Chemical tools to modulate endocannabinoid biosynthesis

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1

General Introduction

Endocannabinoids

Extracts of the plant *Cannabis sativa*, also known as marijuana*,* have been used for recreational and medical purposes for thousands of years.[1,2] Marijuana affects multiple physiological processes, including pain sensation, memory, mood, sleep and appetite.[3] In 1964, the structure of Δ^9-tetrahydrocannabinol (Δ^9-THC, Figure 1) the principal psychoactive component of *Cannabis sativa,* was reported.[4] It took almost 30 years to identify the target protein (termed cannabinoid CB1 receptor) that is activated by Δ^9-THC.[5] The CB_1 receptor belongs to the family of G-protein-coupled receptors and is expressed in neurons, astrocytes and microglial cells in various brain regions, including cerebellum, hippocampus, basal ganglia, cortex, amygdala, hypothalamus, thalamus and brainstem.[6] In neurons, the cannabinoid CB_1 receptor is often located at pre-synaptic membranes, possibly also in mitochondria, and its activation by Δ^9-THC results in reduction of intracellular cAMP-levels, activation of inward-rectifying K^+-channels and inhibition of voltage-sensitive Ca^{2+}-channels, thereby inhibiting neurotransmitter release and modulation of synaptic plasticity. A second Δ^9-THC-binding protein, the cannabinoid CB_2 receptor was identified in 1993.[7] It is primarily found in peripheral immune cells,[8,9] such as B-cells, macrophages and monocytes. Activation of the CB_2 receptor exerts immunosuppressive effects.[10]

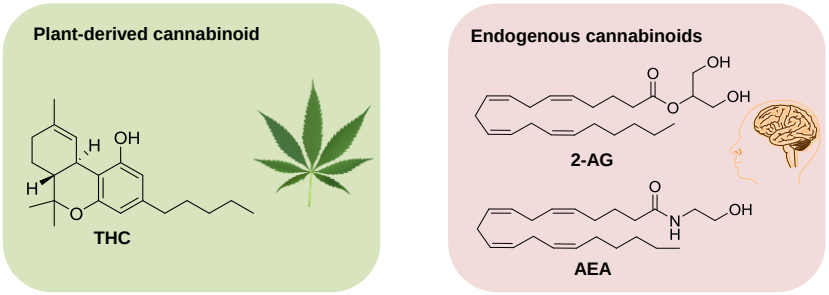

Figure 1. Chemical structures of THC and the two most abundant endocannabinoids: 2-arachidonoylglycerol (2-AG) and anandamide (*N*-arachidonoylethanolamine, AEA).

The discovery of cannabinoid CB_1 receptor initiated the search for endogenous compounds in mammals that could activate this protein. In 1992, the first endogenous ligand was isolated and named anandamide (*N*-arachidonoylethanolamine; AEA, Figure 1), which is derived from the Sanskrit word for bliss.[11] Three years after the discovery of AEA, 2-arachidonoylglycerol (2-AG, Figure 1), a common intermediate in phospholipid and triglyceride metabolism, was reported as the second endogenous lipid that modulated cannabinoid CB_1 receptor function.[12] 2-AG and AEA are the most abundant endogenous ligands of the cannabinoid receptors and are termed "endocannabinoids". Some other lipids, such as 2-arachidonoylglycerylether (noladin ether), *O*-arachidonoylethanolamine (O-AEA, virodhamine) and *N*-arachidonolyl-dopamine (NADA), have also been reported to activate the cannabinoid receptors, but their role as endocannabinoids is under debate.[13,14] (Figure 2).

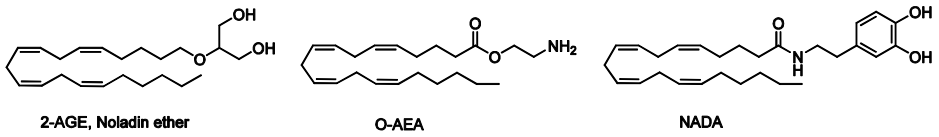

Figure 2. Chemical structures of some other putative endocannabinoids: Noladin ether, virodhamine (O-AEA) and *N*-arachidonoyldopamine (NADA).

AEA and 2-AG are often found together, but their individual levels vary between cell types, brain regions, tissues, species, developmental stages and pathological conditions.[15-17] Endocannabinoids play an essential role in the brain by activating the cannabinoid CB_1 receptor in different brain cells. They modulate neurotransmitter release (Figure 3) and regulate many physiological processes, including pain perception, learning and memory, energy balance, emotional states (anxiety, fear), and

reward-related behaviour.[18] The exact contribution of each individual endocannabinoid in specific brain regions to these (patho)physiological functions remains, however, poorly understood.

Continuous activation of the CB_1 receptor by endocannabinoids is associated with nicotine addiction, obesity and metabolic syndrome.[19,20] Endocannabinoids play also an important role during neurodegeneration and inflammation. All of these are major risk factors for illness and death. The CB_1 receptor antagonist rimonabant was effective in obese patients, but was withdrawn from the market due to unacceptable psychiatric side effects (depression and suicidal ideation in some individuals).[21] This highlights the medical need to understand modulation of the endocannabinoid levels in the brain in a more detailed manner. Inhibitors of the biosynthetic enzymes of the endocannabinoids would provide valuable tools to study the role of each endocannabinoid in the various physiological processes. This thesis will focus on the enzymes that control 2-AG levels. Activity-based protein profiling is applied as a chemoproteomic method to identify inhibitors of these enzymes in order to modulate cannabinoid CB_1 receptor activation by 2-AG.

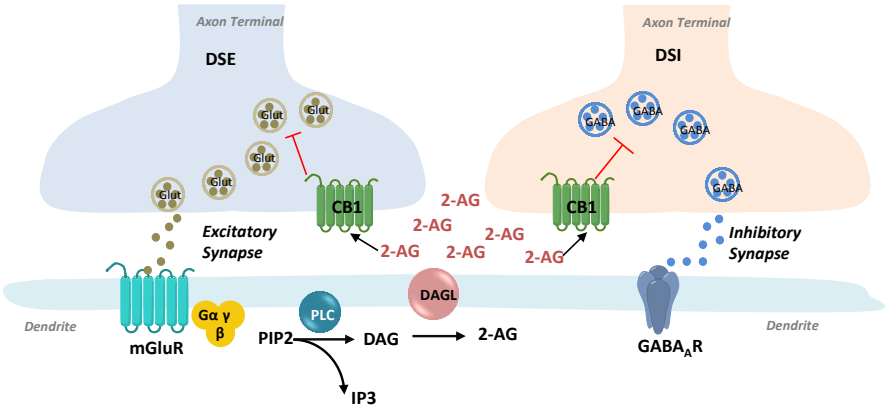

Figure 3. A schematic view of endocannabinoid signaling. Glutamate released from the excitatory axon terminal activates type I metabotropic glutamate receptor (mGluR), which stimulates 2-AG production through the phospholipase C (PLC) and diacylglycerol lipase (DAGL) pathway. 2-AG then crosses the synaptic cleft and activates presynaptic CB_1 receptors, which induces the suppression of glutamate or *γ*-aminobutyric acid (GABA) release.

Diacylglycerol lipases

2-AG is produced from membrane phospholipids via a two-step process starting with *sn*-2 arachidonoyl phosphatidylinositol 4,5-bisphosphate (PIP_2) (Figure 3 and 4).[22] In the first step, PIP_2 is hydrolyzed into arachidonyl-containing diacylglycerol (DAG) species by phospholipase Cβ (PLCβ), which is activated by various G-protein-coupled receptors. The second step is catalyzed by diacylglycerol lipase (DAGL), in which DAG is converted

into 2-AG in a *sn*-1 specific manner.[23,24] In addition, there are some other proposed pathways for 2-AG synthesis.[25,26] For example, hydrolysis of 2-arachidonoyl-LPA by an LPA phosphatase may also provide 2-AG (Figure 4).[25]

The rate-limiting step in 2-AG production is controlled by two homologous isoforms of DAGLs, DAGLα (120 kDa) and DAGLβ (70 kDa).[23] Both proteins are multi-domain membrane-spanning enzymes that belong to the serine hydrolase family and differ from each other by the presence of a long C-terminal tail (~300 amino acids) in DAGLα. This C-terminal tail is involved in the regulation of the catalytic activity of the enzyme.[27-29]

Genetic studies with DAGL knockout mice have demonstrated that DAGLα and DAGLβ regulate 2-AG production in a tissue type dependent manner.[28,30] DAGLα is the principal regulator of 2-AG formation in the nervous system, whereas DAGLβ is the dominant enzyme for 2-AG production in peripheral tissues such as the liver. Interestingly, basal brain anandamide levels are also reduced in DAGLα$^{-/-}$ mice, but not in DAGLβ$^{-/-}$ mice.[31,32] Therefore, a pharmacological agent to modulate DAGLα or DAGLβ activity in an acute and temporal manner would provide an important counterpart for DAGLα$^{-/-}$ or DAGLβ$^{-/-}$ mice to study the physiological functions of DAGLs in complex biological systems.

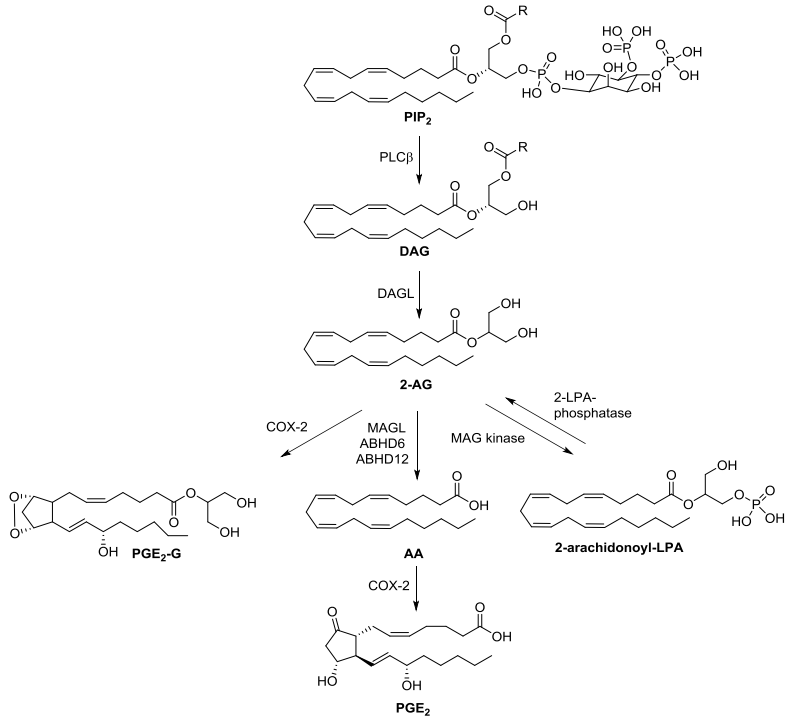

Figure 4. Biosynthetic and metabolic pathways of 2-arachidonoylglycerol (2-AG).

Inhibitors of diacylglycerol lipases

In early studies, the general lipase inhibitors tetrahydrolipostatin (THL, Orlistat) and RHC-80267, a bis-oximino-carbamate have been reported to inhibit DAGL-mediated 2-AG production using a radiometric assay with 1-[^{14}C]oleoyl-2-arachidonoylglycerol as natural substrate. They are, however, poorly active and/or lack the selectivity over other serine hydrolases (Figure 5).[33-35] In 2006, Bisogno *et al.* discovered fluorophosphonate inhibitors against DAGLα (O-3640 and O-3841). These compounds are active *in vitro* systems, but are not suitable for *in vivo* studies due to their poor stability and lack of cell permeability.[68] Further structure-activity relationship studies of fluorophosphonate inhibitors led to the discovery of O-5596, which is a relatively stable and potent DAGLs inhibitor.[36] However, O-5596 cross-reacts with several off-targets, which prohibits its use as a specific DAGLs inhibitor. Recently, the α-ketoheterocycles LEI104 and LEI105 were disclosed as a new chemotype of selective, reversible DAGLs inhibitors, but no *in vivo* activity was reported. In 2012, Hsu *et al.* published the first *in vivo* active DAGLβ inhibitor KT109 (Figure 5), which is based on a triazole urea scaffold.[37] KT109 was ~60-fold selective over DAGLα. However, KT109 does not cross the blood-brain barrier.

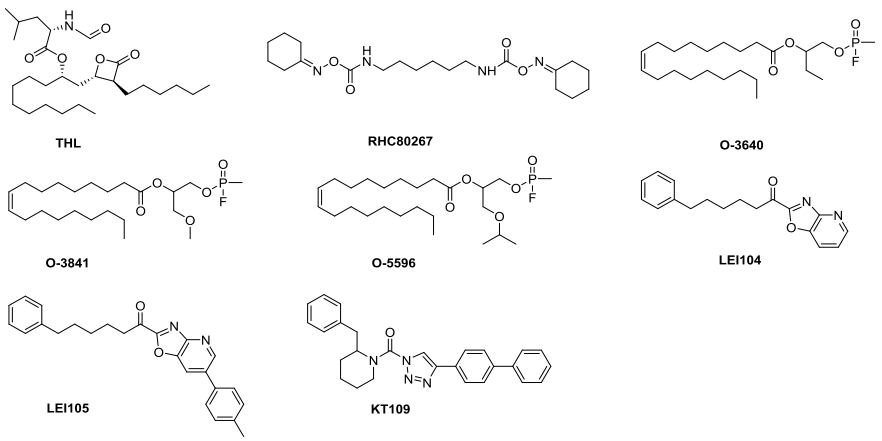

Figure 5. Chemical structures of known DAGL inhibitors. RHC80267, THL, O-3640, O-3841 and O-5596 are first generation DAGL inhibitors, which were non-selective, not potent, or not *in vivo* active. LEI105 is a reversible inhibitor with high selectivity. KT109 is an *in vivo* active DAGLβ inhibitor with good selectivity.

Monoacylglycerol lipase

Monoacylglycerol lipase (MAGL) is the main responsible enzyme for terminating 2-AG signaling by catalyzing the hydrolysis of the ester bond, thereby producing arachidonic acid and glycerol. Other serine hydrolases, including ABHD6, ABHD12 and FAAH, play a

minor and cell-type specific role in the metabolism of 2-AG. MAGL is primarily located at pre-synaptic membranes.[38,39] Studies using MAGL$^{-/-}$ mice and pharmacological tools showed dramatically elevated 2-AG levels in the brain and peripheral tissues.[40] 2-AG can also be transformed into other bioactive lipids, such as prostaglandin-glyceryl esters by cyclooxygenase-2,[41] which are involved in the inflammatory responses. Furthermore, lipid kinases such as monoacylglycerol kinases can phosphorylate 2-AG, thereby producing lysophosphatidic acid.[42] Several MAGL inhibitors disclosed in the literature include URB602, *N*-arachidonoyl maleimide (NAM) and OMDM169 (Figure 6). These compounds have low potency, cross-react with FAAH and other enzymes. Thus, they are not suitable for the functional study of MAGL *in vivo*.[43-45] In 2009, Long *et al.* reported JZL184 as the first, highly selective, *in vivo* active MAGL inhibitor. JZL184 contains a piperidine carbamate as electrophile that reacts to the catalytic serine of MAGL to form a stable and irreversible carbamate adduct. Using competitive activity-based protein profiling, JZL184 was shown to be 100-fold selective over FAAH and other serine hydrolases.[46] JZL184 is less active on rat MAGL than mouse MAGL. The new MAGL inhibitor KML29 does not suffer from this species-dependent activity and shows high potency against rat MAGL resulting increased 2-AG levels in rats.[47] MAGL inhibitors display anti-inflammatory and neuroprotective effects in multiple mice models of neurodegenerative disorders.[48] In addition, Nomura *et al.* demonstrated that inhibition of MAGL activity in aggressive cancer models impaired cell survival, migration and tumor growth.[49] Thus, MAGL inhibitors may have potential therapeutic utility in various diseases.

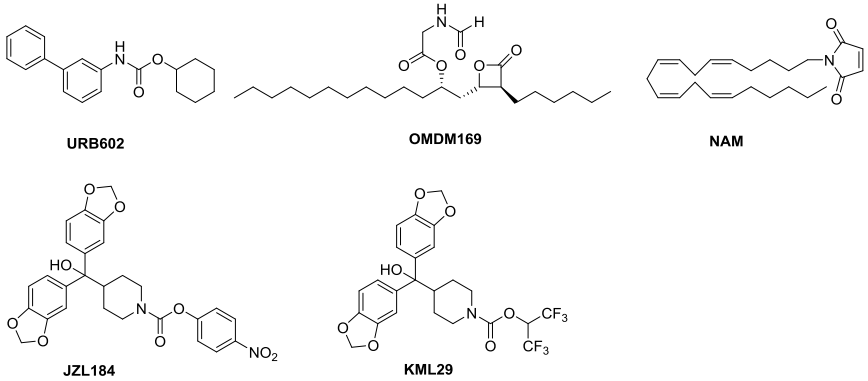

Figure 6. Chemical structures of known MAGL inhibitors. URB602, OMDM169 and NAM are first-generation inhibitors with poor selectivity and potency. JZL184 and KML29 are selective MAGL inhibitors with high *in vivo* potency.

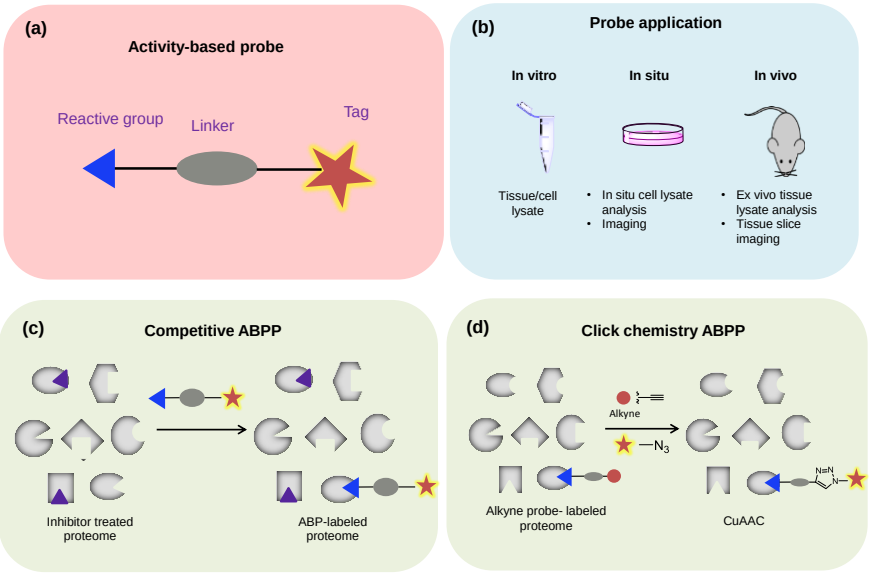

Figure 7. Schematic overview of activity-based protein profiling (ABPP). (a) Representative cartoons of activity-based probes: reactive group (blue), linker (gray) and reporter tag (red) (e.g. fluorophore or biotin affinity tag). (b) ABPs can be used in various biological systems, including cell/tissue lysates *in vitro*, living cellular systems, and *in vivo* animal models. (c) In competitive ABPP, proteomes are pre-incubated with inhibitors, followed by co-incubation with an ABP. (d) Two-steps probes (Click chemistry ABPP) provide a post-detection of protein labeling.

Activity-based protein profiling

Activity-based proteome profiling (ABPP) is a chemical proteomics method that allows the study of proteins and their perturbation by small molecules in their native cellular context.[50,51] ABPP will only visualize active proteins, takes all post-translational modifications (PTMs) into account and by this virtue is complementary to other techniques that detect messenger RNA or polypeptides/proteins (that is, *in situ* hybridization and immunohistology, respectively). ABPP makes use of organic molecules, termed activity-based probes (ABPs) to label the active site of a protein (Figure 7a). ABPs are compounds that covalently and irreversibly inhibit enzymes and that are equipped with a tag (fluorophore, biotin, bioorthogonal tag) through which the target enzyme, or enzyme family, is visualized by fluorescence microscopy, or enriched to enable identification and characterization using chemical proteomics methodology by mass spectrometry. In comparative ABPP two biological samples are interrogated with ABPs. Differences in enzyme activities are monitored and identified with various ABPs. Comparative ABPP allows the discovery of targets and validation of drug-target interaction in live cells, tissue lysates, and sometimes in animals (Figure

7b). In competitive ABPP a small molecule is pre-incubated with a biological sample and residual enzyme activities are subsequently monitored with an ABP (Figure 7c). Activity and selectivity of the small molecules is easily visualized in a complex proteome across the complete protein family. Competitive ABPP can also be used to determine target engagement *in situ* and *in vivo*. In both experimental set ups, two different type of probes can be used. Broad-spectrum ABPs target a whole (or to a large extent) family of proteins, whereas tailor-made ABPs are designed to target a specific protein of interest. The latter type of probe can also be used to validate the target in different therapeutic animal models and serve as a biomarker for target engagement in clinical trials. In case the ABP fall short and do not work due to a lack of bioavailability or enzyme specificity, two-step ABPs can be applied. Two-step ABPs do not constitute a reporter tag, but instead carry a small ligation handle, which can be conjugated to a biotin or fluorescent tag via bio-orthogonal ligation chemistry, only after the ABP has covalently reacted with the target of interest (Figure 7d). These combined ABPP technologies provide a highly attractive platform, both to discern aberrant enzyme functioning in physiological processes, and to identify compounds able to correct for this.

Aim and outline of the thesis

The aim of this thesis is to design, synthesize and apply chemical tools to study the physiological roles of DAGLα/β and MAGL in vitro and in vivo.

Chapter 2 reports on the design, synthesis and *in vitro* characterization of DH376 as a new dual DAGL inhibitor. In **Chapter 3** the discovery of DH379 as a tailor-made activity-based probe for DAGLα/β and the effects of acute pharmacological blockade of DAGLs by DH376 in healthy and lipopolysaccharide-treated mice on brain lipid networks and neuroinflammation is reported. **Chapter 4** describes the efficacy of DH376 in refeeding behavior of fasted mice. In **Chapter 5** the development of the first DAGL PET ligand [^{18}F]DH439 is disclosed. The structure-activity relationship of disubstituted piperidinyl ureas as DAGL inhibitors is reported in **Chapter 6**. The design, synthesis and application of a highly selective tailor-made activity-based imaging probe for MAGL is discussed in **Chapter 7**. Finally, **Chapter 8** provides a summary of the results described in the thesis and proposes some directions for future research.

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