Covalent fluorescent probes for 2-arachidonoylglycerol metabolic pathways

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MINI REVIEW

Covalent fluorescent probes for 2-arachidonoylglycerol metabolic pathways

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Abstract

Covalent fluorescent probes have emerged as versatile chemical tools to visualise active enzymes in complex biological systems. When tailored for specific applications, ranging from activity-based protein profiling for drug development to high spatiotemporal resolution imaging of enzymatic activities, these probes provide unique insights into rapid lipid metabolism. The recent development of reverse-designing optimised inhibitors into probes, together with improved analytical techniques, is rapidly advancing our ability to resolve enzyme function in great detail. In this mini-review, we summarise the current landscape of covalent fluorescent probes for the 2-arachidonoylglycerol (2-AG) metabolising enzymes (diacylglycerol lipase, monoacylglycerol lipase, ABHD6 and ABHD12), highlighting the discoveries they have enabled and discussing strategies to address gaps where probes remain lacking.

KEYWORDS
activity-based probes, chemical biology, diacylglycerol lipases, fluorescent probes, monoacylglycerol lipase

1 | INTRODUCTION

The development of covalent fluorescent probes has greatly expanded the pharmacological toolkit by enabling precise visualisation of enzyme activity within complex biological systems. These probes have yielded insights into both normal physiology and disease-related changes, whilst also accelerating drug discovery (Baggelaar et al., 2017; Niphakis & Cravatt, 2014). Unlike immunohistochemistry, they can be highly tailored to specific applications and directly report enzyme activity in a space and time, rather than protein abundance alone. By incorporating an electrophilic warhead that covalently reacts, either reversibly or irreversibly, with a nucleophilic catalytic residue in the active site, these probes function as activity-based probes (ABPs). This versatility supports applications ranging from chemoproteomic mapping via activity-based protein profiling (ABPP) to in situ imaging of enzyme activity (Baggelaar et al., 2017; Hentsch, Guberman, Radetzki, Kaushik, Huizenga, He, et al., 2025). Such imaging provides high spatiotemporal resolution, particularly valuable for complex biological systems such as the brain, where rapid signalling and regional heterogeneity shape function.

The endocannabinoid system (ECS) exemplifies the need for temporal and spatial resolution (Straub et al., 2025). The ECS regulates mood, appetite, pain, and reward processing by modulating neurotransmission in the central nervous system (CNS) (Maccarrone et al., 2023). Its principal signalling lipids, 2-arachidonoylglycerol (**2-AG**) and *N*-arachidonoylethanolamine (**AEA**, anandamide) activate

Abbreviations: 2-AG, 2-arachidonoylglycerol; AA, arachidonic acid; ABHD, α/β-hydrolase domain-containing protein; ABP, activity-based probe; ABPP, activity-based protein profiling; DAGL, diacylglycerol lipase; ECS, endocannabinoid system; MAGL, monoacylglycerol lipase.

the cannabinoid type 1 receptor (**CB_1R**) at neuronal synapses (Devane et al., 1992; Matsuda et al., 1990; Mechoulam et al., 1995). These lipid pools are tightly regulated by serine hydrolases: diacylglycerol lipases (**DAGLα** and **DAGLβ**) synthesise 2-AG, which is primarily degraded by monoacylglycerol lipase (**MAGL**) with minor contributions from α,β-hydrolase domain-containing proteins **ABHD6** and **ABHD12** (Figure 1) (Bisogno et al., 2003; Blankman et al., 2007; Dinh et al., 2002). AEA biosynthesis involves **ABHD4** and phospholipase **PLA2G4E**, whereas its degradation is mediated by fatty acid amide hydrolase (**FAAH**). Dysregulation of these pathways has been linked to neurological and psychiatric disorders, including Alzheimer's disease, Parkinson's disease, depression and anxiety (Maccarrone et al., 2023).

Recent advances in selective covalent probes, together with improved analytical methods, now offer unprecedented opportunities to study these complex processes. This rapidly evolving toolkit is already reshaping ECS research (Punt et al., 2022). In this mini-review, we summarise the current landscape of covalent fluorescent probes targeting 2-AG-metabolising lipases, describe their design principles and highlight key discoveries enabled by their use. We also discuss gaps in probe availability for certain lipases and potential approaches to address them.

We classify covalent fluorescent probes into two main categories of activity-based proteins (ABPs) (Figure 2): (i) *broad-spectrum ABPs*, probes that label many enzymes, useful for mapping global activity and cross-reactivity in a protein family; and (ii) *tailored ABPs*, probes with chemical features that direct them towards a specific enzyme. These probes enable visualisation and pharmacological validation of single proteins.

2 | DAGL-TARGETED PROBES

DAGLα is predominantly expressed in the CNS, whereas DAGLβ is more found in peripheral tissues and the immune system (Gao et al., 2010). Dysfunction of these enzymes has been linked to non-motor symptoms of Parkinson's disease and an increased risk of mental disorders (Kim et al., 2023; Doherty & Williams, 2025), making them important targets to study the disease pathology. Both isoforms contain a Ser-His-Asp catalytic triad, yet unlike many serine hydrolases they are poorly detected by conventional broad-spectrum ABPs such as fluorophosphonate-rhodamine (**FP-Rh**, compound 1; see Figure 2) (Hoover et al., 2008; Hsu et al., 2012). This gap created the need for tailored covalent probes to study their activity.

To address this, the Cravatt lab developed **HT-01** (compound 2), the first DAGL-directed fluorescent ABP in 2012 (Hsu et al., 2012). Based on the established triazole urea chemotype, HT-01 retains the core scaffold, but the piperidyl moiety was replaced by a

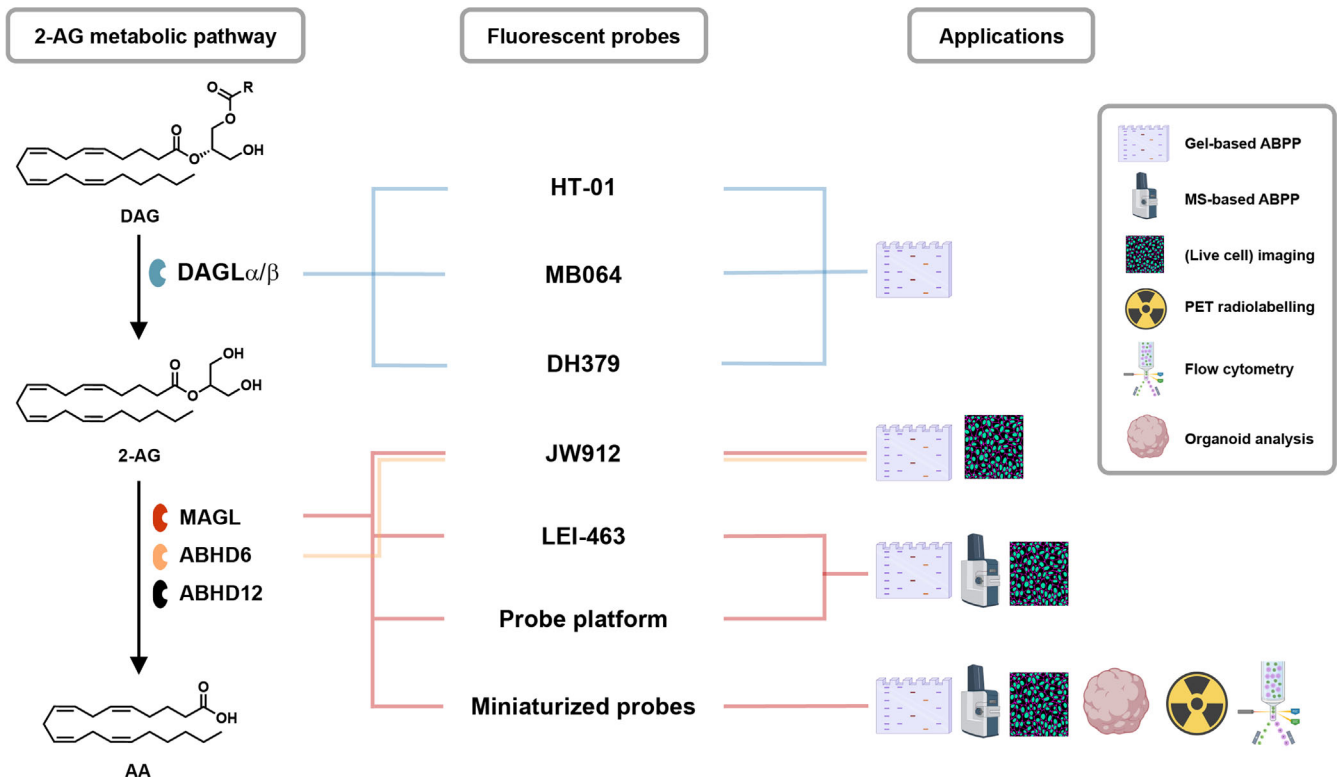

FIGURE 1 **Overview of the 2-arachidonoylglycerol (2-AG) metabolic pathway and associated tailored fluorescent probes with their reported applications.** Diacylglycerol (DAG) is hydrolysed by diacylglycerol lipase (DAGLα and DAGLβ) to produce the endocannabinoid 2-AG, which is degraded by monoacylglycerol lipase (MAGL) and α,β-hydrolase domain-containing proteins ABHD6 and ABHD12 to arachidonic acid (AA). Tailored fluorescent probes discussed in this review are shown in relation to their target enzyme and direct translational applications.

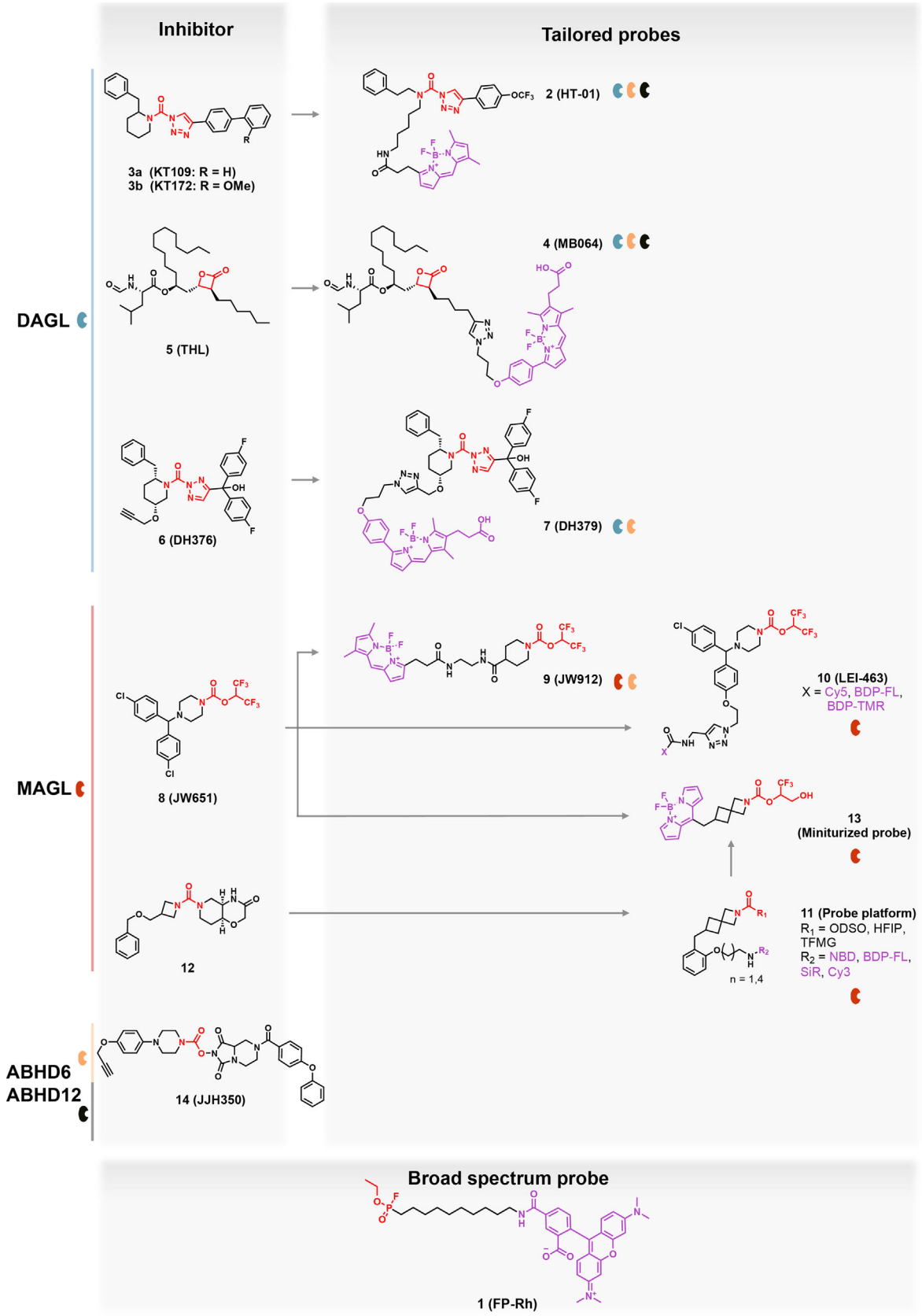

FIGURE 2 Legend on next page.

FIGURE 2 **Overview of covalent fluorescent probes for 2-AG metabolic lipases.** Representative probes are shown for diacylglycerol lipases (DAGLα/β), monoacylglycerol lipase (MAGL), and the α/β-hydrolase domain containing proteins (ABHD6 and ABHD12). Reactive warheads are highlighted in red, reporter groups in purple, and grey arrows indicate original reverse-design scaffold. Enzymes labelled by the tailored ABPs other than their target enzymes are colour coded (blue: DAGL; red: MAGL; orange: ABHD6; black: ABHD12). Abbreviations: BDP, boron-dipyrrolemethene; HFIP, hexafluoro-isopropanol; NBD, nitrobenzoxadiazole; ODSO, 7-oxa-2,5-diazaspiro[3,4]octan-6-one; SiR, silicon rhodamine; TFMG, trifluoromethyl glycerol; TMR, tetramethyl rhodamine.

phenethylamine for potency, and a BODIPY fluorophore linked via a spacer (Figure 2; compound 2). The resulting covalent, irreversible probe enabled fivefold stronger visualisation of endogenous DAGLβ activity in gel-based ABPP experiments using proteomes from Neuro2A cells, primary neurons and mouse brain. HT-01 served as a competitive probe for the discovery and target engagement of DAGL inhibitors **KT109** (compound 3a) and KT172 (compound 3b), and demonstrated ABHD6 as a significant off-target for both inhibitors.

A year later, we introduced MB064 (compound 4), the first DAGLα-directed fluorescent ABP (Baggelaar et al., 2013). MB064 was derived from the clinically-used drug **tetrahydrolipstatin** (orlistat; compound 5), a lipase inhibitor containing a β-lactone warhead. Introducing an alkyne at the short alkyl tail enabled attachment of a BODIPY as a fluorescent reporter via click chemistry. MB064 provided robust readouts of DAGLα activity in gel-based ABPP experiments using mouse brain membranes (Deng et al., 2017). Competitive ABPP using MB064 facilitated the discovery of **LEI-105**, a highly selective, potent, and reversible DAGL inhibitor (Baggelaar et al., 2015). Both HT-01 and MB064 remain essential tools for profiling DAGL activity and guiding inhibitor discovery; however, they cannot be used as imaging probes to visualise DAGL activity in cells and tissue due to their lack of selectivity.

Building further on the triazole urea scaffold, **DH376** (compound 6) and **DO34**, were developed with nanomolar potency against DAGLα (IC_{50}: ~6 nM) and DAGLβ (IC_{50}: 3–8 nM), as determined by a natural substrate assay and ABPP experiments (Ogasawara et al., 2016). The alkyne in DH376 enabled its use as a two-step ABP for in vivo target engagement studies, which revealed the short half-life of DAGLα. Both DH376 and DO34 showed good in vivo activity and were instrumental in studying the physiological processes involving 2-AG biosynthesis, such as synaptic plasticity in the hippocampus and cerebellum, **cocaine** seeking, **alcohol** addiction, food intake, neuroinflammation, anxiety and stress, learning and memory, pain sensation and voluntary movement (Maccarrone et al., 2023). It should be noted that DO34 and DH376, but not LEI-105, also inhibited a few other serine hydrolases, such as ABHD6, butyrylcholinesterase (**BCHE**) and CES1C (mouse homologue of **CES1**), as determined by chemical proteomics (Ogasawara et al., 2016; Van Rooden et al., 2018). Exploiting its alkyne handle, DH376 was converted into DH379 (compound 7), a tailored fluorescent probe with a BODIPY reporter, which allowed for gel-based ABPP of both DAGL isoforms (Ogasawara et al., 2016). In summary, a versatile set of broad-spectrum and tailored ABPs for DAGL have been developed, which allowed in vivo target engagement and drug discovery studies. No specific ABPs or PET tracers are currently available to visualise DAGL activity in cells, organoids, tissue slices, intact animals or humans.

3 | MAGL-TARGETED PROBES

MAGL is the principal enzyme that hydrolyses 2-AG to **arachidonic acid**, a precursor for pro-inflammatory lipid mediators, thereby regulating CB_1R-mediated processes and inflammation in the brain (Baggelaar et al., 2018). Owing to this central role in lipid signalling, MAGL has attracted considerable interest as a therapeutic target (Van Egmond et al., 2021). This has spurred extensive efforts to develop selective inhibitors, some of which have been redesigned into fluorescent probes (Figure 2).

In 2013, Cravatt's lab performed a chemoproteomic analysis of carbamate electrophiles across serine hydrolases using ABPP (Chang et al., 2013). This revealed O-hexafluoro-isopropyl (HFIP) carbamates as particularly selective for the 2-AG hydrolases (MAGL and ABHD6). Guided by this and previous structure–activity relationship studies, they developed MAGL-selective HFIP carbamate JW651 (compound 8) (ABPP IC_{50}: ~38 nM; substrate assay IC_{50}: 4.5 nM), which elevated brain 2-AG levels ~10-fold in mice. Using the same motif, they generated JW912 (compound 9), the first fluorescent covalent probe for MAGL and ABHD6. JW912 enabled in-gel ABPP at 100 nM but lacked selectivity; however, co-incubation with inhibitors such as JW651 (for MAGL) or KT-195 (for ABHD6) allowed reporting of either enzyme's cellular distribution. This requirement for pre-treatment with inhibitors, however, may perturb the physiological system under study.

JW651 was further adapted with an alkyne (JW651yne) to act as a two-step probe in ABPP experiments using click chemistry. We leveraged this handle to attach a Cy5 fluorophore, producing LEI-463 (compound 10), the first selective, covalent, and irreversible fluorescent probe for MAGL (Prokop et al., 2021). Combining a highly selective covalent MAGL ligand with the bright Cy5 fluorophore enabled pharmacoSTORM super-resolution imaging, yielding the first nanoscale, subcellular maps of MAGL. Pretreatment with the covalent MAGL inhibitor **JZL184** greatly diminished the LEI-463 signal, whereas immunostaining remained bright, demonstrating the selective visualisation of active MAGL. This super-resolution imaging enables visualisation of subcellular drug-target interactions, thereby linking nanoscale target-engagement with pharmacological effects.

More recently, we applied the same probe LEI-463, and related analogues LEI-463BDP-TMR/BDP-FL, in activity-based histology of fresh-frozen human brain tissue (Van Der Vliet et al., 2025). This study enabled the first high-resolution spatial mapping of MAGL in human brain tissue, illustrating how legacy covalent inhibitors can be repurposed with modern imaging techniques.

In parallel, Nazaré and co-workers developed a modular probe platform (compound 11) for MAGL using reverse-design and structure

activity relationship studies from compound 12 (Hentsch, Guberman, Radetzki, Kaushik, Huizenga, Paul, et al., 2025). The pharmacophore core tolerated interchangeable headgroups (irreversible carbamates or reversible ureas) and a rigid exit vector for conjugation to various fluorophores (nitrobenzoxadiazole, NBD; boron-dipyrromethene, BODIPY; silicon rhodamine, SiR; and cyanine 3, Cy3). These probes retained high potency (IC_{50}: 0.09–1.15 nM) and high selectivity as determined by gel-based ABPP and were applied to subcellular MAGL imaging of neuronal and cancer cells as well as quantification in patient-derived peripheral blood mononuclear cells as a biomarker.

The same research group miniaturised the probe by cleverly embedding the BODIPY fluorophore directly into the hydrophobic scaffold (compound 13), thereby avoiding long linkers that may impair drug-like properties (Hentsch, Guberman, Radetzki, Kaushik, Huizenga, He, et al., 2025). These compact probes retained nanomolar potency (IC_{50}: 0.14–0.28 nM), whilst improving physiochemical properties such as solubility. Beyond imaging in brain organoids, they enabled fluorescent polarisation assays, flow cytometry and autoradiography. The latter was facilitated by facile radiolabelling of the BODIPY in compound 13 with ^{18}F, allowing autoradiographic mapping of MAGL in mouse brain slices.

In summary, MAGL-targeted probes combine excellent selectivity, potency and chemical versatility enabling powerful imaging applications. Because they rely on covalent active site trapping, they inactivate the enzyme, which is not compatible with real-time imaging of MAGL in intact cells without disturbing its function. A future direction would be to develop probes that report on MAGL activity without permanent inactivation, for example, through ligand-directed approaches (Tamura & Hamachi, 2025).

4 | ABHD6 AND ABHD12-TARGETED PROBES

Alongside MAGL, the serine hydrolases ABHD6 and ABHD12 also contribute to 2-AG hydrolysis, with ABHD6 predominantly active in postsynaptic sites and ABHD12 in the microglia (Lau et al., 2024; Marrs et al., 2010). Despite their relevance, probe development for these enzymes remains limited. To date, the only dedicated tool is the dual ABHD6- and ABDH12-targeted two-step ABP **JJH350** (compound 14), and no selective fluorescent probes have been reported for either enzyme.

The promiscuous active site of ABHD6 has, however, allowed incidental labelling by other tailored probes. DAGL-directed probes such as HT-01, MB064 and DH379 as well as the MAGL-directed JW912 all display ABHD6 activity. This property was exploited by imaging ABHD6 in live cells with JW912 in the presence of a selective MAGL inhibitor (Chang et al., 2013). Broad spectrum probes such as FP-Rh also label both ABHD6 and ABHD12, albeit with limited specificity. For ABHD12, the introduction of JJH350 enabled a two-step ABPP workflow to support structure–activity relationship studies, ultimately leading to the discovery of **DO264**, a potent (IC_{50}: 11 nM), selective and in vivo active ABHD12 inhibitor (Ogasawara et al., 2019).

In summary, ABHD6 and ABHD12 remain underexplored compared with DAGL and MAGL. Existing probes rely largely on cross-reactivity or two-step strategies, limiting their application for high-resolution imaging. Future efforts should focus on adapting scaffolds such as JJH350 or DO264 into fluorescent probes, enabling direct visualisation of ABHD6 and ABHD12 activity in cells and tissues.

5 | CONCLUSIONS AND OUTLOOK

Covalent fluorescent probes have become indispensable tools for investigating 2-AG signalling, enabling direct visualisation of enzyme activity from in vitro assays to intact brain slices. In turn, yielding pharmacological insights through direct measurement of target engagement, resolution of isoform selectivity and linking enzyme inhibition with physiological and spatiotemporal changes in vivo. This field has progressed from broad-spectrum probes such as FP-Rh to increasingly tailored-designs for DAGL and MAGL. Each subclass of covalent probes (broad or tailored) offers distinct advantages, with the most selective probes now supporting high resolution spatiotemporal studies. Recent innovations, including miniaturised drug-like probes and super-resolution imaging applied to intact brain tissues, underscore the rapid pace of development.

Despite these advances, several challenges remain. For DAGL, achieving selectivity without cross-reactivity with ABHD6 is difficult. Structural insights on DAGL from crystallography, cryo-EM or AlphaFold are likely to accelerate probe development, as has already occurred for MAGL. More broadly, probes capable of reporting on enzyme activity without irreversible inactivation are needed to enable dynamic tracking in live cells and tissues, with ligand-directed approaches offering promising solutions. Additionally, ABHD6 and ABHD12 still lack dedicated fluorescent probes.

Beyond 2-AG metabolism, the anandamide branch of the ECS remains largely unexplored with covalent fluorescent ABPs. A small set of inhibitors, including LEI-301 for PLAAT 1–5, **LEI-401** for **NAPE-PLD** and **PF-04457845** for FAAH, could serve as starting points for reverse-design into fluorescent probes (Johnson et al., 2011; Mock et al., 2020; Zhou et al., 2020). By contrast, other enzymes in the AEA pathway, such as PLA2G4E, ABHD4 and the GDE family, still lack suitable inhibitor scaffolds (Alexander et al., 2025). Addressing these gaps would complete the chemical biology toolbox for precise interrogation of the enzymes of the ECS to assist in drug discovery and development.

5.1 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/22 (Alexander et al., 2021).

AUTHOR CONTRIBUTIONS

Nick D. F. Puijmbroeck: Writing—review and editing. **Mario van der Stelt:** Writing—review and editing.

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CONFLICT OF INTEREST STATEMENT

M. v. d. S. holds a patent covering DH376.

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