Activity-based protein profiling of diacylglycerol lipases

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CHAPTER 8

Summary and Future Prospects

The aim of the work described in this thesis is the identification, development and application of chemical tools and methodologies to study the activity of endocannabinoid hydrolases in complex proteomes. Of particular interest were the diacylglycerol lipases. Activity-based protein profiling (ABPP) was used as the main technique for the identification and characterization of small molecule inhibitors for these enzymes.[1] **Chapter 1** introduces this technique and its position within the wide landscape of approaches available to scientists to study enzyme function. ABPP is a chemical proteomic technique that revolves around active site directed small molecules, termed "activity-based probes" (ABPs). They consist of a warhead, a linker/recognition region and a reporter tag (Figure 1). The warhead reacts in a mechanism-based fashion, thereby establishing a covalent bond with only the active form of the target enzyme. The recognition element generally directs the ABP to its target enzyme and the reporter tag ensures readout of the enzymatic activity. ABPP is arguably unique in its ability to directly monitor the activity of proteins in their native environment. This is important because the activity of many proteins is regulated by posttranslational modifications, protein-protein interactions and endogenous inhibitors.[2] In addition, an ABP that interacts with a specific protein provides a direct activity assay independent of prior annotation of the targeted enzyme. These characteristics makes ABPP, especially when performed with broad-spectrum ABPs, an excellent technique to study and identify inhibitors for proteins with unknown functions in native proteomes.[3]

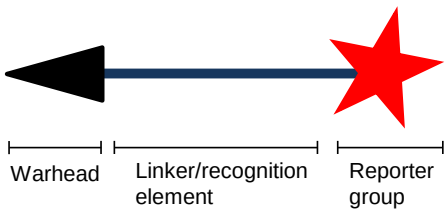


Figure 1. Schematic representation of an activity-based probe (ABP)

The endocannabinoid system is a signaling system that consists of the cannabinoid type 1 and type 2 (CB_1 and CB_2) receptors, lipid messengers termed endocannabinoids and the enzymes responsible for the biosynthesis and metabolism of the

lipid messengers.[4] 2-Arachidonoylglycerol (2-AG) and anandamide (AEA) are the two main endocannabinoids (Figure 2). The CB_1 receptor is highly expressed in the brain and the CB_2 receptor plays and important role in immune cells. Activation of the cannabinoid receptors by their endogenous ligands plays an important role in various patho(physiological) processes, such as learning and memory,[5] pain sensation,[6] energy balance[7] and inflammation.[8] Levels of 2-AG and AEA are tightly regulated by the enzymes responsible for their biosynthesis and catabolism.

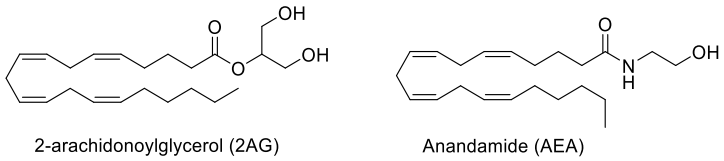


Figure 2. Structures of the endocannabinoids 2-AG and AEA.

Chapter 2 introduces the endocannabinoid system and describes the physiological role of 2-AG in the brain. Modulation of its biosynthesis and catabolism has provided insight in 2-AG-mediated physiology. According to classical models, virtually all physiological processes influenced by 2-AG are mediated by the CB receptors. Although the 2-AG-CB receptor axis is involved in many physiological processes, other biological roles of 2-AG have emerged. 2-AG has been identified as a key metabolic intermediate towards arachidonic acid (AA) and downstream pro-inflammatory prostaglandins. In addition, the function of other proteins, including GPR55, TRPV1, $GABA_A$ and Adenosine $A_{3,}$ might also be modulated by 2-AG.

In view of the important role of 2-AG in multiple pathological processes, the enzymes responsible for its biosynthesis and degradation are considered interesting drug targets. Novel tools that can selectively target specific enzymes involved in 2-AG biosynthesis and catabolism are highly desired and could serve as leads for the development of small molecule based therapies to treat human diseases, such as metabolic syndrome, pain and neuroinflammation.

Diacylglycerol lipase-alpha (DAGL-α) is the main enzyme responsible for the production of the endocannabinoid 2-AG in the central nervous system. It is a potential drug target for the treatment of obesity and neurodegenerative diseases.[9] **Chapter 3** describes the development of MB064 as an ABP for this enzyme (Figure 3).[10] This ABP enables the study of DAGL-α in native proteomes and can serve as a tool to identify new inhibitors for this enzyme.

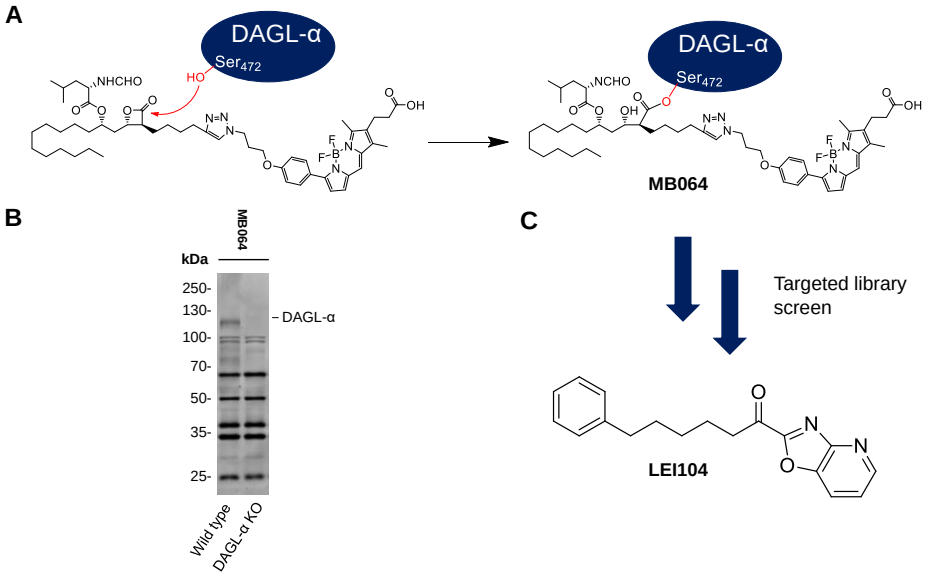


Figure 3. Schematic representation of chapter 3. (*A*) THL based ABP MB064 covalently labels DAGL-α at catalytic serine 472 by opening of the β-lactone and forming a stable ester bond. (*B*) Fluorescent labeling of DAGL-α knockout (KO) and wild type (WT) mouse brain membrane proteome by ABP MB064. A fluorescent band at the molecular weight of DAGL-α (~120 kDa) is visible in the WT proteome but absent in the KO, indicating DAGL-α labeling. (*C*) A targeted library screen led to the identification of the novel DAGL-α inhibitor LEI104.

The design of MB064 is based on THL, which is a known non-specific covalent inhibitor of DAGL-α. THL was equipped with an alkyne handle, and subsequently ligated to a bodipy-based fluorophore for visualization and a biotin group for enrichment and subsequent LC-MS/MS analysis. MB064 labeled recombinantly expressed hDAGL-α at the catalytic Ser-472 (Figure 3). In addition, the ABP also labeled native DAGL-α in the mouse brain proteome. MB064 was used in combination with a biochemical assay to screen a targeted library to identify novel DAGL-α inhibitors. The library consisted of inhibitors that were selected for their ability to inhibit enzymes that recognize similar structures as 2-AG. LEI104 was identified as a covalent reversible α-ketoheterocyclic DAGL-α inhibitor. LEI104 was an *in situ* active and highly selective DAGL-α inhibitor. Fatty acid amide hydrolase (FAAH) was detected as its only off-target. FAAH is the main enzyme responsible for hydrolysis of anandamide (AEA) towards ethanolamine and AA. Selectivity over FAAH is important to dissect the signaling roles of 2-AG and AEA.

Chapter 4 describes identification of a highly selective DAGL inhibitor using a structure-guided and a chemoproteomics strategy to characterize the selectivity of the inhibitor in complex proteomes.[11] Key to the success of this approach was the use of comparative and competitive ABPP in which broad-spectrum fluorophosphonate-based and

β-lactone-based ABPs are combined to report on the inhibition of a protein family in its native environment. Competitive ABPP with broad-spectrum fluorophosphonate-based probes and β-lactone-based probes led to the discovery of α-ketoheterocycle LEI105 as a potent, highly selective, and reversible dual DAGL-α/DAGL-β inhibitor. LEI105 did not affect other enzymes involved in endocannabinoid metabolism including α,β-hydrolase domain-containing protein 6 (ABHD6), α,β-hydrolase domain-containing protein 12 (ABHD12), monoacylglycerol lipase (MAGL), FAAH and did not display affinity for the cannabinoid CB_1 receptor. Targeted lipidomics revealed that LEI105 reduced 2-AG levels in a concentration-dependent manner, but did not affect AEA levels, in Neuro2A cells. It was shown that CB_1-receptor-mediated short-term synaptic plasticity in mouse hippocampal slices could be reduced by LEI105. Hereby, LEI105 provided new pharmacological evidence to support the "on demand biosynthesis" hypothesis for retrograde endocannabinoid signaling in the CNS.

Chapter 5 describes the efforts to improve the potency and physicochemical properties of LEI105. A series of LEI105 analogues was made in which the *p*-tolyl group of LEI105 was exchanged for phenyl groups bearing different substituents or replaced by heterocycles. This resulted in the identification of compound **16**. This inhibitor displayed improved activity in the ABPP activity assay compared to LEI105. In addition, it had the highest lipophilic efficiency (LipE) amongst the 18 tested compounds and reduced FAAH labeling with less that 25% at 10 μM. The inhibitory activity against *h*DAGL-α was tested in a natural substrate assay and **16** had a pIC_{50} of 7.6 ± 0.1.

Towards in vivo active inhibitors

It is anticipated that the inhibitors, tools and methodologies developed in Chapters 3, 4 and 5 will enable the advancement of our understanding of the (patho)physiological role of DAGL. This will allow the evaluation of DAGLs as potential drug targets for treatment the metabolic syndrome, drug abuse or neuroinflammatory diseases, such as Alzheimers's disease, Parkinson's disease and multiple sclerosis (MS). An important next step to achieve this goal will be to study the *in vivo* activity of the α-ketoheterocyclic inhibitors.

The structures described in chapter 5 are part of a larger collaborative effort to optimize LEI105. A complete list of compounds and their biological activities are described in a patent.[12] A noteworthy modification of LEI105 that is described in this patent and which might improve its pharmacokinetic properties, is the replacement of the phenylpentyl by a 4,4,4-trifluoropropyl tail (Figure 4). This modification significantly improved its lipophilic efficiency. In addition, the 4,4,4-trifluoropropyl group is likely to provide metabolic stability. Although, *in vivo* active inhibitors for DAGL have been reported,[13] these reversible α-ketoheterocycles are a valuable addition to the currently known irreversible DAGL inhibitors. Covalent irreversible inhibition could lead to idiosyncratic toxicity. In addition, adverse psychiatric side effects upon complete blockade of DAGL-α are a

significant risk.[14] Reversible α-ketoheterocyclic DAGL inhibitors may provide insight whether a therapeutic window for DAGL inhibition can be established.

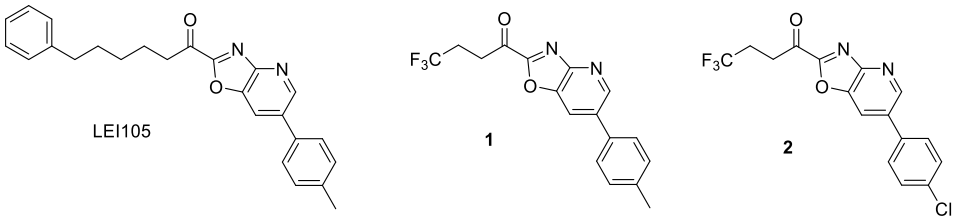


Figure 4. Analogues of LEI105 with potentially better pharmacokinetic and pharmacodynamics properties. LEI105 pIC_{50} 8.52 ± 0.06, **1** pIC_{50} 7.83 ± 0.13, **2** pIC_{50} 8.57 ± 0.07. pIC_{50} was determined by the colorimetric PNP-butyrate *h*DAGL-α assay. N=2; n=2.

DAGL-α and DAGL-β subtype selective inhibitors

DAGL-α and DAGL-β display a tissue specific distribution and appear to regulate different 2-AG pools. DAGL-α, but not DAGL-β, regulates the major forms of 2-AG mediated synaptic plasticity.[15,16] In addition, DAGL-α has the highest activity in neurons, while DAGL-β showed the highest activity microglia.[17] Interestingly, genetic disruption of DAGL-β attenuates neuro-inflammatory events *in vivo* independently of broader effects on 2-AG levels. Subtype selective inhibitors will be instrumental to investigate the contributions of each subtype to 2-AG biosynthesis in specific (patho)physiological processes.

Subtype specific inhibitors could enable better spatiotemporal control over 2-AG biosynthesis and circumvent or reduce adverse effects. It would be interesting to evaluate DAGL-β specific inhibitors in models of neurodegenerative disorders, such as Parkinson's disease, Alzheimer's disease and Multiple Sclerosis.

In the colorimetric assay based on hydrolysis of *para*-nitro-phenylbutyrate, LEI105 showed a high potency for both DAGL-α (pIC_{50} of 8.5 ± 0.06; n=4) and DAGL-β (pIC_{50} 8.1 ± 0.07; n=4). The inhibitors synthesized in chapter 5 were, however, not tested for their activity on DAGL-β. It is important to investigate if the inhibitors in these new series show some preference for DAGL-α over DAGL-β or vice-versa. DAGL-α and DAGL-β show an extensive homology, but differ in the length of a C-terminal tail.[18] Currently no crystal structures of DAGL-α and DAGL-β are available to guide the design of subtype selective inhibitors. Crystal structures of DAGL-α and DAGL-β will be an important breakthrough for the design of subtype selective inhibitors. Alternatively, homology models of DAGL-α can be used, but a homology model of DAGL-β is still lacking.

Full interaction landscape of reversible inhibitors

The competitive and comparative chemoproteomic selectivity assay developed in chapter 4 is a powerful technique to study the selectivity of reversible DAGL inhibitors over a broad panel of serine hydrolases. In addition, it provides confirmation of target engagement of reversible inhibitors in native proteomes. Although a good coverage of relevant off-targets is obtained, the technique remains limited to screening proteins that are targeted by the ABPs at hand. Other non-related off-targets can not be excluded. Covalent irreversible inhibitors are commonly equipped with a reporter tag and used as an ABP to monitor all interacting proteins to which the inhibitor covalently binds.[13] This is not feasible for reversible inhibitors. Photoaffinity-based protein profiling is an alternative method to gain a more comprehensive map of the interaction landscape of reversible inhibitors. This technique does not require a covalent irreversible bond between a catalytic nucleophile of the target protein and the ABP. Instead, photoaffinity labeling utilizes a photoreactive group to establish a covalent bond with target proteins in response to light. Figure 5 depicts two photoaffinity probes based on the structure of LEI105 wich could be used to investigate selectivity of LEI105. Photoaffinity probe **PAP1** relies on an aryl azide, and probe **PAP2** on a diazirine as photocrosslinker. These probes can be used to monitor their interaction partners directly, or in a competitive setting. This method is not limited to the targets of MB108 and FP-Biotin, but can also detect interactions with proteins that do not have a catalytic nucleophile, such as transporters and receptors.

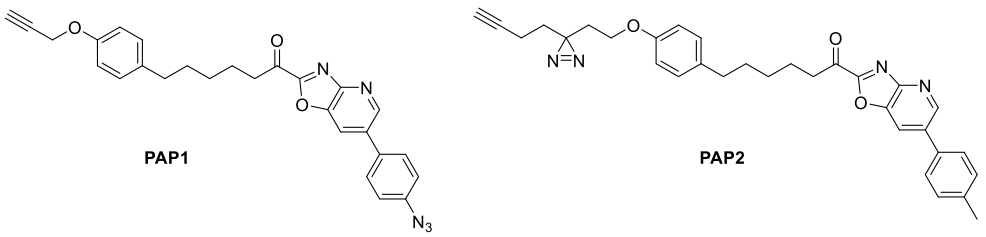


Figure 5. Two proposed photoaffinity-based probes **(PAP1** and **PAP2**) based on LEI105 which could be used to monitor the full interaction landscape of LEI105.

Chapter 6: TAMRA-FP and MB064 do not only detect DAGL-α, but react with multiple endocannabinoid hydrolases. Therefore, they are excellent tools to study the endocannabinoid regulatory machinery. The biosynthetic and metabolic enzymes of the endocannabinoids tightly regulate endocannabinoid-mediated activation of the CB_1 receptor. Monitoring the activity of these endocannabinoid hydrolases in different brain regions is, therefore, key to gain insight in spatiotemporal control of CB_1 receptor-mediated physiology.

Chapter 6 describes the development of a comparative chemical proteomics approach to quantitatively map the activity profile of endocannabinoid hydrolases in

various mouse brain regions at the same time. To this end, two different activity-based probes: fluorophosphonate-biotin (FP-biotin), which quantifies FAAH, ABHD6 and MAG-lipase activity, and MB108 that detects DAGL-α, ABHD4, ABHD6 and ABHD12 were used. Both probes were applied to four different brain regions (frontal cortex, hippocampus, striatum and cerebellum).

Comparison of endocannabinoid hydrolase activity in the four brain regions revealed that FAAH activity was highest in hippocampus, MAGL activity was most pronounced in the frontal cortex, whereas DAGL-α was most active in cerebellum. ABHD4, 6 and 12 activities were equally distributed over all brain regions. The enzyme activity profile was compared to a global proteomics dataset and pronounced differences were found. This could indicate that post-translational modification of the endocannabinoid hydrolases is important to regulate their activities. Measured enzyme activity was compared with protein expression and lipodomics from literature. Since, different age and sample handling could lead to variable outcomes, it would be valuable to perform shotgun proteomics and lipodomics on the same brain regions that were used for the comparative chemoproteomics.

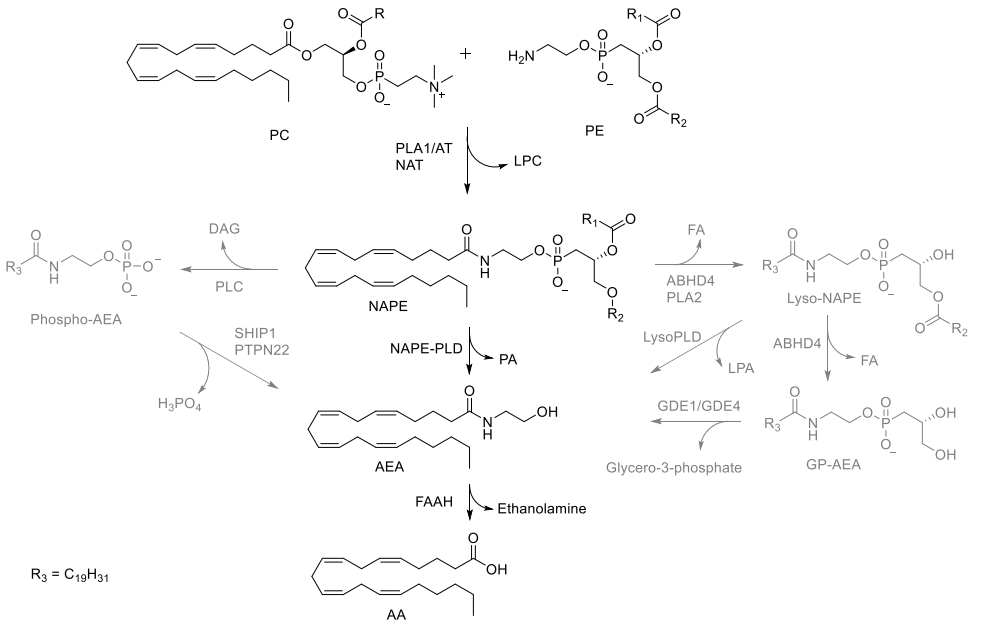
Next, the effect of genetic deletion of the cannabinoid CB_1 receptor on the activity of endocannabinoid hydrolases was studied. No differences in enzymatic activities were observed in the cerebellum, striatum, frontal cortex and hippocampus of CB_1 receptor knockout animals compared to wild type mice. Our results are in line with previous reports and indicate that the CB_1 receptor exerts no regulatory control over the basal production and degradation of endocannabinoids. Genetic deletion of the CB_1 receptor did not induce compensatory mechanisms in endocannabinoid hydrolase activity.

2-AG plays an important role in neuro-inflammation.[17,19] The comparative chemoproteomic method described in Chapter 6 may be extended to study diseases that have a neuro-inflammatory component. Endocannabinoid hydrolase activity in healthy and diseased tissue can be compared to identify dysregulated enzyme activities. This might ultimately lead to the identification of novel drug targets.

AEA biosynthesis

The comparative chemoproteomic method described in Chapter 6 is an efficient method to monitor the activity of both the major biosynthetic and catabolic enzymes for 2-AG in the brain. However, the biosynthesis of AEA, the other important endocannabinoid, can currently not be monitored. The functions of 2-AG and AEA are strongly intertwined and crosstalk between the two lipids has been suggested.[20] In addition, AEA levels decrease when 2-AG biosynthesis is blocked.[13] The underlying mechanism of this effect remains to be elucidated. The identification of tools to modulate and detect the AEA biosynthetic machinery will be instrumental to dissect AEA and 2-AG signaling and study the (patho)physiological role of each endocannabinoid in the endocannabinoid system. Multiple biosynthetic pathways for AEA have recently been uncovered (Scheme 1),[21-23] but

the contribution to AEA biosynthesis of each biological pathway during different physiological and pathological conditions is unknown.



Scheme 1. biosynthetic pathways of AEA. PC: Phosphatidylcholine, PE: Phosphatidylethanolamine, PA: Phosphaditic acid, LPA: Lyso-phosphaditic acid, NAT: N-acyltransferease, LPC: Lyso-phosphatidylcholine, FA: Fatty acid, ABHD4: α,β-hydrolase domain-4, PLA2: Phospholipase A2, GDE1 or 4: Glycerophosphodiesterase 1 or 4, GP-AEA: Glycerophospho-AEA, DAG: Diacylglycerol, PLC: Phospholipase C, PTPN22: protein tyrosine phosphatase, non-receptor type 22. NAPE: N-arachidonoylphosphatidylethanolamine, FAAH: Fatty acid amide hydrolase, AA: Arachindonic acid.

The rate limiting step of AEA formation is transacylation, which is performed by N-acyltransfereses (NATs). They produce a precursor termed N-arachidonoylphosphatidylethanolamine (NArPE),[24] which plays a central role in the current models for AEA generation.[24-26] There are two types of NATs, Ca^{2+}-dependent NAT, which has recently been identified as cytosolic phospholipase A2 epsilon (PLA2G4E) in mouse brain,[27] and Ca^{2+}-independent phospholipase A1/acyltransferases (PLA/AT). It is unknown whether PLA/ATs contribute to NAPE formation in the brain, but they produce AEA in primary sensory neurons in a Ca^{2+}-insensitive manner.[28] AEA can be generated from NArPE via different phospholipase-dependent pathways.[21,29] (*a*) hydrolysis by *N*-acylphosphatidylethanolamine-phospholipase D (NArPE-PLD), a metallo-β-lactamase, producing AEA and a phosphatidic acid in one step.[30] (*b*) Phospholipase A_2 (PLA_2) and α,β-hydrolase domain-containing protein 4 (ABHD4)-mediated conversion to lyso-NArPE, followed by the action of an unknown lysophospholipase D (PLD). Of note, lyso-NArPE can also be converted in a two-step sequence by ABHD4 to glycerophospho-AEA (GP-

AEA) and subsequently hydrolyzed to AEA by glycerophosphodiesterase 1 or 4 (GDE1 or GDE4),[31,32] (*c*) in macrophages NArPE serves as a substrate for an unidentified phospholipase C yielding phospho-AEA. Hydrolysis of the phosphate group by phosphatases PTPN22 or SHIP1 provides AEA.[22,33]
Studies with KO mice lacking these different biosynthetic proteins did not yield conclusive evidence for the preferred pathway in the CNS. This is possibly because of the induction of compensatory mechanisms as a result of congenital deletion of these enzymes.[34,35]

ABPs that monitor and detect activity of the enzymes that catalyze the rate-limiting step towards AEA formation are likely to provide insight in their function and can facilitate inhibitor discovery. In the tissue wide chemoproteomic screen described in Chapter 7, PLA/AT5 activity was detected in the testes, but no other PLA/ATs were found. This could be due to low expression/activity of these enzymes in native proteomes (under basal conditions). Further investigation of the affinity of MB064 for PLA/ATs could lead to the identification of MB064 as a PLA/AT family targeting ABP.

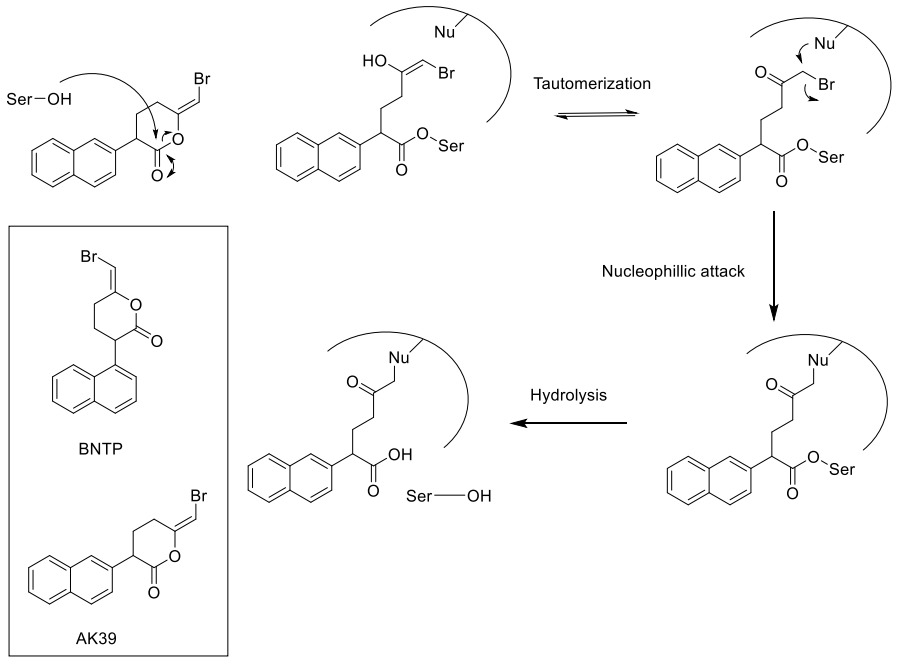
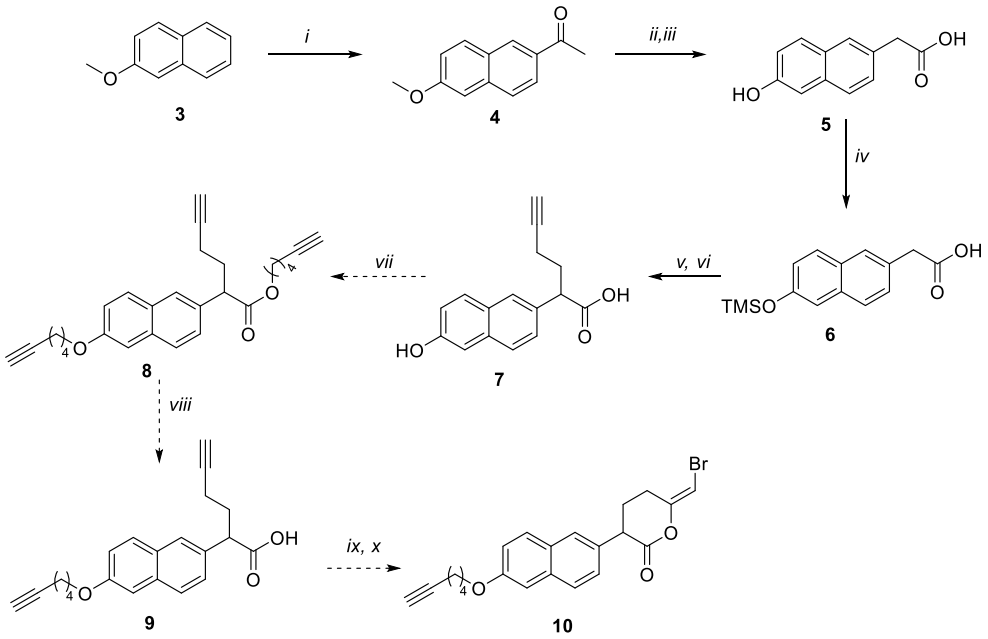


Figure 6. Structures of Ca^{2+}-dependent NAT inhibitors, BNTP and AK39. Proposed mechanism of covalent inhibition of bromoenol lactones.

An ABP or inhibitors for the Ca^{2+}-dependent NAT cannot be based on a THL scaffold, because THL does not inhibit PLA2G4E. Previously, Cadas *et al.* reported the inhibition of Ca^{2+} dependent AEA biosynthesis by the covalent inhibitor (E)-6-(bromomethylene)

tetrahydro-3-(l-naphthalenyl)-2H-pyran-2-one (BTNP) in rat brain (Figure 6). Recombinant PLA2G4E is labeled by the fluorescent fluorophosphonate-based ABP TAMRA-FP. However, the endogenous expression of PLA2GE is very low, therefore a tailored-ABP can be a valuable tool to study endogenous PLA2G4E activity. BNTP can serve as a lead for the development of tailored ABPs for this enzyme. Initial experiments revealed that a BNTP-analogue, AK39, inhibited recombinantly expressed PLA2G4E to over 90% at 10 μM (unpublished results). Scheme 2 depicts a possible synthetic route towards a proposed PLA2G4E directed ABP.



Scheme 2. Proposed synthetic scheme towards Ca^{2+} dependent NAT (PLA2G4E) ABP. Reagents and conditions. (*i*) $AlCl_3$, acetyl chloride, CH_2Cl_2, 0 °C → rt, 28%. (*ii*) Morpholine, S_8, pTsOH, 130 °C, 4 h. (*iii*) HCl (37% aq.), AcOH (gl.), TEBA, 100 °C, on, 72%. (*iv*) TMS-Cl, Et_3N, benzene, 80 °C, 1.5 h, 44%. (*v*) LDA, THF, -10 °C, 45 min. (*vi*) 4-bromo-1-butyne, -10 °C → rt, 2h. 97%. (*vii*) K_2CO_3, DMF, 60 °C. (*viii*) NaOH (5 M aq.), H_2O:MeOH (1:10), rt, 4h. (*ix*) $KHCO_3$, CH_2Cl_2. (*x*) NBS, H_2O.

Chapters 3-6 have shown that MB064 and MB108 target many hydrolases in the mouse brain proteome. The target profile of the β-lactone-based ABPs was, therefore systematically investigated in **Chapter 7** using proteomes from mouse spleen, kidney, liver, heart, lung, pancreas, brain and testes. This revealed that the ABPs acted as highly effective broad spectrum probes for proteins with a α,β-hydrolase fold. The ABPs targeted ~50% of the α,β-hydrolase fold protein family and multiple other hydrolases and transferases. Therefore, these probes are considered as suitable ABPs for the identification of new inhibitors for the α,β-hydrolase fold protein family. The value of MB108 and MB064 for the identification of new inhibitors was demonstrated. A protein library of

ABHD2, 3, 4, 6, 11, 12, and 16A was compiled and screened against a focused library of 207 lipase inhibitors. This library versus library screen allowed evaluation of both selectivity and activity of the inhibitors in one experiment. The screen led to the identification of compounds **60** and **183** as novel inhibitors for ABHD3 and ABHD2, respectively. Their selectivity was further investigated in native proteome by comparative and competitive ABPP. This revealed that both inhibitors showed a highly restricted off-target profile. It is anticipated that inhibitors **60** and **183** will be instrumental to study the physiological role of ABHD2 and ABHD3.

In conclusion, ABPs MB064 and MB108 are the first tools that enabled the study of DAGL-α activity in native biological systems. These probes in combination with the development of the ABPP-based assays described in this thesis have greatly facilitated the identification and characterization of novel DAGL-α inhibitors. Inhibitors for DAGL-α are potential leads for the development of therapeutics to battle the metabolic syndrome and neurodegenerative diseases such a Parkinson's disease, Alzheimer's disease and Multiple Sclerosis.
ABPs MB064 and MB108 that were designed to target DAGL-α revealed to be broad spectrum probes that target many members of the α,β-hydrolase fold protein family. Therefore they represent powerful tools for the identification for new inhibitors for many members of this enzyme family. This finding demonstrates that it is important to analyze the full target profile of an ABP to optimally exploit its properties.

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