rather than by a singular enzyme. The hydrolysis of neutral lipids such as TG, which are commonly stored in cytosolic lipid droplets (LD), involves adipose triglyceride lipase (ATGL; *PNPLA2*), hormone sensitive lipase (HSL; *LIPE*), as well as monoacylglycerol lipase (MGL; *MGLL*) in a catalytic cascade. The first enzyme in this pathway, ATGL, produces DG from TG resulting in the release of a free fatty acid (FA). The generated DG is further hydrolysed to MG and FA by HSL, which can also hydrolyse TG and CE, however, to a lesser extent. In the last step, MG is broken down into glycerol and FA by MGL (Figure 1A).[8]

While ATGL is regarded as the rate-limiting enzyme of lipolysis in adipocytes (controlled by its co-activator CGI58 as well as its inhibitor G0/G1 switch 2 (G0S2)[14]), in other tissues such as liver and the intestine, TG might also be degraded by enzymes classified as members of the carboxylesterase family (EC 3.1.1.1; such as CES3 in mice and CES1/2 in humans).[15] Furthermore, in lysosomes, TG, CE and RE are degraded predominantly by lysosomal acid lipase (LAL; *LIPA*) (Figure 1B).[16] In addition to HSL, in brain and pancreas, DG can also be hydrolysed by diacylglycerol lipase α (DAGLA), while in bone marrow and liver DAGL β (DAGLB) is the major DG lipase.[17] The main role of DAGL enzymes is the production of the neuro-modulatory endocannabinoid 2-arachidonoylglycerol (2-AG) which, as an MG, is also an evident substrate for MGL.[17b] Next to MGL, a number of other MG lipases have been reported, including α/β hydrolase domain containing protein-6 (ABHD6) and α/β hydrolase domain containing protein-12 (ABHD12).[8, 18] Another important lipase group involved in lipid signalling are phospholipases, including phospholipases A1, A2, B, C and D.

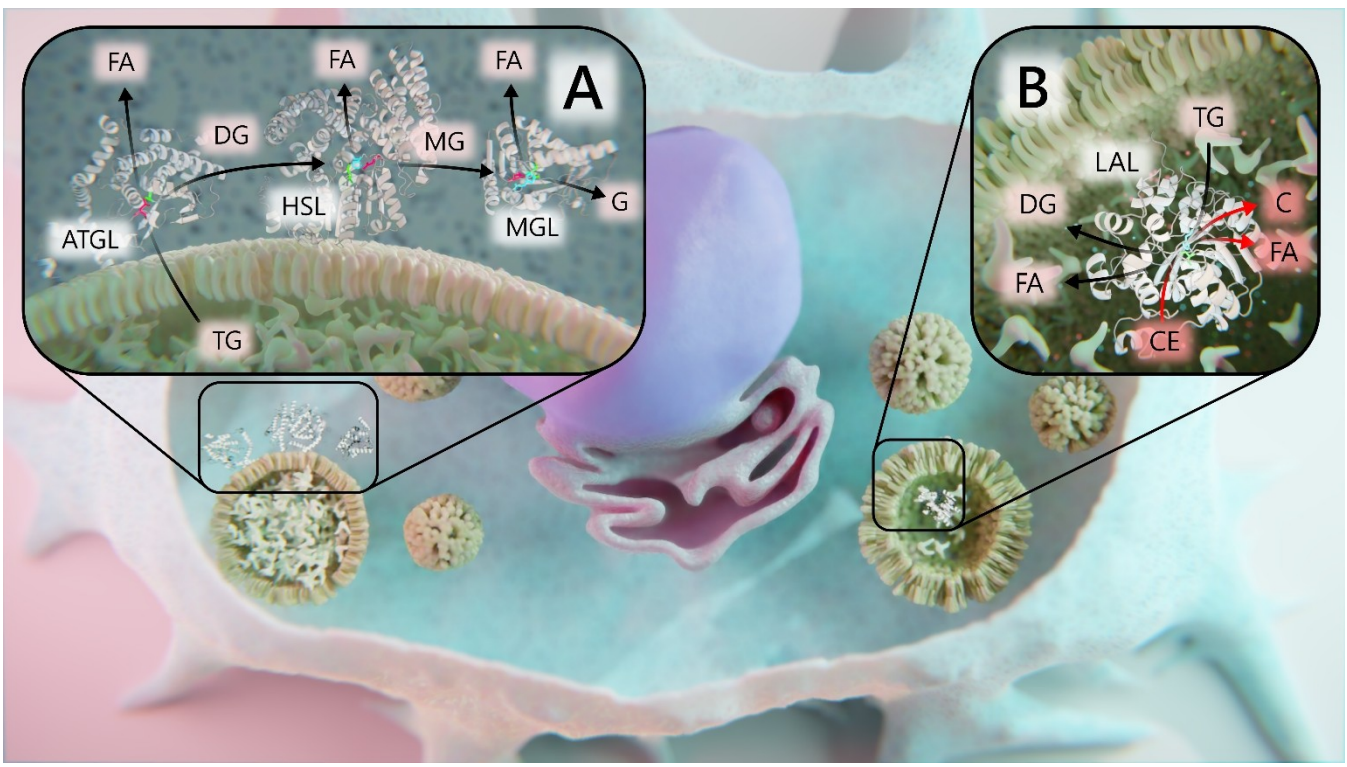

Figure 1. Location and enzymatic function of several lipid hydrolases. TG: triglyceride, DG: diglyceride, MG: monoglyceride, FA: free fatty acid, CE: cholesterol ester, C: cholesterol, G: glycerol. (A) Lipolytic cascade on lipid droplets from left to right: adipocyte triglyceride lipase (ATGL; *PNPLA2*) hydrolysing TG; hormone sensitive lipase (HSL; *LIPE*) hydrolysing DG; monoglyceride lipase (MGL; *MGLL*) hydrolysing MG. (B) Lysosome located lysosomal acid lipase (LAL; *LIPA*) hydrolysing TG and CE.

A1, A2 and B are acyl hydrolases, while C and D are phosphodiesterases. Phospholipases B cleave FA from diacyl- or lysophospholipids at *sn*-1 and *sn*-2 positions, while Phospholipases A1 hydrolyse phospholipids at *sn*-1 position only. Phospholipases A2, arguably the most prominent subclass of phospholipases, cleave polyunsaturated FA from *sn*-2 position of phospholipids, releasing precursors for synthesis of eicosanoids (and other signalling lipids) which, together with residual lysophospholipid moieties, can affect a variety of biological processes.[19]

As indicated by the name of some of the enzymes, most lipid hydrolases share a distinct α/β-hydrolase fold with conserved active site signatures. With only a few exceptions (e. g., phospholipases C and D[20]), lipid hydrolases belong to the large enzymatic superfamily of serine hydrolases, which accounts for more than 200 members and spans across different enzymatic subfamilies.[17b] All serine hydrolases are characterized by the presence of a serine as the main nucleophile in the active site, which is embedded in the conserved pentapeptide G–X–S–X–G. There is, however, variety in participating amino acids that make up the catalytic core of the enzyme. In addition to the nucleophilic serine, many lipid hydrolases carry a catalytic acid (Asp or Glu) and/or a histidine residue in a highly conserved geometry, forming a catalytic triad (Ser–Asp/Glu–His) or catalytic dyad (Ser–Asp or Ser–His).

The mechanism of action of serine lipid hydrolases is a nucleophilic attack of the catalytic serine on the substrates' carbonyl group (e. g., on a triglyceride). If present in the active site, histidine can accept the leaving proton of the hydroxyl group of serine. An oxyanion tetrahedral transition state (TS1) is formed, which is stabilized by the hydrogens of two backbone amine groups, while aspartate or glutamate additionally stabilize the protonated histidine. Electron rearrangement results in a more stable acyl-enzyme intermediate, while an alcohol, amine or thiol group is cleaved. In aqueous environment a second nucleophilic attack occurs on the carbonyl group of the newly formed serine acyl-enzyme (serine-substrate complex), causing the release of a fatty acid and restoration of the catalytic serine.[20] The activity of a lipase is in most cases regulated with a "lid", a flexible element consisted of one or two short α-helices able to hide or expose the substrate binding site.[21] As activity-based profiling of lipid hydrolases is predominantly based on employing serine hydrolase probes, they will therefore be the focus of this review.

2.2 Activity-Based Probes Targeting Lipid Hydrolases

The key element of activity-based proteomic profiling are activity-based probes (ABP). These small molecule probes are designed to covalently modify the active site of the enzymes of interest, which allows for downstream applications such as identification, quantitation, or imaging of active enzymes.

The structure of an activity-based probe consists of several parts: a reactive group, a binding group (BG), a leaving group (LG) and a reporter tag or linker (RT/Linker) (Figure 2A).

The reactive group – or warhead – is the core of every activity-based probe and determines the reactivity with the enzyme or enzyme group. Over the last decades, a number of different electrophiles have been described as inhibitors or probes of serine hydrolases. These include phosphonates, isocoumarins, sulfonyl fluorides, beta-lactams and -lactones as well as triazole ureas. Phosphonate probes are selective towards serine hydrolases and are among the most common probes for broad screening of serine hydrolases. Fluorophosphonate probes (e.g. biotinylated fluorophosphonate (FP-biotin (Figure 2B)) or the fluorescent FP-TAMRA) were first described by the Cravatt group in 1999,[5] and while they still find application today their toxicity does not permit enzyme tagging in living cells. Aryl-phosphonate probes have emerged as less toxic alternative to target lipid hydrolases,[22] allowing labelling in living cells. While 4-chloro-isocoumarins have primarily been used as probes for serine proteases in the past,[23] some have been proposed as inhibitors for pancreatic cholesterol esterase (Figure 2B),[24] and more recently, a 4-chloroisocoumarin probe (Figure 2B) was developed to target acyl-protein thioesterases.[25] Sulfonyl fluorides have been known to covalently inhibit serine proteases for decades, but have found application in the realm of lipid hydrolases in more recent years. Tam and colleagues designed a triacylglycerol lipase-specific sulfonyl fluoride probe – M352 (Figure 2B) – for triacylglycerol lipase screening,[26] and competitive ABPP revealed a sulfonyl fluoride-based inhibitor – AM3506 (Figure 2B) – to be selective for fatty-acid amide hydrolase (FAAH).[27] Beta-lactam and -lactone probes have been widely used for screening of antibacterial proteins[28] and less for screening of lipid hydrolases. However, the pan-lipase inhibitor orlistat is based on a beta-lactone structure, as is the DAGLA inhibitor MB064 (Figure 2B) and its biotinylated counterpart MB108 that can be employed as a DAGLA-specific ABP.[29] Carbamates also make up an important class of serine hydrolase ABPs or inhibitors that can be fine-tuned to be highly selective for lipid hydrolase enzymes as exemplified by the ABP JW912 (Figure 2B), which is selective towards the MG lipases MGL and ABHD6.[30] Triazole ureas have been described as potent inhibitors against a plethora of serine hydrolases, including several lipases.[31] One example of a triazole urea inhibitor is the dual lysophospholipase 1/2 (LYPLA1/2) inhibitor ML211 (Figure 2B).[32] More recently triazole urea-derived N-acyl pyrazoles have been proposed as potential and selective inhibitors for serine hydrolases.[6e]

The highly nucleophilic serine residue in the active site of most lipid hydrolases mediates the nucleophilic attack on the electrophilic inhibitor or ABP,[33] first forming an oxyanion transition state, followed by a more stable enzyme-probe/inhibitor-complex (Figure 2A). In order to readily undergo this addition reaction, the probes or inhibitors are equipped with a good leaving group (LG) – such as p-nitrophenyl or fluoride –

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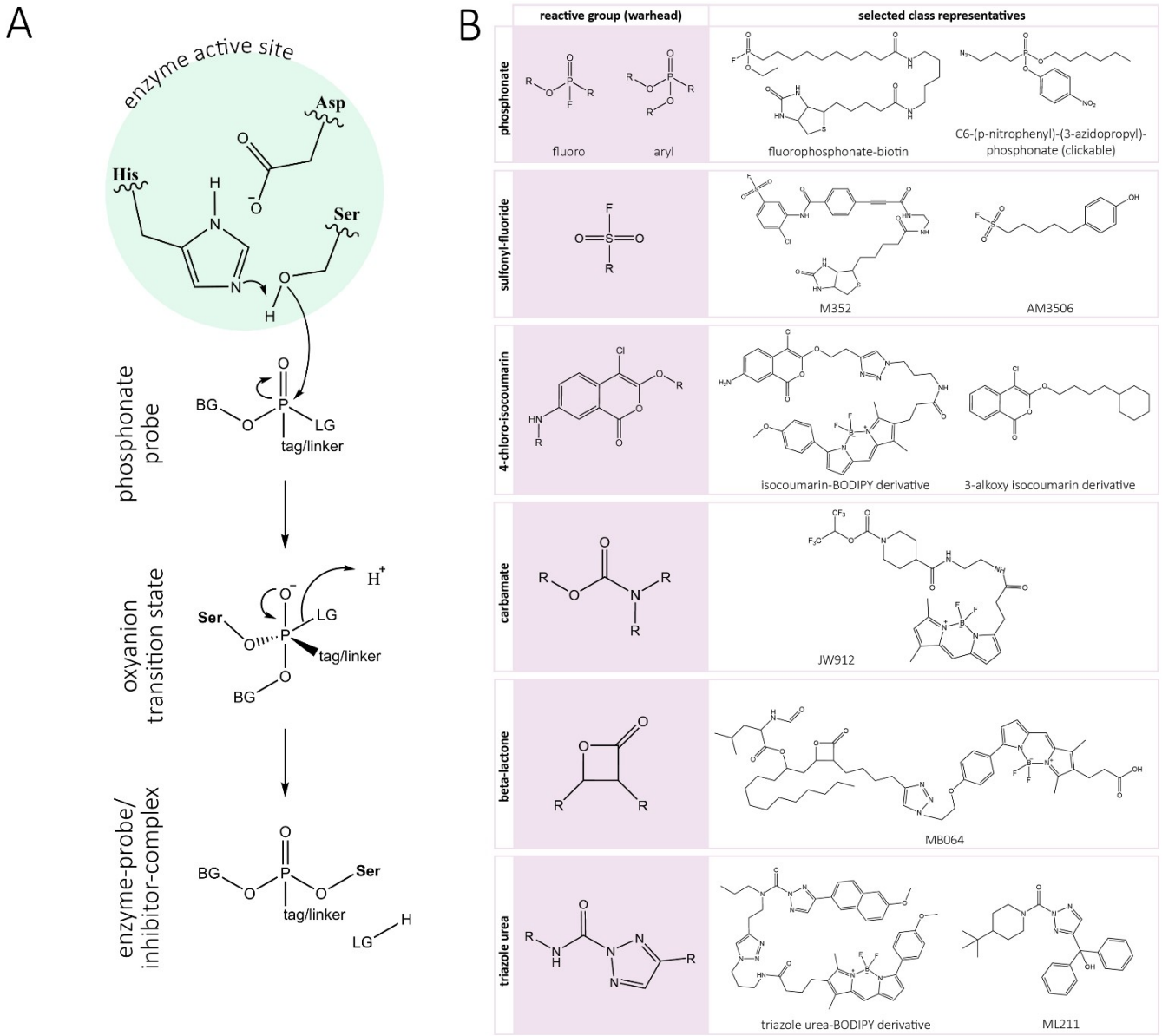

Figure 2. Serine hydrolase catalytic mechanism and structures of activity-based probes and lipase inhibitors (A) Serine-Aspartate-Histidine catalytic triad active site of a serine hydrolase (blue circle). Reaction mechanism of nucleophilic attack by an activated serine residue on a phosphonate probe. (B) chemical structures of a selection of serine/lipid hydrolase probes and inhibitors categorized based on reactive group (warhead): phosphonates: fluorophosphonate-biotin,[5] C6-(p-nitrophenyl)-(3-azidopropyl)-phosphonate (clickable);[22b] sulfonyl-fluoride: M352,[26] AM3506;[27] 4-chloro-isocoumarin: isocoumarin-BODIPY derivative,[25] 3-alkoxy isocoumarin derivative;[24] carbamate: JW912;[30] beta-lactone: MB064;[29a, 50] triazole urea: triazole urea-BODIPY derivative,[75] ML211.[32] clickable probes contain an bioorthogonal functional group (e. g. azide) that can undergo click-chemistry reaction to attach the reporter tag in a subsequent step.

which foster the covalent attachment of the probe to the active site of the enzyme.[34]

Schmidinger *et al.* have shown that a differentiation between lipases, esterases and cholesterol esterases – all belonging to the serine hydrolase family – is possible by modifying the binding group and position of the reporter tag on p-nitrophenyl phosphonates.[35] The binding group can be different alkyl chains or lipid analogues and can be modified to fine-tune the selectivity of the reactive group, e. g., specifically for lipid hydrolases within the serine hydrolase superfamily of enzymes.[36] Depending on the application, the binding group can be fine-tuned to achieve selectivity for a whole enzyme class, a subpopulation, or individual enzymes.

Finally, reporter tags (RT) or linkers enable subsequent detection or enrichment of the labelled enzymes. These tags can vary based on the downstream application and may

include fluorophores, such as TAMRA; or affinity tags, such as biotin.

Good ABPs have to fulfil certain criteria: high selectivity of the probes to the targets without interactions to other cell components; high reaction kinetics, which guarantee rapid binding at low intracellular concentrations; stability and functionality of the probes under physiological conditions; and non-cytotoxic mode of action at appropriate intracellular concentrations. In order for probes to achieve sufficient intracellular concentration, they must be membrane permeable, which limits their charge-to-hydrophobicity balance.[37] This is especially important for the use of ABPs on living cells and tissues and for *in-vivo* studies. Because reporter tags can be bulky and cause steric hindrance at the enzymes' active sites or decrease the ABPs' membrane permeability, two-step labelling approaches can be used to attach the reporter tag in a subsequent step, i.e., after labelling of the enzymes with the probe. This is achieved by equipping the ABP with a chemical handle – or bioorthogonal functional group – that is non-native and non-perturbing to the cell.[38] Prominent examples are azides or terminal alkynes, which react with activated alkynes or azides in aqueous solution, respectively. Affinity tags or fluorophores can then be added in a subsequent step through click-chemistry reaction – typically Copper-Catalyzed Azide-Alkyne Cycloaddition (CuAAC) or Strain-Promoted Azide-Alkyne Cycloaddition (SPAAC).[37a, 39]

2.3 Discovery, Profiling and Imaging of Lipid Hydrolases by ABPP

2.3.1 Identification and Quantitation

The use of activity-based probes in mass spectrometry (MS)-based proteomics aims to identify and quantify enzymes based on their activity as opposed to their abundance. A major challenge of MS-based proteomics is the vast dynamic range of protein abundance in most biological samples, ranging from single copies to millions of copies per cell.[40] In most tissues, lipid hydrolases are among the lower abundant proteins of a cell, which makes them challenging targets. Additionally, their activity is oftentimes regulated at the post-translational level through post-translational modifications (e.g., phosphorylation, acylation, etc.) or interaction with other proteins, and thus their abundance does not reflect their functionality. These two challenges in the identification and quantitation of lipid hydrolases are overcome by ABPP. This functional proteomics method employs probes that covalently bind to the active site of enzymes – only when they are catalytically active at the timepoint of labelling – resulting in functional information of the enzyme. The subsequent binding of an affinity reporter tag (e.g., biotin) enables the enrichment of the labelled enzymes, which mitigates the problem of sample complexity (Figure 3).[33] Next to MS-based identification and quantification,

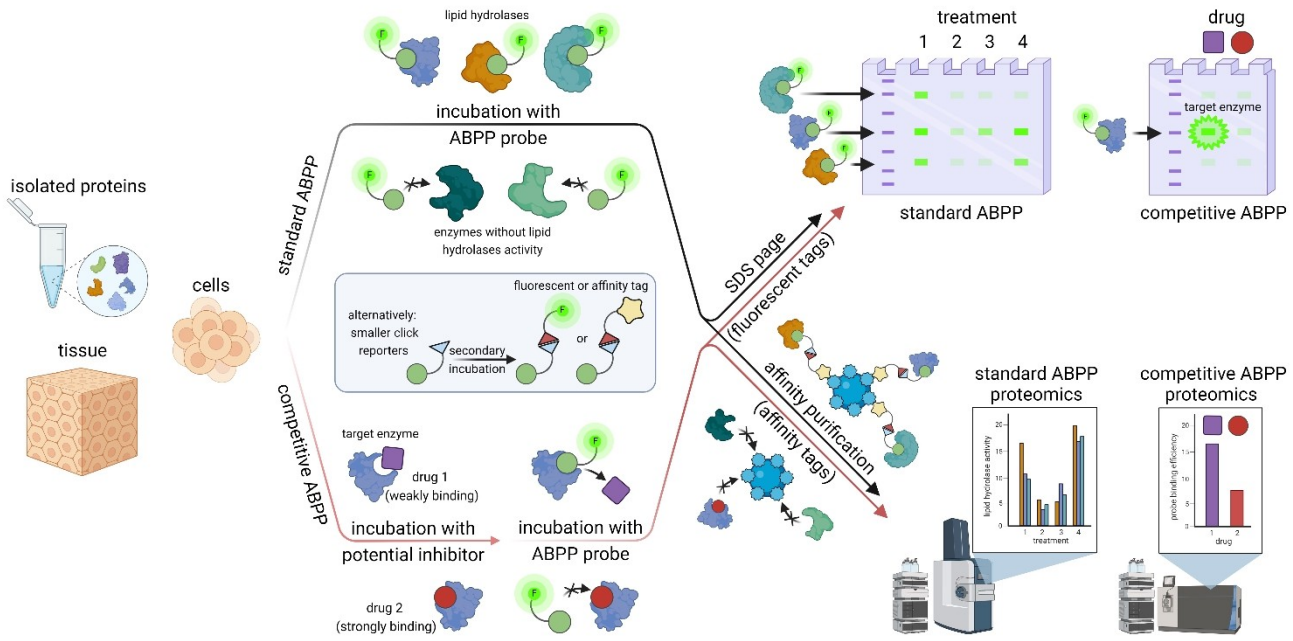

Figure 3. Schematic summary of standard and competitive ABPP. From left to right: **black pathway** illustrating standard ABPP workflow from sample collection (left) via incubation with ABPP probe to read-out via gel-based (fluorescence, top) or MS-based quantification (bottom). **Red pathway** depicting competitive ABPP workflow starting from sample collection. Target enzymes are first treated with potential drugs/inhibitors and subsequently incubated with ABPP probes. Lastly, samples are quantified using either gel-based (fluorescence, top) or MS-based quantification (bottom).

gel-based fluorescent detection is usually employed. A two-dimensional differential activity-based gel electrophoresis method was designed to allow multiplexed detection and quantification of lipolytic and esterolytic proteins.[41] Moreover, protein and ABP microarrays were introduced as tools for functional on-chip screening of lipolytic enzymes.[42]

Employing ABPP has enabled the identification of several lipid hydrolases. A prominent example of successful identification of novel enzymes with the help of ABPP is adipose triglyceride lipase (ATGL), which is now known to be a key player in lipid metabolism by playing a crucial role in the hydrolysis of TGs.[22a, 43] ABPP was used to profile crude pancreatic lipase preparations,[44] revealed the lipolytic proteomes of adipose tissue[22a] and cells[45] as well as liver tissue.[46]

Dominguez and colleagues applied ABPP together with phenotypic profiling in an attempt to map and functionally characterize enzymes in response to a library of small molecule probes. Using this approach, they identified novel carbamate inhibitors for carboxylesterase 3 (Ces3 or Ces1d) and were further able to show the implication of Ces3 in adipocyte differentiation and lipid storage.[47]

In recent years, ABPP has been employed in several studies to investigate the enzymes involved in the endocannabinoid system. The mammalian endocannabinoids – N-arachidonoyl-ethanolamine (anandamide) and 2-arachidonoyl-glycerol (2-AG) – have been shown to play roles in a plethora of physiological and pathological pathways ranging from neuronal development to cardiovascular function.[48] In the endeavour to study the endocannabinoid system during neuronal differentiation, the Van der Stelt group employed ABPP to identify enzymes responsible for the production of the endocannabinoid 2-AG upon retinoic acid-induced neurite outgrowth of Neuro-2a cells. They identified the lipid hydrolase ABHD6 as an alternative DG hydrolase that contributes to 2-AG production in Neuro-2a cells and thus suggest its involvement in neuronal differentiation.[48] The same group aimed to better understand the involvement of endocannabinoids and their metabolic enzymes in cardiac ischemia. To do so they employed lipidomics in combination with ABPP in human failing hearts and identified reduced activity of MGL, which is a 2-AG hydrolysing enzyme, amongst several other hydrolases whose activities were reduced in human failing hearts.[50]

In an effort to better understand transient storage of dietary TGs in enterocytes as well as their remobilization and secretion through chylomicrons, Schittmayer *et al.* used ABPP to screen for lipases along the murine small intestine. Given the uneven distribution of chylomicron secretion along the small intestine, they aimed to find lipases whose activity profile fits the observed chylomicron secretion pattern, and through ABPP they identified several lipases whose activity patterns correlate with TG mobilization from cytosolic LDs in enterocytes, among which were the carboxylesterase Ces2e as well as the arylacetamide deacetylase Aadac.[22b]

2.3.2 Imaging

ABPP techniques used for imaging enzyme activity *in-vitro* and *in-vivo* usually rely on reporter tags that can subsequently be used for visualization (e.g., fluorophore, bioorthogonal functional group).[37a, 51] Most reported ABPP imaging protocols use probes directly on living cells or tissue samples to label the target, thus making permeabilizing or lysing steps obsolete.[51-52]

The use of activity-based probes to localize and image enzymes, among other things, has the advantage that even targets that are exposed to a number of post-translational modifications can be analysed. Comparable imaging methods such as immunofluorescence imaging sometimes do not provide the desired information due to this fact. Another advantage of ABPP imaging is a low probe concentration (typically low µM scale) which is sufficient to result in a highly specific activity-based stain.[51, 52c]

To this day, most groups still report ABPP imaging via confocal fluorescence microscopy in conjunction with a fluorophore as reporter tag. Commonly used fluorophore tags are BODIPY, Cy5 or TAMRA based compounds.[30a, 51-52] Extensive research in the field of ABPP imaging led to the emergence of high-resolution fluorescence imaging methods which can be used to visualize global serine hydrolase activity not only in complex tumorous tissue sections but also in healthy regions of mammalian brains.[51] To image specific lipases such as MGL or ABHD6, Chang *et al.* used probes with a narrower reactivity towards serine hydrolase family members.[30a] In cell models (H29, NeuroA2 and PC3) they observed exceptional selectivity of their probe for MGL and ABHD6 with minimal cross-reactivity for other proteins. Similar results were published by Chang and Moellering who used ABPP imaging to visualize and locate the serine hydrolase KIAA1363 in three aggressive human cancer cell lines (PC3, 231MFP and SKOV3).[52c] These and further examples are steppingstones on the way to establish methods for precision medicine by visualization of highly expressed serine hydrolases in primary tumours.

The probes used by Viertler and Schittmayer to image three cytosolic lipases and one ER residing lipase in living cells made use of a bioorthogonal functional group that was coupled to a fluorophore via CuAAC click-chemistry.[37a] Their reported method allowed them to combine *in-vivo* labelling with *in-situ* detection of lipases using fluorescence microscopy. Van Dalen *et al.* applied an activity-based probe for cathepsin S in combination with multicolour correlative light-electron microscopy to investigate the localization of cathepsin S activity at an ultrastructural level in bone marrow-derived dendritic cells.[53]

In addition to fluorescence-based ABPP imaging methods, MS imaging can be used. Yang *et al.* described a novel mass spectrometry technology using serine hydrolase specific probes bearing mass tags.[52d] Tissue sections were imaged for active enzymes using Matrix-Assisted Laser Desorption/Ionization (MALDI)-MS via the release and measurement of

these mass tags upon laser irradiation. However, spatial resolution of this method was reported to be around 80–100 μm, which is limited by the ionizing laser beam on target. While this resolution is good enough for tissue or organ-based research and precision medicine, it is failing to resolve single cells and far below the resolution achievable by high-resolution fluorescence imaging methods.

3. Competitive ABPP Screening for Lipid Hydrolase Inhibitor Discovery and Validation

ABPP probes are selective for certain enzymes and compete, on the one hand, with endogenous substrates for the enzyme's active site by displacing the substrate and blocking the active site terminally by covalently binding to it. On the other hand, inhibitors that target an enzyme limit the probe's access to the active site. This competing behaviour is a useful feature in competitive ABPP, where decreased probe binding efficiency is inversely proportional to a given inhibitor's affinity for a specific enzyme (Figure 3). This enables discovery and characterization of potential inhibitors/drugs in tissue, live cells, and protein isolates. A particular strength of the method is that the selectivity of a drug is assessed when additional enzymes are present, e. g., in a cellular or tissue context, which allows for the identification of potential off-targets.

Competitive ABPP has been used for more than 20 years and is featured in many reviews since, including several focusing on serine hydrolases.[—43b, 54] Many competitive ABPP studies rely on gel-based enzyme discovery, where proteins are separated in SDS gels and subsequently detected by fluorescent or horseradish peroxidase (HRP) tags.[29, 55] This low-throughput approach requires only basic equipment but is dependent on differences in molecular weight and highly selective probes for protein identification. Scanning enzyme libraries e. g., serine hydrolases, versus potential drug libraries (library vs. library) on the other hand, requires high throughput approaches such as standard and multiplex MS proteomics (Figure 3). In this method, labels for sample multiplexing are added to affinity purified enzymes, allowing for parallel measurement of several conditions.[56]

For even higher throughput, the polarization of fluorescently labelled phosphonate probes can be assessed during probe binding. As the orientation of the small, unbound probe rapidly changes, binding the probe to the target enzyme decreases movement and thereby increases fluorescence polarization. Changes in polarization can therefore be used to identify probe binding intensity in competitive ABPP. This approach has been used in identifying the lysophospholipase 2 (LYPLA2) inhibitor ML349.[32]

Multiplexed MS-based proteomics is used to identify and quantify proteins in a sample and allows for the simultaneous analysis and comparison across several samples, making it a highly efficient technique for studying protein expression. In the case of competitive ABPP, multiplexed MS-based proteomics allows the identification and quantification of all the targets and off-targets in a given proteome based on treatment with inhibitors. Activity-based fluorescent polarization, on the other hand, is usually applied for individual enzymes to study their activity change based on treatment with several inhibitors, which allows for fast, high-throughput screening of inhibitor libraries.[57] This method is useful for studying the activity of a specific enzyme in response to an inhibitor, rather than identifying a range of enzymes as targets of a specific inhibitor.

Many inhibitors for notable members of the serine hydrolase family have been discovered via competitive ABPP. Among these, JZL184[58] has been shown to be an effective and selective inhibitor against MGL. Its discovery was achieved by using fluorophosphonate probes on a mouse brain membrane fraction proteome, where a decrease in MGL activity was detected by in-gel fluorescence after incubation with the inhibitor JZL184. Other similar enzymes (FAAH, ABHD6) were not affected by JZL184. This inhibitor has since been improved, and a newer version – KML29 – was again discovered and characterized via competitive ABPP.[6b] ABX-1431, the latest installation of ABPP discovered MGL inhibitors is an orally administrative, blood-brain-barrier penetrating drug, which is currently under investigation for human trials.[59] For another member of the intracellular lipolytic cascade, HSL, only nonspecific inhibitors could be identified via competitive ABPP,[60] and selective human ATGL inhibition has recently been achieved by NG-497, although via different means of discovery.[61]

Many inhibitors of the ABHD protein family are summarized by Bononi *et al.*,[62] several of which have been identified through ABPP. Recently, ABPP enabled the discovery of a new ABHD6 inhibitor that improves selectivity over MGL and FAAH.[63]

In a recent publication on the immunomodulatory lipid networks in innate immune cells, the Cravatt lab used DAGLB inhibitors DO34 and DH376 as well as the MGL inhibitor MJN110 to investigate the activity and contribution of these enzymes in human monocyte-derived macrophages.[6a] They employed competitive ABPP to confirm that these inhibitors work in primary cell lines as well as to rule out off-targets. Strikingly, they found that MGL was not only inhibited by MJN110 but also by DO34. Due to this previously unknown off-target of the DAGLB inhibitor they switched to a different inhibitor – DH376 – that did not cross-react with MGL.[6a] More recently (2022), our group has employed competitive ABPP in a collaborative effort with Bradic and colleagues to confirm suspected off-target effects of the LAL inhibitors lalistat-1 and lalistat-2 that were shown to also have inhibitory effects on neutral lipases.[6c]

These studies highlight the importance of understanding cross-reactivity and potential off-targets of inhibitors. Inhibitor specificity can vary between tissues and species and thus characterization of an inhibitor in the experimental setting is important to rule out unwanted off-target effects. Furthermore, defining the off-targets and understanding the mechanism of a

given inhibitor not only prevents wrong interpretation of data but also enables improvement of the inhibitor.[6d]

4. Lipid Hydrolase Active-Site Mapping

The mode of action of ABP also makes them formidable tools to directly study the active site of the targeted enzymes and gain insight into substrate binding and the catalytic mechanism. In the case of serine hydrolases, ABP allow to map the region of catalysis down to the active site serine. Bottom-up proteomics is an ideal tool to identify active sites modified with activity-based probes as the mass of the ABP modified active site peptide can be calculated. However, the challenge of identifying peptides of interest in a complex sample consisting of tens to hundreds of thousands of peptides has to be addressed. Initially the target enzymes had to be laboriously overexpressed and purified to reduce sample complexity[64] and make the detection of active sites feasible. However, the Cravatt group quickly developed the methodology to further enable enrichment of ABP labelled enzymes by the incorporation of affinity or immunogenic labels[5] and soon reported successful mapping of several active sites employing TOP-ABPP (i.e., tandem orthogonal proteolysis activity-based proteome profiling) workflows,[65] albeit on different enzyme classes. This was further evolved to a precise quantitative approach in the isoTOP-ABPP workflow[66] and, in collaboration with the Gygi lab, by implementing stable isotope labels and cleavable linkers yielding the CAPE (catch and release activity profiling of enzymes) method.[67] Finally, the applicability of this method was further expanded and applied by the Nomura group to map all hyper-reactive or functional hotspots in proteins by the quantitative readout of site labelling under varying probe concentrations.[68] In one recent example these techniques have been employed to identify and map the active site of 43 serine hydrolases during rice seed germination out of which 54% were associated with the lipid metabolic process according to GO-molecular function.[69]

Another useful application of ABP bound to active sites is the study of enzyme-protein interactions as demonstrated by Saaria *et al.* for the membrane protein FAAH.[70] They employed ABP of different lengths and also harbouring a photo-crosslinker to find new protein interactors of FAAH. Moreover, for enzymes with highly flexible domains ABP can be employed to lock the enzyme conformation in a more defined state for structural analysis, greatly facilitating crystallization and improving resolution of x-ray diffraction. One example is bacterial monoacylglycerol lipase, which despite harbouring a lid/cap region with a very high degree of conformational plasticity was resolved to 1.7 Å but only when complexed with an ABP ligand.[71] The same approach was also successful for the *S. cerevisiae* monoglyceride lipase Yju3p, but in this case an overlong ABP, preventing the lid closing fully, was employed.[72]

5. Summary and Future Directions

Lipid hydrolases are key components of lipid metabolism pathways, and thus play an important role in the development and progression of several lipid-associated human diseases including diabetes, cardiovascular diseases and cancer. Studying lipid hydrolases, however, comes with some challenges: their localization at lipid/water interphases or within biological membranes makes enzymatic activity studies more difficult[12] and naturally low abundance and/or hydrophobicity of some lipid hydrolases makes proteomics studies more demanding.

ABPP has emerged as a technique that is applied for the investigation of lipid hydrolases as it eases some of the aforementioned challenges. This technique uses probes that mimic the substrate of a single enzyme, enzyme group or whole enzyme class. However, in contrast to the substrate, which leaves the active site again, the probe binds covalently to the active site of an enzyme and will thereby label individual active enzymes or active enzymes of a whole enzyme class. This labelling enables downstream processes such as imaging or enrichment coupled to MS-based proteomics for identification and quantitation of the covalently bound enzymes, dependent on which tags/linkers are used.

By employing small molecule probes on tissues or living cells, the enzymes of interest are acting on the probes in their native environment, which yields functional information of an enzyme's activity while it is in its native state. Because activity-based probes are designed to bind covalently to the active site of enzymes, they can be coupled to downstream enrichment prior to MS-based proteomic identification and quantitation which can help identify and quantify low abundant enzymes that require enrichment in order to be measured within the complex proteome.

Several lipid hydrolases have been discovered and characterized in numerous ABPP studies.[22, 43a, 44-50] While identification and characterization of enzymes is one application of ABPP, enzyme libraries can be screened against potential inhibitor libraries in a competitive ABPP manner, which allows for the identification of lipid hydrolase inhibitors.[56] Important lipid hydrolase inhibitors have been identified through this method,[6b, 32, 58-59] and competitive ABPP is further used in the characterization of inhibitors, e.g., to identify or rule out off-targets.[6a, c]

As with many other bioanalytic techniques, ABPP has certain limitations regarding sample input amount as well as sample throughput. The requirement of generally high starting sample amounts is especially challenging for precious clinical samples that often cannot easily be scaled up. More recently, proteomics workflows have thus been optimized to reduce input sample amount,[73] however, the reduction of sample amounts in ABPP studies remains a challenge.

Moreover, (competitive) ABPP studies would immensely benefit from increasing the sample throughput. However, ABPP workflows require many additional steps in the sample preparation as compared to classic proteomics workflows, which poses additional challenges for increasing the through-

put. Jones and colleagues have recently developed a semi-automated workflow for ABPP termed high-throughput-compatible activity-based protein profiling (ABPP-HT), which allows for up to ten times higher throughput compared to classical ABPP and at the same time preserving the enzyme profiling characteristics.[74] By employing their ABPP-HT workflow, they were able to test several inhibitors of deubiquitylating enzymes at various concentrations in a cellular context, thus allowing for reduced sample preparation time as well as MS time, which ultimately also saved cost for the experiment.[74] However, this method has (to our knowledge) not yet been employed for ABPP of lipid hydrolases, and a future direction of competitive and classic ABPP of lipid hydrolases and their inhibitors remains to improve throughput and required sample amount for these studies.

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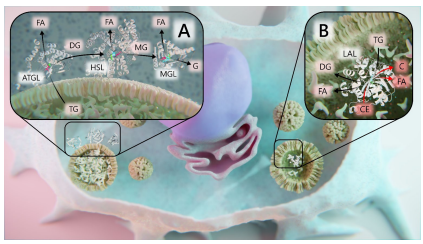

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