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**Author:** Rooden, E.J. van  
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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. The cannabinoid receptors were discovered as the targets of ∆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. 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. 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. ABPP has benefitted from the tremendous developments in proteomics technology over the last twenty years. ABPP can be used to map the interactions between small molecules and enzymes in living systems. 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. Additionally, a comparison of endocannabinoid enzyme activity can be made between healthy and diseased states. Potential inhibitors can be screened for potency and selectivity simultaneously. 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 (DAGLα and DAGLβ) (Fig. 1). Diacylglycerols are generated by phospholipases C-β (PLC-β) from phosphatidylinositol 4,5-bisphosphate (PIP2).

![Figure 1](image1.png)

**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). Several enzymes can catalyze this transfer: phospholipase A2 group IVE (PLA2G4E) is a calcium-dependent N-acyl transferase and the phospholipase A/acyltransferase (PLA/AT) family are calcium-independent N-acyl transferases. From NAPEs, several enzymatic routes can generate N-acyl ethanolamides (NAEs), including AEA. The main route is hydrolysis by N-arachidonoyl phosphatidylethanolamine phospholipase D (NAPE-PLD). Alternative pathways for anandamide synthesis are also proposed, such as via a phospholipase C to form phospho-anandamide, which is subsequently dephosphorylated by phosphatases SH2 domain-containing inositol 5’-phosphatase 1 (SHIP1) or protein-tyrosine phosphatase non-receptor type 22 (PTPN22). Another possible route is via two hydrolysis steps by α/β-hydrolase domain containing protein 4 (ABHD4) and one by glycerophosphodiester phosphodiesterase 1 (GDE1) or GDE4. A fourth proposed route goes from NAPE to NAE via secretory phospholipase A2 (sPLA2) or ABHD4 and subsequent hydrolysis to NAE and phosphatidic acid by GDE4 or GDE7.
Both 2-AG and AEA are hydrolyzed to form arachidonic acid (AA) (**Fig. 3**). 2-AG is mainly hydrolyzed by monoacylglycerol lipase (MAGL), and can also be hydrolyzed by ABHD6 and ABHD12 to form AA and glycerol. Anandamide is hydrolyzed by fatty acid amide hydrolase (FAAH) to AA and ethanolamine. At acidic pH, N-acylethanolamine-hydrolyzing acid amidase (NAAA) also hydrolyzes NAEs, including anandamide.

**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. 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, develop selective inhibitors for ECS enzymes, and compare the activity of ECS enzymes in healthy and diseased states.
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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 (PLCβ1, PLCβ4, sPLA2). 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). FP-TAMRA inhibits DAGLβ, but not DAGLα. MB064 was the first fluorescent probe reported for DAGLα. MB064 is based on the inhibitor tetrahydrolipstatin and has a β-lactone as electrophilic trap. 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. The carbamate JW912 is a dual ABHD6/MAGL probe. For the cysteine hydrolase NAAA, a fluorescent probe with a β-lactam electrophilic trap is reported.

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

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<th>Enzyme</th>
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Table 2 | The reported fluorescent activity-based probes for endocannabinoid metabolizing enzymes feature distinct chemotypes.

<table>
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<td>DH379</td>
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<td>JW912</td>
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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 DAGLα inhibitor DH376, which was previously studied using dimethyl-labeling techniques, 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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References


General introduction


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