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Activity-based proteomics of the endocannabinoid system

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Chapter 1

General introduction

The endocannabinoid system (ECS) consists of the cannabinoid receptors CB₁ and CB₂, their endogenous ligands and the enzymes that regulate the levels of these ligands.¹ The cannabinoid receptors were discovered as the targets of Δ^9 -tetrahydrocannabinol (THC), the psychoactive constituent in marijuana. The main endogenous ligands of these receptors are the lipid signaling molecules 2-arachidonoylglycerol (2-AG) and anandamide (AEA). Numerous neurological processes are regulated by the ECS, including learning, memory, pain perception, feeding and reward behaviors.

The medicinal use of preparations of the plant *Cannabis sativa* has a long history. Currently, the endocannabinoid system is viewed as a promising target for the discovery of novel therapeutics.²⁻⁵ However, the psychoactive properties of THC and other CB₁ agonists are undesirable for patients, and these as well as the addictive properties have led to their regulated use and restricted access. The adverse side-effects can potentially be avoided if the CB₁ receptor is not activated, by using antagonists or inverse agonists instead. Rimonabant was the first (and only) inverse agonist on the CB₁ receptor that was approved as an anti-obesity drug. Due to severe psychiatric side effects Rimonabant was removed from the market. The case of Rimonabant illustrates that targeting the ECS is a delicate balancing act due to its involvement in many physiological processes.⁶ Therefore, an alternative approach to modulate CB₁ receptor activity is to target the endocannabinoid metabolizing enzymes. Small molecule inhibitors can block the activity of enzymes temporarily and change the levels of the receptor's endogenous ligands. This approach is promising for more controlled tuning of the ECS and finding a suitable therapeutic window.

Activity-based protein profiling (ABPP) is an excellent method for the discovery and optimization of drug candidates to target the ECS.¹ ABPP has benefitted from the tremendous developments in proteomics technology over the last twenty years.⁷ ABPP can be used to map the interactions between small molecules and enzymes in living systems.⁸ The fundamental biological role of the endocannabinoid enzymes can be studied by measuring when and where these proteins are active *in vivo* by using ABPP.⁹ Additionally, a comparison of endocannabinoid enzyme activity can be made between healthy and diseased states.¹⁰ Potential inhibitors can be screened for potency and selectivity simultaneously.¹¹ ABPP

also has the potential to be used for personalized medicine by guiding the drug and dose selection after measuring the level of enzyme activity in an individual patient.

Endocannabinoid enzymes

A variety of enzymes are involved in the biosynthesis and degradation of both 2-AG and AEA and are therefore potential targets for therapeutic intervention strategies aimed at modulating endocannabinoid levels. 2-AG is mainly generated by the hydrolysis of diacylglycerols, as catalyzed by two diacylglycerol lipases (DAGL α and DAGL β) (**Fig. 1**).¹² Diacylglycerols are generated by phospholipases C- β (PLC- β) from phosphatidylinositol 4,5-bisphosphate (PIP2).^{13,14}

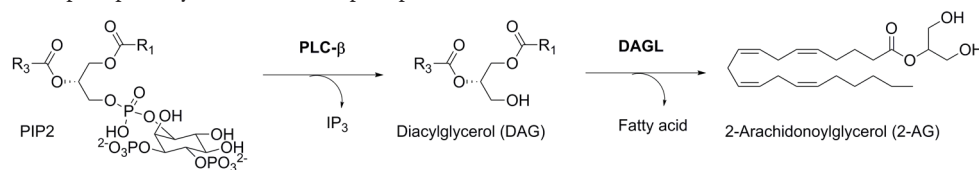


Figure 1 | Biosynthesis of 2-AG. DAGL: diacylglycerol lipase. IP3: inositol 1,4,5-trisphosphate. PIP2: phosphatidylinositol 4,5-bisphosphate. PLC: phospholipase C.

Anandamide is generated by N-acyl transferases that transfer arachidonic acid from the *sn*-1 position of membrane phospholipids to the primary amine of phosphatidylethanolamine (PE) to form N-arachidonoyl phosphatidylethanolamine (NAPE) (**Fig. 2**).¹⁵ Several enzymes can catalyze this transfer: phospholipase A2 group IVE (PLA2G4E) is a calcium-dependent N-acyl transferase^{16,17} and the phospholipase A/acyltransferase (PLA/AT) family are calcium-independent N-acyl transferases.¹⁸ From NAPEs, several enzymatic routes can generate N-acyl ethanolamides (NAEs), including AEA.¹⁹ The main route is hydrolysis by N-arachidonoyl phosphatidylethanolamine phospholipase D (NAPE-PLD).²⁰ Alternative pathways for anandamide synthesis are also proposed, such as via a phospholipase C to form phosphoanandamide, which is subsequently dephosphorylated by phosphatases SH2 domain-containing inositol 5'-phosphatase 1 (SHIP1) or protein-tyrosine phosphatase non-receptor type 22 (PTPN22).^{21,22} Another possible route is via two hydrolysis steps by α/β -hydrolase domain containing protein 4 (ABHD4)²³ and one by glycerophosphodiester phosphodiesterase 1 (GDE1)²⁴ or GDE4.²⁵ A fourth proposed route goes from NAPE to NAE via secretory phospholipase A2 (sPLA2)²⁶ or ABHD4 and subsequent hydrolysis to NAE and phosphatidic acid by GDE4²⁵ or GDE7.²⁷

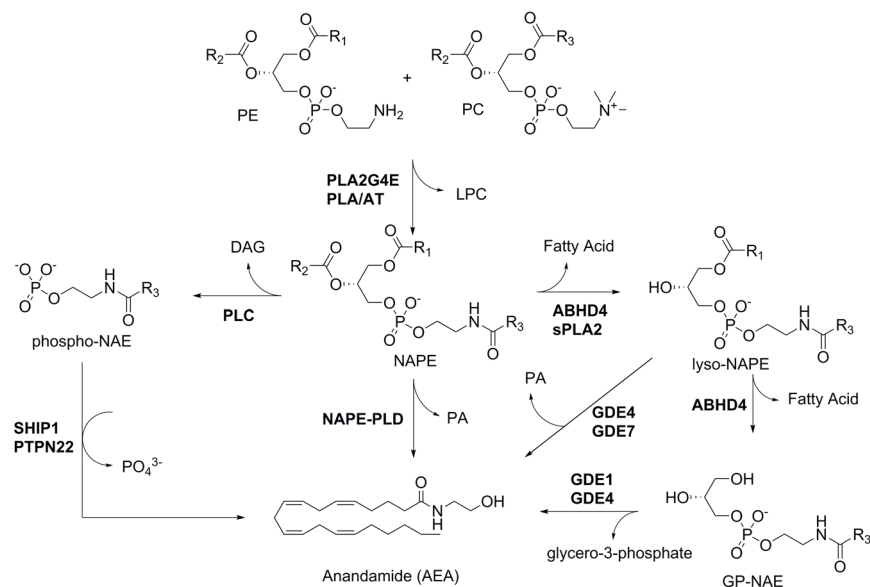


Figure 2 | Biosynthesis of anandamide. $R_{1/2}$: alkyl, R_3 = arachidonoyl. ABHD4: α/β -hydrolase domain containing protein 4. DAG: diacylglycerol. GDE: glycerophosphodiester phosphodiesterase. GP-NAE: *sn*-glycero-3-phospho-N-acylethanolamine. LPC: lysophosphatidylcholine. NAE: N-acylethanolamine. NAPE: N-arachidonoyl phosphatidylethanolamine. PA: phosphatidic acid. PC: phosphatidylcholine. PE: phosphatidylethanolamine. PLA/AT: phospholipase A/acyltransferase. PLA2G4E: phospholipase A2 group IVE. PLC: phospholipase C. PLD: phospholipase D. PTPN22: protein-tyrosine phosphatase non-receptor type 22. SHIP1: SH2 domain-containing inositol 5'-phosphatase 1. sPLA2: secretory phospholipase A2.

Both 2-AG and AEA are hydrolyzed to form arachidonic acid (AA) (**Fig. 3**). 2-AG is mainly hydrolyzed by monoacylglycerol lipase (MAGL),²⁸ and can also be hydrolyzed by ABHD6 and ABHD12 to form AA and glycerol.²⁹ Anandamide is hydrolyzed by fatty acid amide hydrolase (FAAH) to AA and ethanolamine.³⁰ At acidic pH, N-acylethanolamine-hydrolyzing acid amidase (NAAA) also hydrolyzes NAEs, including anandamide.³¹

Activity-based probes for the endocannabinoid system

ABPP relies on chemical probes that react with the catalytic nucleophile of target enzymes in their native biological environment.³² These probes form a covalent and irreversible bond with the target enzyme and report on the abundance of active enzymes. Therefore, these chemical probes are called activity-based probes (ABPs). ABPP has been successfully applied to discover new enzymes involved in the ECS,^{16,29,33} develop selective inhibitors for ECS enzymes^{1,34-39} and compare the activity of ECS enzymes in healthy and diseased states.¹⁰

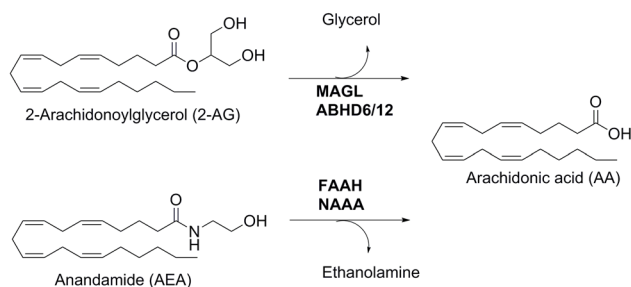


Figure 3 | Hydrolysis of 2-AG and anandamide. ABHD: α/β -hydrolase domain containing protein. FAAH: fatty acid amide hydrolase. MAGL: monoacylglycerol lipase. NAAA: N-acylethanolamine-hydrolyzing acid amidase.

The majority of enzymes involved in the endocannabinoid system are serine hydrolases (**Table 1**). A few enzymes have a catalytic cysteine residue: the PLA/AT family and NAAA. The phosphatase PTNP22 also has a cysteine in the active site, but it is as yet unclear if this cysteine acts as a nucleophile during catalysis. Several other enzymes employ active site histidines for an acid/base mechanism with water in the active site as nucleophile (PLC β 1, PLC β 4, sPLA2⁴⁰). GDE1, GDE4, GDE7 and NAPE-PLD are metallohydrolases and therefore these enzymes cannot be targeted with classical ABPs. The exact identity of some of the proposed ECS enzymes is still unknown (PLC).

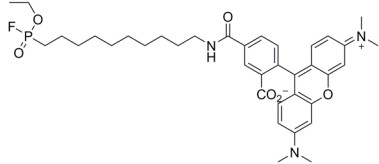
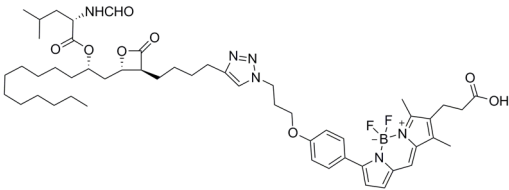
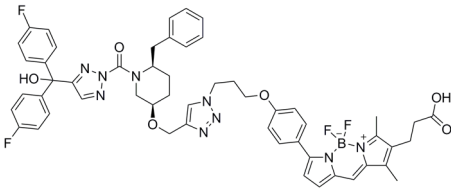
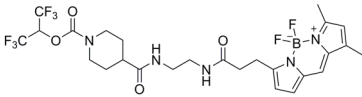
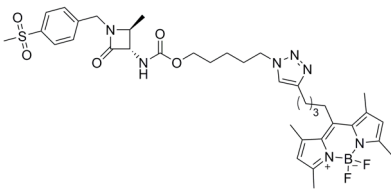
Most of the serine hydrolases of the ECS are targeted by broad-spectrum probes with a fluorophosphonate electrophilic trap, such as FP-TAMRA (**Table 2**).³² FP-TAMRA inhibits DAGL β , but not DAGL α . MB064 was the first fluorescent probe reported for DAGL α . MB064 is based on the inhibitor tetrahydrolipstatin and has a β -lactone as electrophilic trap.⁴⁴ MB064 targets several other enzymes from the α,β -hydrolase fold family. The triazole urea DH379 is a more selective fluorescent probe for diacylglycerol lipases.⁴⁵ The carbamate JW912 is a dual ABHD6/MAGL probe.⁴¹ For the cysteine hydrolase NAAA, a fluorescent probe with a β -lactam electrophilic trap is reported.⁴³

Table 1 | Human endocannabinoid enzymes and reported activity-based probes.

Enzyme	Uniprot accession	Catalytic nucleophile	Probe	Route
DAGL α ¹²	Q9Y4D2	S	MB064, DH379	2-AG biosynthesis
DAGL β ¹²	Q8NCG7	S	MB064, DH379	
PLC β 1 ¹³	Q9NQ66			
PLC β 4 ¹⁴	Q15147			
MAGL ²⁸	Q99685	S	FP, carbamates	2-AG hydrolysis
ABHD6 ²⁹	Q9BV23	S	FP, carbamates, ⁴¹ MB064, DH379	
ABHD12 ²⁹	Q8N2K0	S	FP, MB064	
NAPE-PLD ²⁰	Q6IQ20			Anandamide biosynthesis
PLA2G4E ¹⁶	Q3MJ16	S	FP	
PLA/AT1-5 ¹⁸	Q9HDD0, Q9NWW9, P53816, Q9UL19, Q96KN8	C		
ABHD4 ²³	Q8TB40	S	FP, MB064	
GDE1 ²⁴	Q9NZC3			
GDE4 ²⁵	Q8N9F7			
GDE7 ²⁷	Q7L5L3			
PLC				
PTPN22 ²¹	Q9Y2R2	C?		
SHIP1 ²²	Q92835			
sPLA2 ²⁶	P04054			
FAAH ³⁰	O00519	S	FP	Anandamide hydrolysis
FAAH2	Q6GMR7	S	FP	
NAAA ³¹	Q02083	C	β -lactam ^{42,43}	

Chapter 1

Table 2 | The reported fluorescent activity-based probes for endocannabinoid metabolizing enzymes feature distinct chemotypes.

Name	Chemotype	Structure
FP-TAMRA	fluorophosphonate	
MB064	β -lactone	
DH379	triazole urea	
JW912	carbamate	
NAAA-probe	β -lactam	

Aim and outline

The aim of this thesis is to use activity-based proteomics to further our understanding of the endocannabinoid system. This thesis describes the development of a method for the label-free quantification of enzyme activity using proteomics. Furthermore, the development of new activity-based probes for the endocannabinoid enzymes DAGL and ABHD6 is described.

In **Chapter 2**, the activity-based protein profiling method is explained. An overview of available probes, analytical techniques and applications is given. ABPP is applied in **Chapter 3** to study the role of the ECS in the lysosomal storage disorder Niemann-Pick Type C. Both gel-based and mass-spectrometry-based ABPP is used to compare serine hydrolase activity between healthy and diseased brain tissue from a transgenic mouse model. In this study, both dimethyl-labeling and label-free quantitative mass spectrometry is used. This led to the development of an ABPP method with label-free quantification for which a protocol is described in **Chapter 4**. The well-characterized DAGL α inhibitor DH376, which was previously studied using dimethyl-labeling techniques,⁴⁵ was studied with this new method. **Chapters 5 and 6** describe the design and synthesis of new probes. In **Chapter 5**, the synthesis and characterization of quenched activity-based probes for DAGL and ABHD6 is described. In **Chapter 6**, the design and synthesis of new two-step probes for DAGL is described. **Chapter 7** provides a summary and points at directions for future research.

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