Chemical tools to modulate 2-arachidonoylglycerol biosynthesis

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Abstract

2-Arachidonoylglycerol (2-AG) is an important endogenous signaling lipid that activates the cannabinoid receptors (CB_1R and CB_2R), thereby regulating a diverse range of physiological processes including anxiety, appetite, inflammation, memory, pain sensation, and nociception. Diacylglycerol lipases (DAGLs) are the principle enzymes responsible for 2-AG biosynthesis. Recently, the (patho)physiological functions of DAGLs have been explored by both genetic methods and chemical tools. This review will focus on the recent efforts to develop highly selective and *in vivo* active DAGLs inhibitors using activity-based protein profiling. Volume 65, Number 1, Pages 9–15, 2018

Keywords: *activity-based probe, activity-based protein profiling, 2-arachidonylglycerol, diacylglycerol lipase, endocannabinoids, inhibitors*

1. Introduction

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; Fig. 1), the principal psychoactive component of *C. sativa,* was reported [4]. It took almost 30 years to identify the target protein (termed cannabinoid CB_1 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, and thalamus [6]. In neurons, the cannabinoid CB_1 receptor is often located at presynaptic membranes, possibly also in mitochondria [7], 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 [8]. It is primarily found in peripheral immune cells [9, 10], such as B-cells, macrophages, and monocytes. Activation of the CB_2 receptor exerts immunosuppressive effects [11].

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 discovered and named anandamide (*N*-arachidonoylethanolamine, AEA; Fig. 1), which is derived from the Sanskrit word for bliss [12]. Three years after the discovery of AEA, 2-arachidonoylglycerol (2-AG; Fig. 1), a common intermediate in phospholipid and triglyceride metabolism, was reported as the second endogenous lipid that modulated cannabinoid CB_1 receptor function [13]. 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*-arachidonoyl-dopamine (NADA), have also been reported to activate the cannabinoid receptors, but their role as endocannabinoids is under debate [14, 15]. (Fig. 2).

Abbreviations: *ABP, Activity-based probe; ABPP, Activity-based protein profiling; AEA, anandamide; 2-AG, 2-arachidonoylglycerol; DAGL, diacylglycerol lipase; DSE, depolarization-induced suppression of excitation; DSI, depolarization-induced suppression of inhibition; FAAH, Fatty acid amide hydrolase; LPS, lipopolysaccharide; MAGL, monoacylglycerol lipase; NADA, N-arachidonoyl-dopamine; O-AEA, virodhamine; PKC, protein kinase C; THC, tetrahydrocannabinol; THL, tetrahydrolipstatin; TNF, tumor necrosis factor.*

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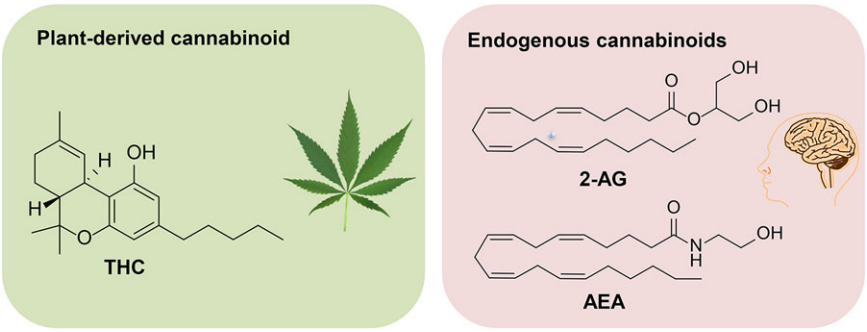

FIG. 1 *Chemical structures of THC and the two most abundant endocannabinoids: 2-AG and AEA.*

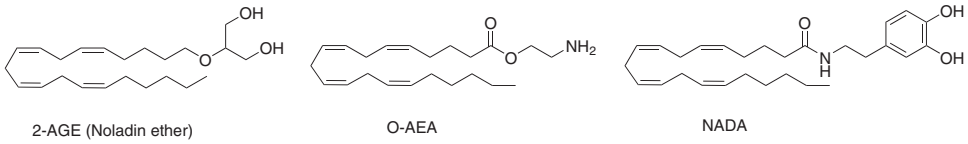

FIG. 2 *Chemical structures of some other putative endocannabinoids: Noladin ether, virodhamine (O-AEA), and NADA.*

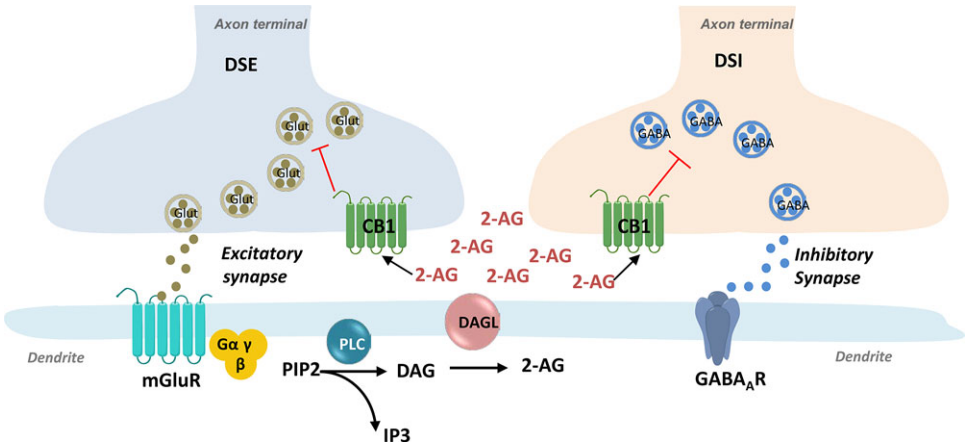

FIG. 3 *A schematic view of endocannabinoid signaling. Glutamate released from the excitatory axon terminal activates type I metabotropic glutamate receptor (mGluR), which stimulates 2-AG production through the phospholipase C and DAGL pathway. 2-AG then crosses the synaptic cleft and activates presynaptic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

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 [16–18]. Endocannabinoids play an essential role in the brain by activating the cannabinoid CB_1 receptor in different brain cells. They modulate neurotransmitter release (Fig. 3) and regulate many physiological processes, including pain perception, learning and memory, energy balance, emotional states (anxiety, fear), and reward-related behavior [19]. The exact contribution of each individual endocannabinoid in specific brain regions to these (patho)physiological functions remains, however, is poorly understood.

Continuous activation of the CB_1 receptor by endocannabinoids is associated with nicotine addiction, obesity, and the metabolic syndrome [20, 21]. Endocannabinoids play also an important role during neurodegeneration and inflammation [22–24]. 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) [25]. This highlights the medical need to understand modulation of the endocannabinoid levels in the brain in a more detailed manner. Inhibitors of the

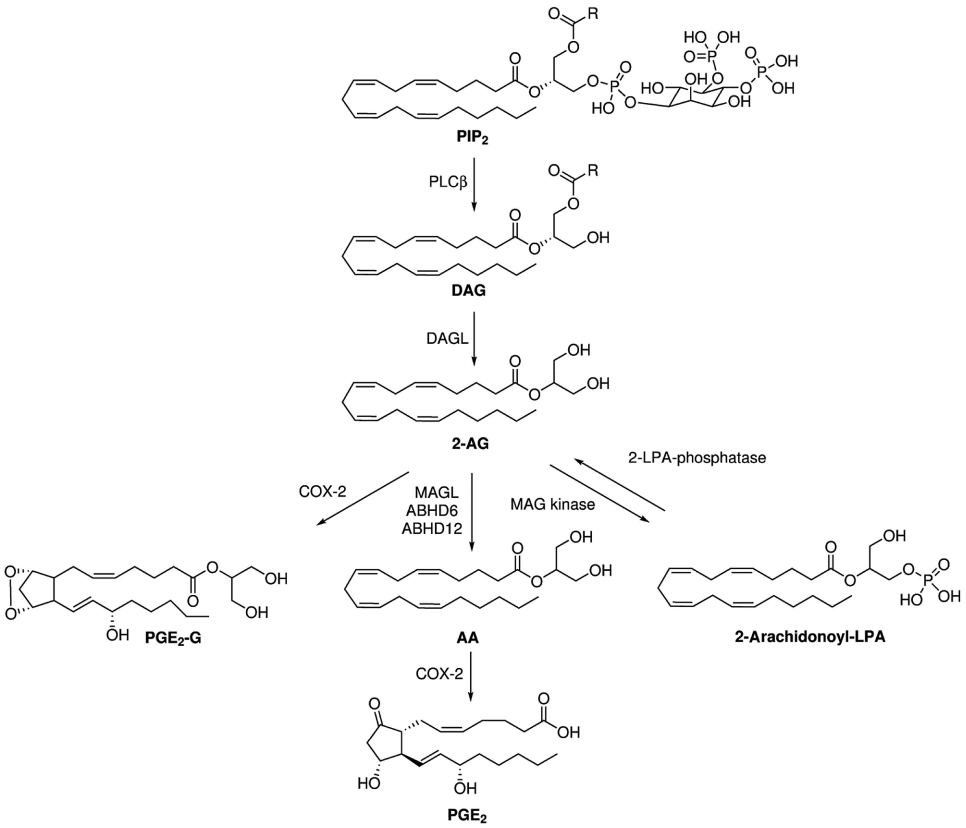

FIG. 4 *Biosynthetic and metabolic pathways of 2-AG.*

biosynthetic enzymes of the endocannabinoids would provide valuable tools to study the role of each endocannabinoid in the various physiological processes. This review will focus on the enzymes that control 2-AG production. Activity-based protein profiling (ABPP) is applied as a chemoproteomic method to identify inhibitors of these enzymes to modulate cannabinoid CB_1 receptor activation by 2-AG.

2. 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) (Figs. 3 and 4) [26]. 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 [27, 28]. In addition, there are some other proposed pathways for 2-AG synthesis [29, 30]. For example, hydrolysis of 2-arachidonoyl-LPA by an LPA phosphatase may also provide 2-AG (Fig. 4) [29].

The rate-limiting step in 2-AG production is controlled by two homologous isoforms of DAGLs, DAGLα (120 kDa) and DAGLβ (70 kDa) [27]. Both proteins are multidomain 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 [31–33].

Genetic studies with DAGL knockout mice have demonstrated that DAGLα and DAGLβ regulate 2-AG production in a tissue-type-dependent manner [32, 34]. 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 DAGL$\alpha^{-/-}$ mice, but not in DAGL$\beta^{-/-}$ mice [35, 36]. Therefore, a pharmacological agent to modulate DAGLα or DAGLβ activity in an acute and temporal manner would provide an important counterpart for DAGL$\alpha^{-/-}$ or DAGL$\beta^{-/-}$ mice to study the physiological functions of DAGLs in complex biological systems.

3. Activity-Based Protein Profiling for DAGL Inhibitor Discovery and Optimization

In early studies, the general lipase inhibitors tetrahydrolipstatin (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 (Fig. 5) [37–39].

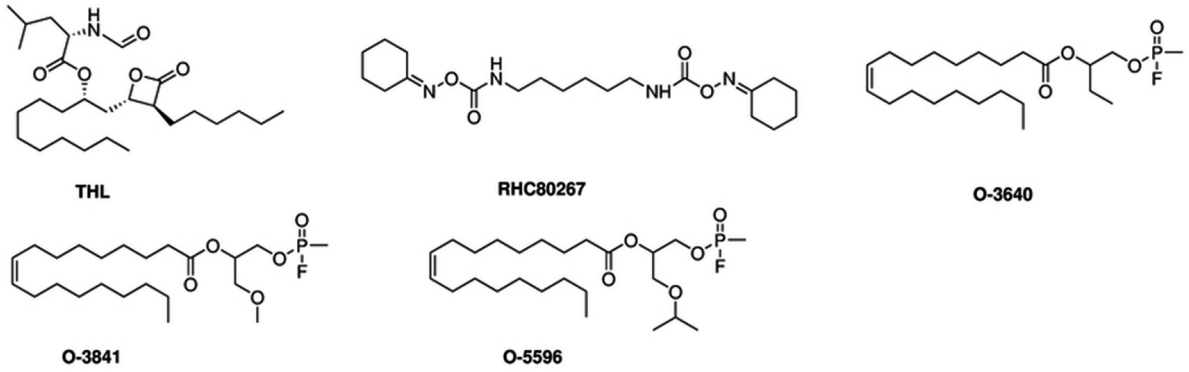

FIG. 5 *Chemical structures of earlier reported DAGL inhibitors: RHC80267, THL, O-3640, O-3841, and O-5596.*

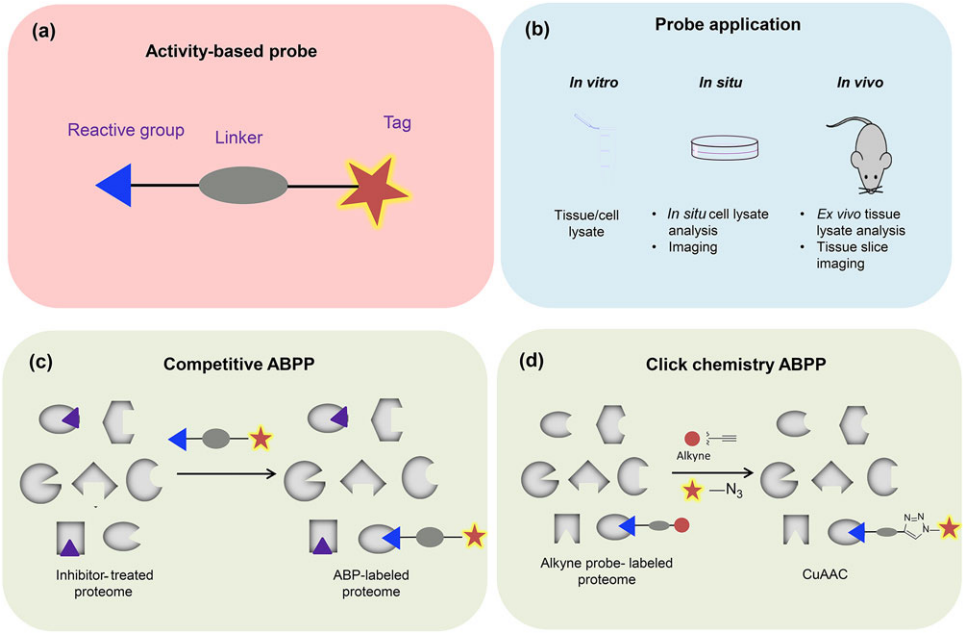

FIG. 6 *Schematic overview of ABPP. (a) Representative cartoons of ABPs: reactive group (blue), linker (gray), and reporter tag (red) (e.g., fluorophore or biotin affinity tag). (b) ABPs can be used in various biological systems, including cell/tissue lysates in vitro, living cellular systems, and in vivo animal models. (c) In competitive ABPP, proteomes are preincubated with inhibitors, followed by coincubation with an ABP. (d) Two-steps probes (click chemistry ABPP) provide a postdetection of protein labeling.*

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 [40]. However, O-5596 cross-reacts with several off-targets, which prohibits its use as a specific DAGLs inhibitor.

To study the function of DAGL in native biological systems, high selectivity, potent, and *in vivo* active DAGL inhibitors are required. ABPP, a chemical proteomic technology, has greatly accelerated the discovery and development of selective and *in vivo* active DAGL inhibitors. ABPP allows the study of the functional state of proteins by chemical probes in a native cellular context [41, 42]. In contrast to other techniques that detect messenger RNA or polypeptides/proteins (i.e., *in situ* hybridization and immunohistology, respectively), ABPP only visualizes active proteins and takes all posttranslational modifications into account. ABPP makes use of organic molecules, termed activity-based probes (ABPs) to label the active site of a protein (Fig. 6a). ABPs are tool 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

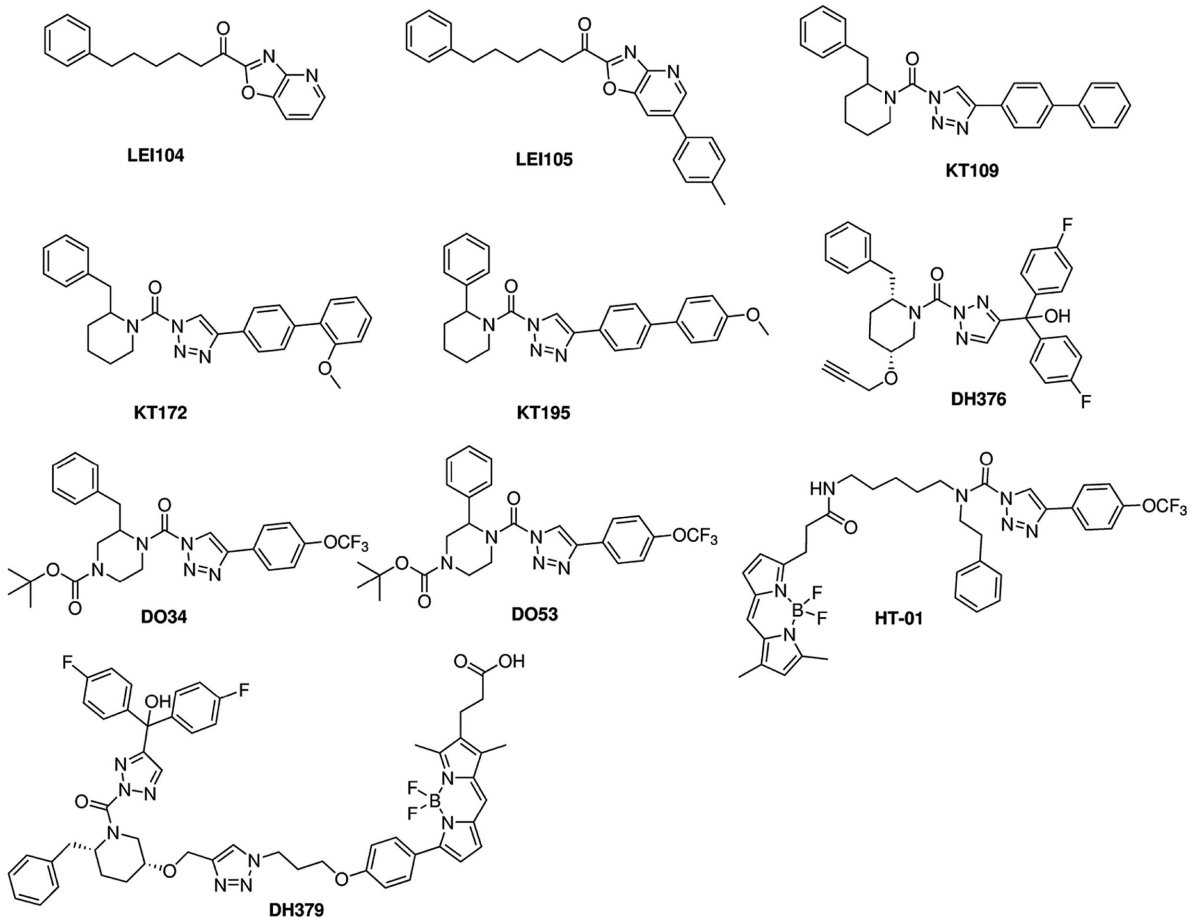

FIG. 7 *DAGL inhibitors with high selectivity and/or in vivo activity. LEI104 and LEI105 are reversible inhibitors with high selectivity. KT109 and KT172 are peripherally restricted in vivo active DAGLβ inhibitors, DH376 and DO34 are CNS-active DAGLs inhibitors. KT195 and DO53 are control compounds. HT-01 and DH379 are DAGL-tailored ABPs.*

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 (Fig. 6b). In competitive ABPP, a small molecule is preincubated with a biological sample and residual enzyme activities are subsequently monitored with an ABP (Fig. 6c). Competitive ABPP can also be used to determine target engagement *in situ* and *in vivo*. 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 (Fig. 6d). 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.

Using an ABPP screen, Baggelaar et al. discovered α-ketoheterocycles LEI104 and LEI105 (Fig. 7) as a new chemotype of selective, reversible DAGLs inhibitors. LEI104 was poorly active in a cellular assay and inhibited fatty acid amide hydrolase (FAAH), the enzyme responsible for the metabolism of anandamide. LEI105, a derivative of LEI104, was a potent and highly selective DAGL inhibitor that did not target other proteins in the endocannabinoid system, including CB_1R, CB_2R, ABHD6, ABHD12, FAAH, and monoacylglycerol lipase (MAGL). However, no *in vivo* activity has been reported for LEI105 to date.

In 2012, Hsu et al. published the first *in vivo* active DAGLβ inhibitors KT109 and KT172 (Fig. 7), which are based on a triazole urea scaffold [43]. KT109 and KT172 were ~60-fold selective over DAGLα as determined by ABPP. Both inhibitors showed high selectivity against DAGLβ over other serine hydrolases, except for ABHD6. To exclude the effects of ABHD6, a control compound KT195 (a close structural analog of KT109 and KT172 that did not inhibit DAGLβ, but did inhibit ABHD6) was applied in their studies. Based on the 1,2,3-triazole urea

scaffold, Hsu et al. also developed an ABP HT-01 for DAGL, which was more sensitive for DAGLβ than DAGLα. KT109 and KT172 selectively inhibit DAGLβ in Neuro2A cells and in mouse macrophages as determined by ABPP using HT-01 and FP-biotin. Acute blockade of DAGLβ by KT109 or KT172 reduced 2-AG, arachidonic acid, and eicosanoid levels in Neuro2A cells, human prostate cancer cells, and mouse peritoneal macrophages. Additionally, DAGLβ blockade decreased the levels of the proinflammatory cytokine tumor necrosis factor α (TNFα) in lipopolysaccharide (LPS)-treated mice. However, KT109 and KT172 did not cross the blood–brain barrier.

Recently, Ogasawara et al. developed the triazole ureas DH376 and DO34 as potent brain-active DAGL inhibitors. They were very selective and displayed only a few off-target activity [44], including ABHD6 (DH376, DO34), CES1C (DH376, DO34), BCHE (DH376), LIPE (DH376), ABHD2 (DO34), PAFAH2 (DO34), and PLA2G7 (DO34). Meanwhile, DO53 was designated as a paired control compound [44], which showed negligible activity against DAGLα or DAGLβ, but cross-reacted with many off-targets of DH376 and DO34. They also reported the development of DH379, a DAGL-tailored ABP. Pharmacological inhibition of DAGLs by DH376 and DO34 induced a rapid reorganization of lipid signaling networks in the brain, including reduction in levels of 2-AG, anandamide, arachidonic acid, eicosanoids (PGD_2 and PGE_2), and an elevation in 1-stearoyl-2-arachidonoyl-sn-glycerol (SAG, C18:0/C20:4) and 1-oleoyl-2-arachidonoyl-sn-glycerol (DAG, C18:1/C20:4). The robust alterations of these brain lipids in DH376- and DO34-treated mice were dose and time dependent and absent in DO53-treated mice. Of note, most lipid changes in DAGL inhibitor-treated mice were mirrored by the lipid changes in DAGL$\alpha^{-/-}$ mice, apart from a reduction in triglycerides. This indicates that chronic, long-term inhibition of DAGLα might be needed for triglyceride metabolism. Interestingly, the authors found that DAGLα is a short half-life protein, which is rapidly degraded and replaced by newly synthesized enzyme. At the moment, it is still unclear how the reduction in anandamide levels, which was observed both in DAGL$\alpha^{-/-}$ mice and in DAGL inhibitor-treated mice, can be explained. It might be due to the strong reduction in arachidonic acid, which could perhaps deplete the pool of arachidonic acid required for the biosynthesis of the precursor of anandamide, but crosstalk between DAG-mediated protein kinase C (PKC) signaling and the enzymes responsible for anandamide biosynthesis is also an interesting option to explore.

Synaptic plasticity regulated by 2-AG signaling, such as depolarization-induced suppression of excitation (DSE) and inhibition (DSI), were fully blocked by both DAGL inhibitors, but not by DO53. Acute inhibition of DAGL also strongly suppressed the LPS-induced neuroinflammation as witnessed by reductions in brain prostaglandin and cytokine levels as well as reversal of anapyrexia [44]. Interestingly, the reduction in cytokine levels was also observed with the negative control compound DO53, suggesting that an additional target may be involved. Furthermore, Deng et al. found that DH376 temporarily reduced fasting-induced refeeding of mice, thereby emulating the effect of cannabinoid CB_1-receptor inverse agonists [45]. This was mirrored by the DAGL inhibitor DO34 but also by DO53. The contribution of the off-target(s) shared by DAGL inhibitors and DO53 in controlling food intake in fasting-induced mice can thus not be excluded. These results indicate that DH376 and DO34, along with the control probe DO53, are important chemical tools for studying diverse aspects of DAGL function in animals and *ex vivo* brain preparations.

To conclude, we have reviewed here the use of ABPP in the development of highly selective DAGL inhibitors, such as DH376 and DO34. Several studies using these chemical tools have shown that DAGLs coordinate crosstalk between different lipid-signaling networks and modulate neuro(immuno)logical functions in the brain. However, to elucidate the specific roles of DAGLα and DAGLβ in (patho)physiological conditions, subtype-selective inhibitors are required. Furthermore, the short half-life of DAGLα also brings some challenges for current set of inhibitors, and improving the pharmacokinetic properties of DAGL inhibitors is required to obtain prolonged DAGLα inhibition *in vivo*.

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