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Reverse Design toward Optimized Labeled Chemical Probes – Examples from the Endocannabinoid System

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Abstract: Labeled chemical probes are of utmost importance to bring drugs from the laboratory through the clinic and ultimately to market. They support and impact all research and discovery phases: target verification and validation; assay development; lead optimization; and biomarker engagement in the context of preclinical studies and human trials. Probes should display high potency and selectivity as well as fulfill specific criteria in connection with absorption, distribution, metabolism, excretion and toxicology (ADMET) profile. Progress in fields such as imaging and proteomics increased the need for specialized probes to support drug discovery. Labeled probes carrying an additional reporter group are valuable tools to meet specific application requirements, but pose significant challenges in design and construction. In the reverse-design approach, small molecules previously optimized in medicinal chemistry programs form the basis for the generation of such high-quality probes. We discuss the reverse design concept for the generation of labeled probes targeting the endocannabinoid system (ECS), a complex lipid signaling network that plays a key role in many human health and disease conditions. The examples highlighted include diverse reporter units for a range of applications. In several cases the reported probes were the product of mutually rewarding and highly cross-fertilizing collaborations among academic and industry research programs, a strategy that can serve as a blueprint for future probe generation efforts.

Keywords: Academia–industry collaboration · Chemical probe · Drug discovery · Endocannabinoid system · Reverse design

Mónica Guberman studied Chemistry at the University of Buenos Aires (Argentina). She performed her doctoral research under the supervision of Prof. Dr. Seeberger (Max Planck Institute of Colloids and Interfaces, Potsdam, Germany), focusing on the synthesis of complex glycans and the development of synthetic methodology, with intensive application of solid-phase automated synthesis and flow chemistry. In August 2020 she joined the Medicinal Chemistry Group of Dr. Nazaré (Leibniz Research Institute for Molecular Pharmacology, Berlin, Germany) as a postdoc, to work on the design and development of chemical probes in collaboration with F. Hoffmann-La Roche Ltd.

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Marc Nazaré studied chemistry at the University of Karlsruhe and obtained his PhD in organic chemistry in 1999 with Herbert Waldmann working on a total synthesis of a natural product. He then joined pharmaceutical industry at Sanofi in Frankfurt as a medicinal chemist. In 2013, Dr. Nazaré started a medicinal chemistry group at the Leibniz Institute for Molecular Pharmacology FMP. His current research interests are focused on the design and optimization of chemical probes and imaging tools for chemical biology.

Uwe Grether received his Diploma in Chemistry at the University of Karlsruhe, Germany, where he subsequently earned his PhD in Organic Chemistry with Professor Herbert Waldmann in 2000. After that, he joined Professor James D. White's group at Oregon State University for his postdoctoral research. In 2001, Dr. Grether started at the Pharma Research and Early Development unit of F. Hoffmann-La Roche Ltd. in Basel, Switzerland. His research interests include medicinal chemistry, late stage functionalization and chemical probes focusing on holistic approaches toward a clinical end-goal.

1. Introduction

Chemical probes address fundamental biological questions associated with cellular targets and link chemistry, biology, and drug discovery. They influence all research and discovery phases. The use of probes can verify whether a specific target is expressed and in turn unravel mechanistic questions during the target validation process. Additionally, chemical probes are instrumental for setting up tailor-made *in vitro* pharmacology assays during hit identification and subsequent lead optimization phases. Moreover, they can be implemented to verify target engagement, identify biomarkers, and support preclinical studies and human trials. The use of chemical probes has been around for quite some time, and they are rather ambiguously defined. Although the history of chemical probes traces back to over four decades, their developments were predominantly empirically guided.[1] Progress in fields such as imaging and proteomics increase the demand to adapt these probes to specific technological requirements to support drug discovery. As a reflection of their ever-growing significance and applications,[2] herein we employ the definition of a labeled chemical probe as a small molecule that is a ligand for the target and bears a reporter unit that allows characterization of ligand-target interactions, optionally connected by a linker (Fig. 1).[3]

Effective and reliable probes should demonstrate defined, relevant, well-characterized mechanism of action as well as potency, and selectivity while precluding off-target effects.[2b] To access intracellular targets, probes need to possess cellular permeability, which in turn delimits a set of physicochemical properties.[4] For their application in late-preclinical and clinical drug discovery, suitable ADMET characteristics are additionally required. Some probes do not fulfill the criteria,[5] and despite their widespread use, the generated data is of limited utility.[4] Traditional approaches typically consider only the potency of a model ligand as the criterion for selection of the recognition element, and thus fall short of the exacting requirements associated with modern research and development drug discovery endeavors.

In the reverse-design approach, the exceeding demands for high-quality probes can be met in a timely manner by leveraging prior optimization performed by medicinal chemists. The development of selective, potent probes with fitting pharmacokinetic properties is, therefore, expedited by the pre-existent structure-activity relationship (SAR) data and inherent modification possibilities for labeling. Moreover, the availability of (co)crystal structures of the biological target with ligand provides a wealth of information. Probe design can be fast-tracked by the identification of exit vectors from the ligand binding site along with appropriate spacers between the recognition element and reporter. Both design features are crucial to prevent detrimental probe–target interactions.

In this article, we showcase the important concepts of reverse-design by focusing on the endocannabinoid system (ECS), a complex lipid-signaling network of paramount therapeutic interest.[6] We discuss current opportunities and future challenges in the field of labeled chemical probes by highlighting diverse probe types, reporter units, and their applications for selected targets within the ECS.

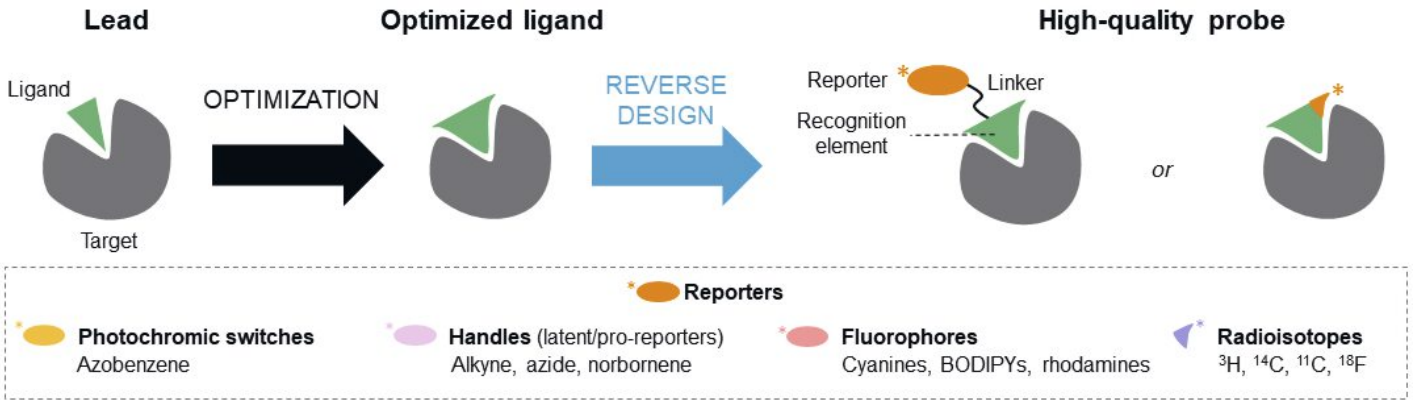


Fig. 1. Reverse-design approach to labeled chemical probes and illustrative examples of reporter units.

1.2 *Probe Types*

Drug discovery processes generally start with new therapy proposals and a subsequent target assessment phase. Lead identification and optimization efforts follow and, if successful, provide development candidates, which are thoroughly evaluated in multiple preclinical investigations, clinical studies, and culminate in product launch. Often market introduction of a new molecular

entity is accompanied by specific labeled chemical probe-based diagnostic tests. Overall, labeled chemical probes have proven to be essential for supporting all research, development, and even post-launch phases as they address a variety of questions and applications. These include unravelling target expression and signaling mechanisms in cell-type, tissue, and disease state-dependent contexts. Applications encompass *in vitro* pharmacology assays and generation as well as translation of information from preclinical studies on non-human species to the patient. Consequently, a large diversity of chemical probes with specific (ADMET) profiles and reporter units matched to their applications are needed.[7]

Radioactive reporter moieties, typically ^{3}H, ^{14}C, ^{35}S, ^{125}I or ^{11}C, ^{18}F, are contained in radioligands and positron emission tomography (PET) tracers, respectively. The radioisotopes emit detectable and quantifiable β-particles, γ–radiation, or positrons. Radioligands are employed for the characterization of their unlabeled counterparts, providing binding affinities[8] and kinetics[9] as well as for the investigation of tissues using autoradiography.[10] The primary aims of PET tracers are the non-invasive determination of receptor expression in tissues along with the distribution and receptor occupancy of drug candidates in patients. Additionally, PET tracers are an indispensable tool in early development of therapeutics.[11]

Fluorescently labeled molecules are versatile tools that have been used extensively *in vitro* and *in vivo*. The major challenge for the design of fluorescent probes includes identification of a

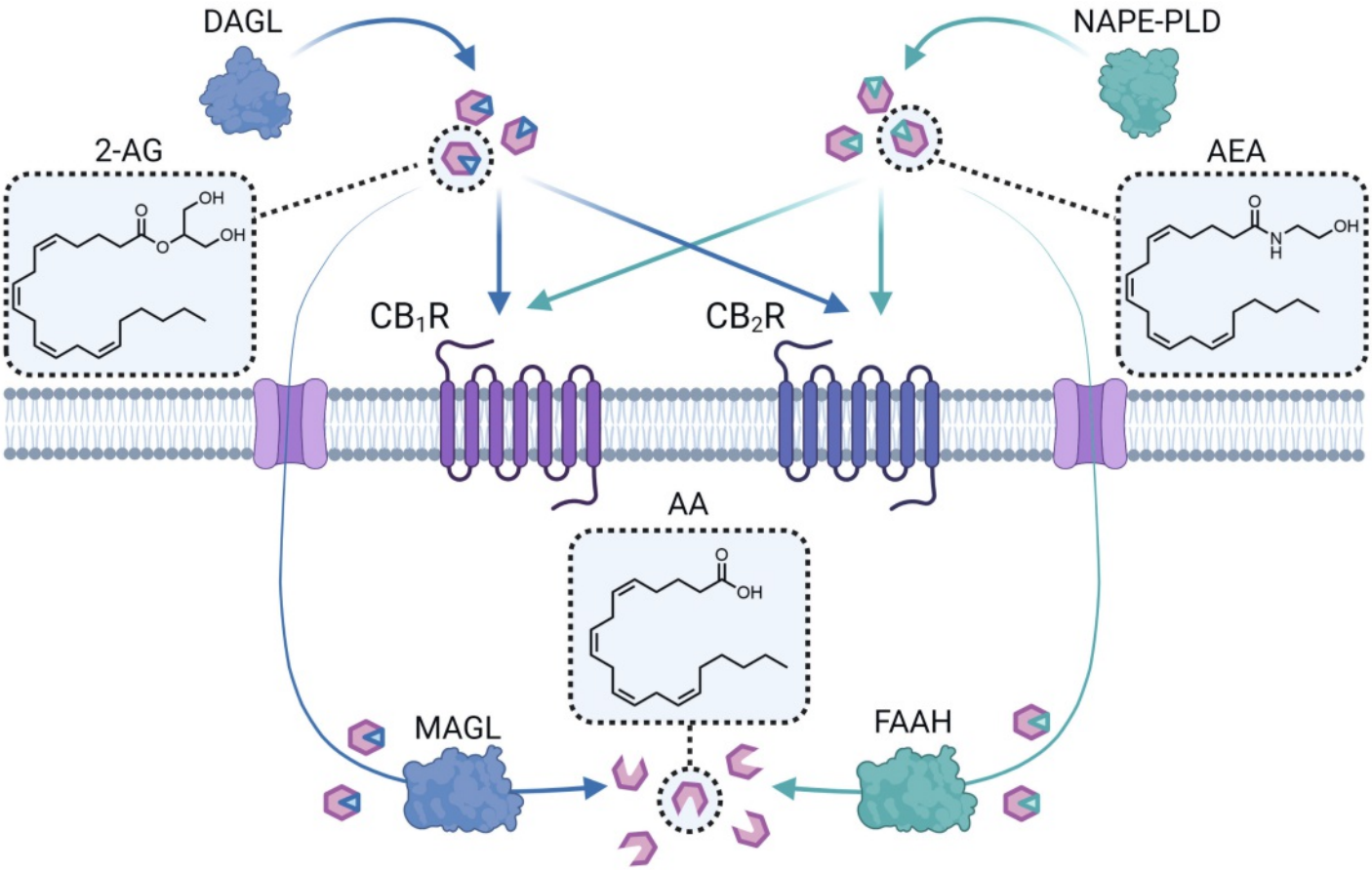


Fig. 2. The fundamental components comprising the endocannabinoid system.[21] For abbreviations see text.

suitable attachment point for chemical modification without compromising affinity and overall probe properties. Incorporation of cell-permeable fluorescent dyes results in small-molecule derived probes capable of visualizing intracellular protein targets, a task which cannot be achieved with traditional antibodies.[12] Their use enables determination of the localization and expression level of targets as well as unravels molecular mechanisms driving pharmacological responses.[13] Techniques such as fluorescence confocal microscopy, flow cytometry and time-resolved Förster resonance energy transfer (TR-FRET) have been successfully applied to study ligand–receptor interactions.

Photoswitchable probes are functional ligands which can be reversibly activated by light. They consist of a core recognition element connected to a chromophore, such as, azobenzenes, diarylethenes or binaphthyls. Irradiation results in temporal geometrical isomerization which subsequently leads to different and temporal functional activities at the target. Photoswitchable ligands can provide exceptional spatiotemporal control over receptor function and, therefore, are valuable tools to study receptor kinetics, dynamics, and function.[14]

Covalent probes are the modality of choice when experimental conditions required for binding site mapping, protein isolation and purification, result in disruption of non-covalent interactions between ligand and target protein. Attachment of the probe to a protein can be achieved by incorporating onto the ligand either a photoactivatable or electrophilic moiety, which ideally reacts with one or more amino acid side chains located at or near the ligand binding site.[15] Photoactivatable or photoaffinity probes contain a photoreactive moiety, such as an azide, diazirine or benzophenone. Upon irradiation with a specific wavelength these

groups form a reactive nitrene, carbene or biradical, respectively, which subsequently covalently bond to a nearby amino acid. By contrast, probes containing electrophilic entities are matched to specific amino acid partners in close proximity and are therefore more difficult to design. However, the design efforts required are counterbalanced by crosslinking yields, which are generally higher than those observed for photoaffinity probes. Accordingly, numerous electrophiles, spanning a wide range of reactivity and amino acid specificity, have been reported.[16] Bifunctional probes that combine a reactive site for covalent attachment and a handle for introducing a second reporter group have proven to be powerful and efficacious in multiple medicinal chemistry applications, such as activity-based protein profiling (ABPP).[17]

1.3 *The Endocannabinoid System (ECS)*

The ECS provides a prime example where the synergy of different types of labeled chemical probes, achieved by reverse design of known binders, was successfully leveraged to study and elucidate the complex signaling network. The ECS is ubiquitous to all vertebrates and consists of membrane receptors responsible for triggering intracellular pathways,[18] their endogenous ligands[19] and biosynthetic as well as hydrolytic enzymes[20] (Fig. 2).

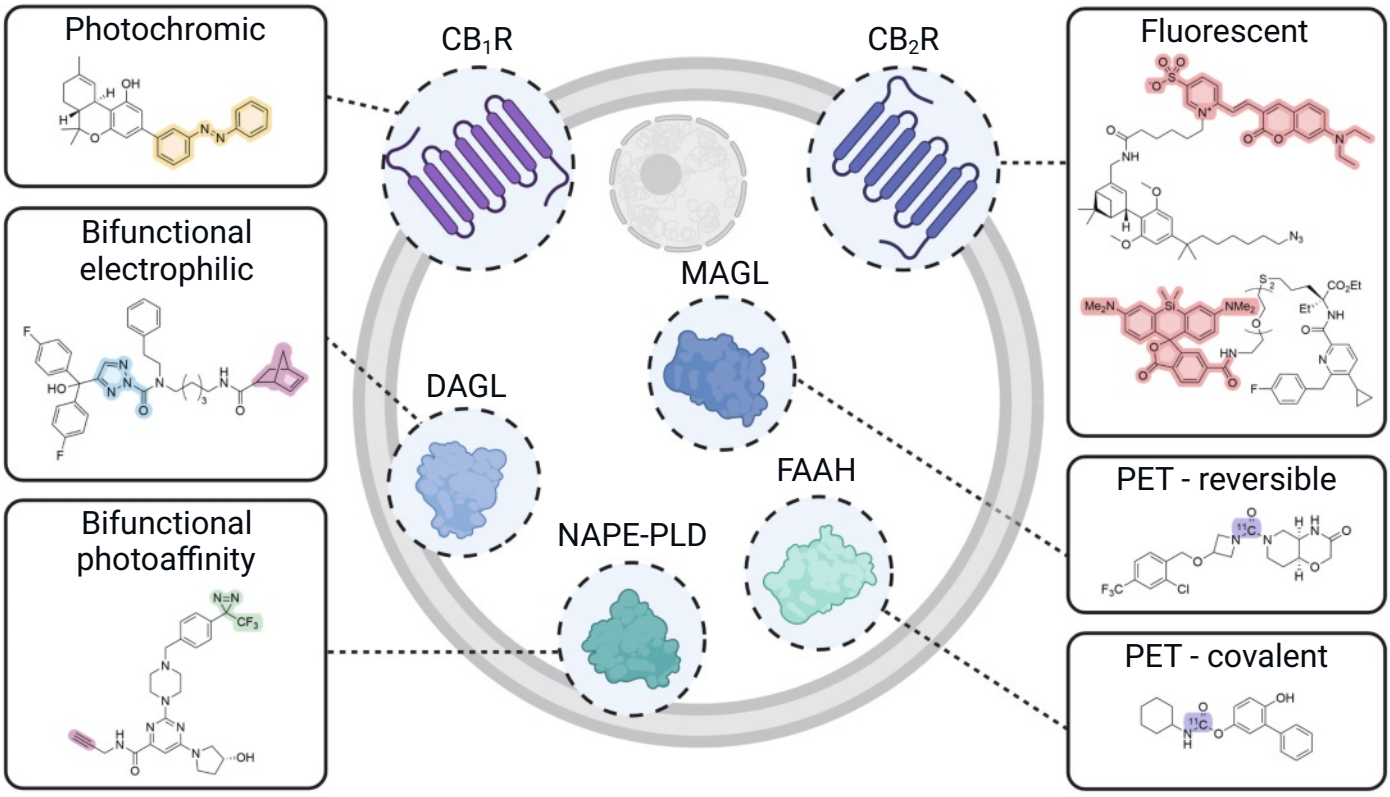
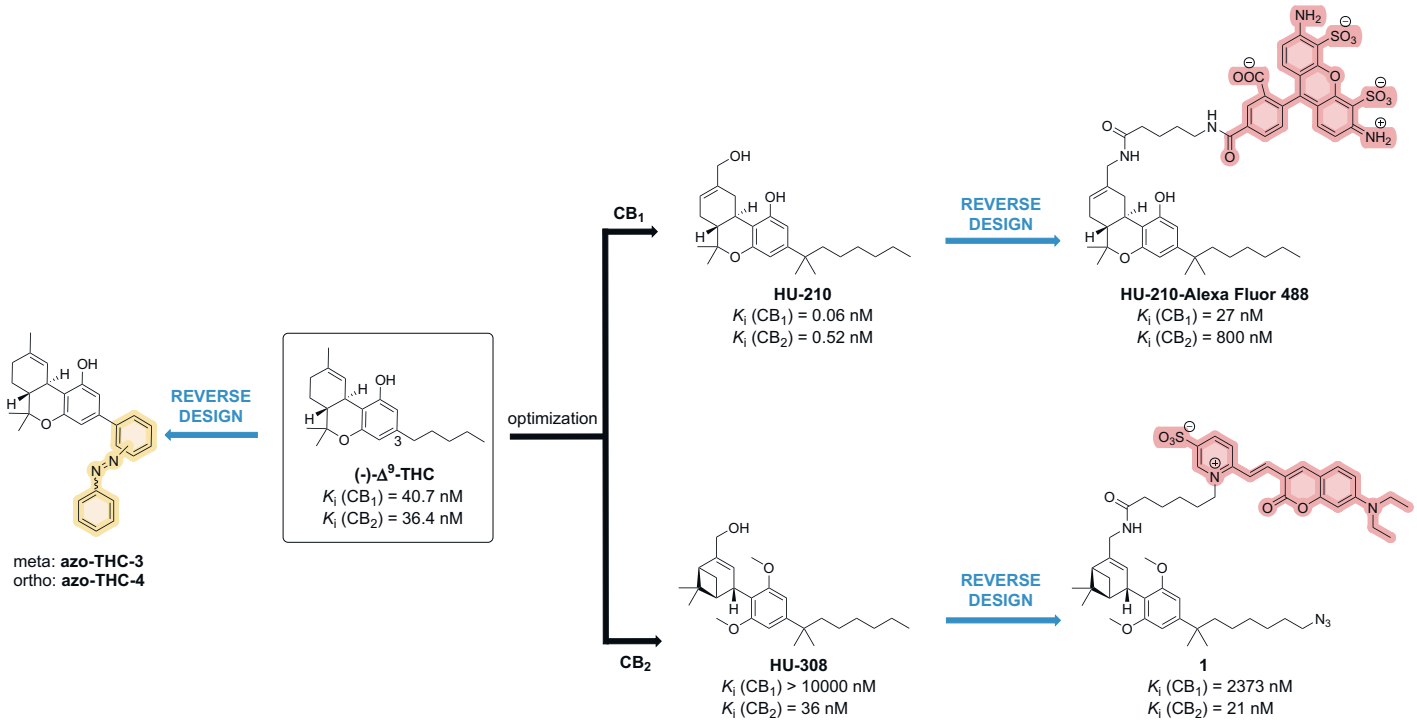


Fig. 3. Overview of the different types of representative labeled chemical probes and their respective targets of the endocannabinoid system.[21]

G-protein coupled receptor (GPCR) cannabinoid receptor 1 (CB_1R) is primarily expressed in the central nervous system (CNS) and responsible for the psychoactive effects of (–)-Δ^9-tetrahydrocannabinol (THC).[22] Cannabinoid receptor 2 (CB_2R) is found mainly in cells of the immune system and has been shown to play a crucial role in immunomodulatory disorders,[23] such as atherosclerosis, and neuroinflammatory disorders, for instance Alzheimer's disease.[24] Arachidonoylethanolamide (AEA) and 2-arachidonoylglycerol (2-AG) are derivatives of arachidonic acid (AA) and the two most prominent endocannabinoid ligands that bind and trigger cellular signaling by acting on both CB_1R and CB_2R.[25] AEA is synthesized on demand by the enzyme *N*-acyl-phosphatidylethanolamine-specific phospholipase D (NAPE-PLD)[26] and acts on membrane-bound receptors, for example, presynaptically located CB_1R to facilitate inhibition of neurotransmitter release.[27] The action of AEA is interrupted upon hydrolysis catalyzed by fatty acid amidohydrolase (FAAH).[28] Diacylglycerol serves as a precursor for the synthesis of 2-AG by the enzyme diacylglycerol lipase (DAGL)[29] and the majority of 2-AG inactivation is catalyzed by monoacylglycerol lipase (MAGL).[30] Despite the hydrolysis of AEA and 2-AG, which terminates action at the cannabinoid receptors, the hydrolysis product AA engages in further signaling cascades, *via* its conversion to prostaglandins and leukotrienes.[31] Consequently, the synthesis, action and catabolism of endocannabinoids is a tightly regulated, complex process whose elucidation requires chemical probes with a wide variety of recognition and reporter elements. With the aid of targets from the ECS, we showcase the best-in-class labeled probes that were constructed by reverse design and allowed to interrogate ECS signaling at the molecular level as well as identify and visualize its individual components.

2. Labeled Chemical Probes Targeting Proteins of the ECS

Owing to the complexity of the ECS a variety of specialized probes was employed to investigate individual contribution of its components to aid in understanding the overall system (Fig. 3). For example, photoswitchable THCs, established by the Carreira group, provide reversible optical control over CB_1R activation with unprecedented spatiotemporal resolution.[32] Fluorescent CB_2R selective probes were developed in a successful academia–industry collaboration between Roche and the groups of Carreira and



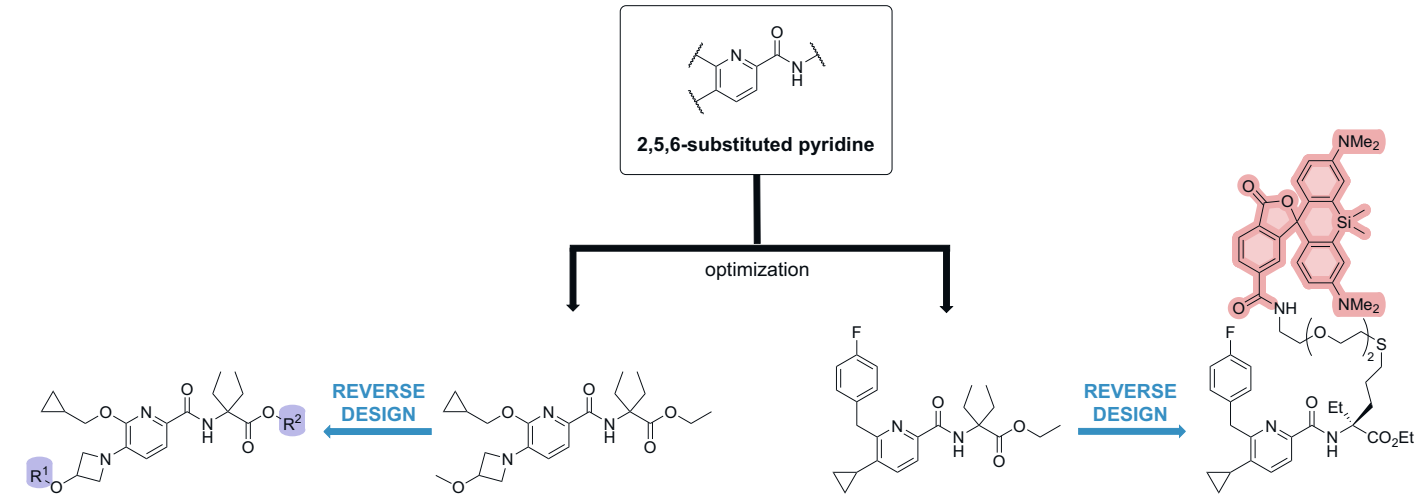
Scheme 1. Reverse design of **THC**-based fluorescent and photoswitchable probes for CB_1R and CB_2R. Binding affinities are reported for human cannabinoid receptors.

Nazaré to visualize CB_2R *in cellulo* and *in vivo*.[12,33] Bifunctional probes, which allow attachment of a reporter unit following reaction with its protein target, were used to study DAGL and NAPE-PLD activity in live cells.[34] Reversible and covalent PET tracers were applied to map the catabolic enzymes MAGL and FAAH in mouse and human brain, respectively.[35]

2.1 *GPCRs (CB_1R and CB_2R)*

Despite their great therapeutic potential, there are only few marketed drugs targeting CB_1R and CB_2R. This could be attributed to the complexity of ECS signaling, high similarity of the orthosteric ligand-binding pockets of CBRs and expression in various cellular systems.[36] Therefore, to avoid off-target or unwanted psychotropic effects, a deep understanding of CB_1R and CB_2R tissue expressions and their signaling mechanism is crucial. To overcome these issues, subtype selective CBR probes seem to be the best suited tools.

One of the well-known natural ligands for the CB_1R receptor is **THC**, the main psychoactive constituent of *Cannabis sativa* and also the active ingredient in marketed drugs such as Nabiximols[37] and Marinol.[38] The SAR of **THC** was the subject of intense studies since its isolation in pure form from *Cannabis sativa* in the early 1960s.[39] These studies led to the discovery of synthetic analogs with higher affinity and in some cases higher receptor selectivity than that observed for **THC**.[40] For instance, **HU-210,** a potent (agonist) classic analog of **THC**, has 678-fold higher CB_1R affinity than its parent compound (Scheme 1).[41] **HU-210** was chosen



Scheme 2. 2,5,6-Substituted pyridine served as a reverse design starting point for designing various CB_2R probes with different functionalities. Binding affinities are reported for human cannabinoid receptors.

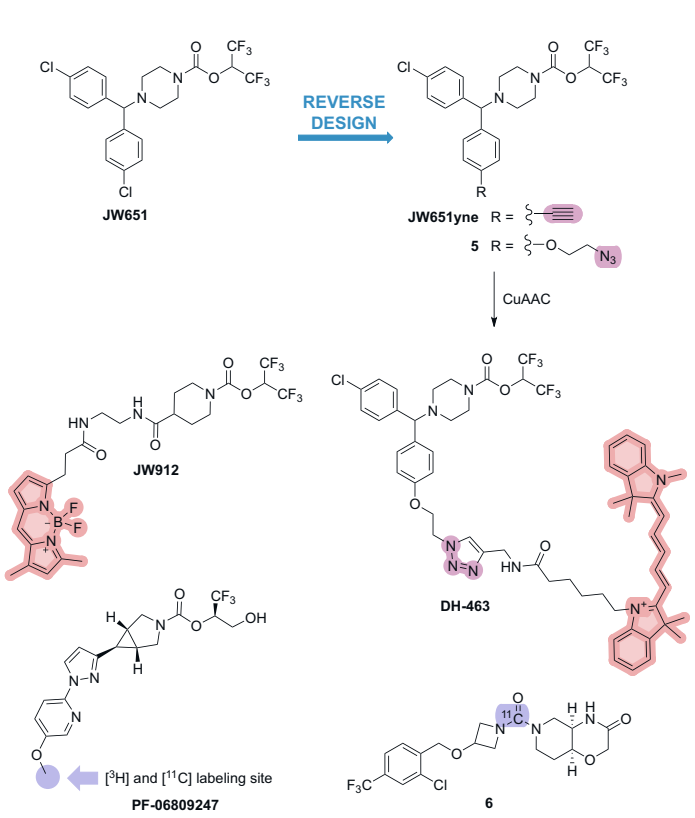
as the recognition moiety for the attachment of a dye (Alexa Fluor 488) and affinity tag (biotin) to obtain suitable chemical probes for the study of CB_1R in various cell lines (*e.g.* microglial cells, hippocampal neurons, blood and tonsil immune cells).[42] The major advantage of using this high-affinity cannabinoid scaffold is that the expected decrease in affinity due to the introduction of a reporter unit would be acceptable. Surprisingly, the attachment of Alexa Fluor 488 to **HU-210** resulted in improved selectivity for CB_1R (K_i hCB_2R/hCB_1R = 29). One of the interesting applications of the highly CB_1R-selective **HU-210-Alexa Fluor 488** was illustrating the CB_1R-expression in immune system cells which were initially assumed to be regulated by only CB_2R-expression. Palomares *et al.* successfully visualized and quantified CB_1R-expressing cells in human blood and tonsil immune-system cells employing fluorescent ligand **HU-210-Alexa Fluor 488**.[42a]

THC itself has also been used as the recognition element for designing labeled probes. One of the challenging steps in probe design is the identification of a suitable site for attachment of the reporter unit. This step can be streamlined by taking advantage of insights provided by previous SAR studies. In 2017 Carreira *et al.* reported photoswitchable **THC** derivatives using azobenzenes as the photochromic moiety.[32] Prior SAR studies had elucidated that the presence of lipophilic and/or bulky substituents at the C(3) side chain of **THC** result in increased affinity and potency of the ligand.[40b] Combined *in silico* docking and insights from SAR studies led to the successful introduction of a photoswitch at C(3). Two photoswitchable probes enabled optical control over GIRK channels by CB_1R modulation and paved the way for further functional studies. **Azo-THC-3** and **azo-THC-4** inhibit cAMP in a light-dependent manner, which holds promise to better understand conformational changes upon receptor activation.

THC-based compounds have also been exploited as the recognition element for the design of CB_2R probes. For example, **HU-308** is a potent agonist reported by Mechoulam *et al.* based on pinene.[43] **HU-308** displayed excellent affinity for CB_2R (Scheme 1) and selectivity over CB_1R (K_i hCB_1R/hCB_2R > 278), likely conferred by the phenolic methyl ether.[43] A thorough in-depth profiling study, in which multiple institutions from academia and industry have been involved, identified **HU-308** as one of the three best suited ligands for studying the pharmacology of CB_2R *in vitro* and *in vivo*.[44] AM841 was developed as a potent (K_i hCB_1R = 9 nM) and unselective ligand towards CBR, bearing the classical cannabinoid scaffold and an isothiocyanate.[45] Identification, evaluation and combination of favorable structural elements of the two cannabinoid ligands served as a starting point for the development of a privileged chimera motif that could be functionalized with a range of fluorophores while retaining excellent affinity and selectivity for CB_2R.[33,46] **1**, bearing a DY480-XL coumarin fluorophore (K_i hCB_1R/hCB_2R = 113), enabled a novel TR-FRET-based assay to characterize equilibrium and kinetic binding of established CB_2R selective ligands, **HU-308** and SR144528, and promised rapid, safe and high-throughput profiling of binding parameters without the need for radiolabels. **1** also allowed real-time visualization of CB_2R in primary, non-transfected murine splenocytes and human macrophages by live-cell confocal fluorescence microscopy.

The high lipophilicity and insufficient water solubility of **THC**-based phytocannabinoid chemical probes contribute to undesired pharmacokinetics and pose challenges in translation of preclinical data. Based on a series of 2,5,6-substituted pyridines[47] (Scheme 2), such as **2** having excellent drug-like properties, *i.e.* high membrane permeability, affinity, potency and selectivity, Nazaré *et al.* reported a series of full agonist pyridine-based fluorescent probes with excellent affinities for both human and mouse CB_2R.[12] **3**, a cell-permeable variant of the probe with a high target specificity was further employed for live cell imaging by super resolution confocal microscopy ranging from native human macrophages to murine splenocytes. This allowed detection and visualization of CB_2R at the cell membrane but also of intracellular receptor pools in live cells. Probe **3** could be used as well for real-time *in vivo* tracing of CB_2R in zebrafish larvae demonstrating the potential to define the functional expression levels of CB_2R in healthy as well as different disease states *in vivo*.

Application of 2,5,6-substitituted pyridine-based ligands has not been limited to design of fluorescent probes. **RSR-056**, a pyridine-based inverse agonist (K_i hCB_1R/hCB_2R = 1040)[48] was functionalized with a variety of radiolabels to allow determination of kinetic binding parameters (**[^{3}H]-RO6957022**)[9] and PET studies mapping receptor expression patterns (**[^{11}C]-RSR-056** and **4**).[48,49] **[^{11}C]-RSR-056** showed high specificity for CB_2R both in *in vitro* autoradiography and *in vivo* PET as well as biodistribution studies using spleen tissue of rats and mice. Analogously, **4** also indicated a high specificity toward CB_2R using rat spleen tissues and successfully detected CB_2R upregulation in human amyotrophic lateral sclerosis spinal cord tissue. These studies highlight the value of reverse-design approach for selecting the most suitable recognition element to successfully design a wide variety of probes.

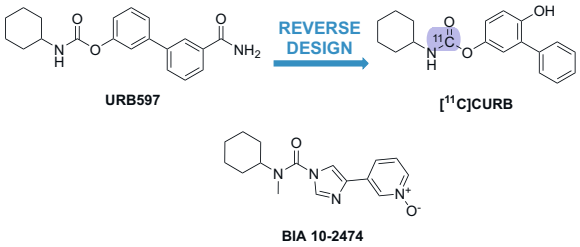


Scheme 3. Selected labeled chemical probes for MAGL.

2.2 *Endocannabinoid-catabolizing Enzymes (MAGL and FAAH)*

MAGL is the main enzyme responsible for the hydrolysis of 2-AG, the most abundant brain endocannabinoid. For lipid-signaling messengers, signal termination relies significantly on its degradative enzymes. Thus, the study of these enzymes is fundamental for understanding the ECS.[50] MAGL is a 33 kDa serine hydrolase that features the characteristic Ser-Asp-His catalytic triad. The availability of reversible and irreversible MAGL inhibitors has proliferated with the identification of its crystal structure.[51] In contrast, only a few examples of labeled chemical probes targeting MAGL are available.

The Cravatt Lab conducted pioneer work on producing active-site directed probes for ECS serine hydrolases.[52] Dual MAGL/alpha/beta-hydrolase domain containing six (ABHD6) activity-



Scheme 4. Reverse design approach for FAAH PET tracer **[^{11}C]CURB** and FAAH inhibitor **BIA 10-2474**.

based probes were developed. The probe design was guided by previously available SAR data for this enzyme family. MAGL-selective irreversible ligand **JW651**[53] was identified as a suitable candidate for the recognition element (Scheme 3). The replacement of one of the chlorine substituents of **JW651** with a sterically-equivalent ethynyl group provided an exit vector for the instalment of an alkyne moiety, which can act as a latent covalent affinity handle. By conjugation with a rhodamine-azide (Rh-N_3) reporter tag using 'click' chemistry, a direct readout of covalent probe-protein interactions through the use of alkyne derivative inhibitor **JW651yne** was possible. Click chemistry (CC)-ABPP of mouse brain proteomes confirmed that MAGL and ABHD6 were primary targets. The click probe could detect MAGL reactivity at probe concentrations as low as 10 nM. *In vitro* and *in vivo* proteome reactivity of a series of irreversible inhibitors was evaluated using a combination of competitive and CC-ABPP.

In the same work, a dual MAGL/ABHD6 activity-based imaging probe was presented. The design of fluorescent probe **JW912** capitalized on the remarkably high selectivity known for hexafluoroisopropanol (HFIP) carbamates. Probe **JW912** detected enzyme activity in mouse and human cells and visualized subcellular distributions of endocannabinoid hydrolases in prostate cancer cells. The lack of selectivity between MAGL and ABHD6 was overcome by administering competitive selective inhibitors.

Recently, a valuable fluorescent PharmacoSTORM probe was presented.[54] Reverse design guided the development of a Cy5-conjugated covalent MAGL inhibitor **DH-463**, in which the recognition element was also derived from **JW651**. The fluorescent reporter was attached to azide-bearing derivative **5** *via* copper-catalyzed azide-alkyne cycloaddition (CuAAC). **DH-463** allowed for the quantitative nanoscale imaging of MAGL in PharmacoSTORM experiments. **DH-463** selectivity was confirmed by competitive ligand-binding measurements with an unlabeled MAGL inhibitor.

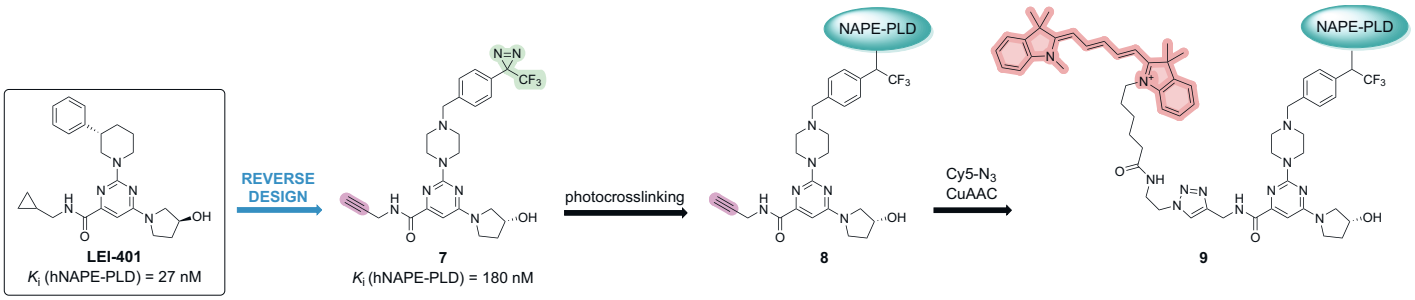
The last decade has witnessed significant efforts that culminated in improved MAGL PET tracers.[35a] These endeavors put significant focus on improving affinity, selectivity, and brain uptake. Although multiple radioligands for MAGL have been published, PET tracers appropriate for human use and their application in clinical research remain challenging. The prevailing bottlenecks are associated with achieving sufficient brain penetration and selectivity over other targets.

Pfizer carried out an exemplary application of the reverse-design approach for the development of covalent ^{11}C-PET and ^{3}H-labeled probes.[55] The high lipophilicity of the HFIP leaving group is disadvantageous for CNS PET as it contributes to extremely low fractions unbound (Fu) in the brain, making non-specific binding a major concern. Previous endeavors for the generation of MAGL inhibitors with more favorable physicochemical properties were leveraged. HFIP was replaced by a novel trifluoromethyl glycol leaving group, which maintained the potency and selectivity of HFIP, but exhibited lower lipophilicity. This resulted in improved physicochemical profile as well as *in silico* calculated Fu (cFu) in the brain. Based on previously reported SAR data, a vector for the introduction of structural variations was identified on a candidate inhibitor. Selection of candidates' analogs was further supported with *in silico* calculated PET-specific properties (*e.g.* clogD, cFu). The potent and selective irreversible MAGL inhibitor **PF-06809247** was finally selected as a suitable radioligand and successfully applied for cross-species measurement of MAGL brain expression, assessment of *in vivo* binding in rat, and non-human primate PET imaging.

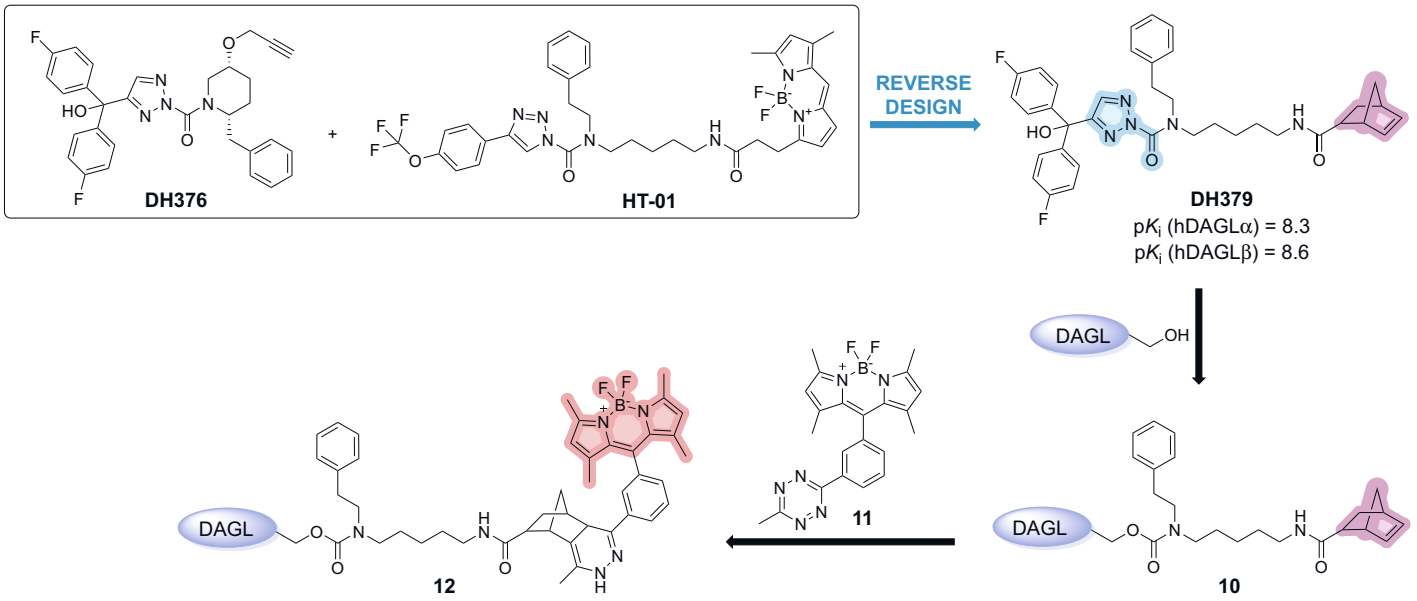
In a complementary manner, researchers at Roche developed reversible MAGL PET tracers by evaluating a selection of morpholine-3-one derivatives as candidates for ^{11}C-labeling.[35b] Candidates were prioritized based not only on their high affinity towards the target, but also on their favorable physicochemical properties for neuroimaging. The developed radiotracers were evaluated in *in vitro* autoradiography studies and exhibited high specificity and selectivity. Novel compound **6** allowed *in vivo* visualization of MAGL in mouse brain, and its specificity was confirmed through comparison of PET imaging in MAGL knockout and wild-type mice. Reversible PET probes are essential to access kinetic modelling in clinical trials.

FAAH is the ECS serine hydrolase responsible for the hydrolysis of AEA equipped with an unusual Ser-Ser-Lys triad. Several years of development led to the discovery and implementation of successful PET tracers that are currently used in clinical research.[35a] Significant improvement in the field was achieved by focusing the radiotracer development on analogs of the potent and selective FAAH inhibitor **URB597** (Scheme 4). ^{11}C-carbonylation *via* [^{11}C]CO_2 fixation was key for the successful development of multiple radiotracers derived from **URB597** that avoid the production of brain-penetrant radiometabolites. This eventually led to the development of **[^{11}C]CURB**, a clinically relevant imaging probe that has been applied to map FAAH in the human living brain. For instance, imaging with **[^{11}C]CURB** concluded that individuals with cannabis use disorder showed reduced brain uptake in certain brain regions in comparison to healthy controls.

Furthermore, chemical probes allowed to shed light on the putative causes of a fatal phase I clinical trial with FAAH inhibitor **BIA 10-2474**.[56] During the study, mild-to-severe neurological symptoms were reported by four volunteers as well as a fatality. After other possible causes were discarded, the currently accepted



Scheme 5. Potent inhibitor **LEI-401** served as the reverse-design starting point of NAPE-PLD photoprobe **7**.



Scheme 6. Generation of two-step activity-based DAGL probe **DH379** from inhibitor **DH376** and fluorescent probe **HT-01**, subsequent covalent attachment to the Ser residue of the catalytic triad and IEDDA with BODIPY-tetrazine **11**.

hypothesis for the clinical neurotoxicity of **BIA 10-2474** was potential off-target activity. Firstly, derivatives of **BIA 10-2474** carrying an alkyne handle were successfully applied for fluorescent labeling of FAAH in human and mouse brain lysates. This aided confirmation of an irreversible mode of action for **BIA 10-2474**. Next, the widespread serine hydrolase fluorophosphonate (FP) probe[5] was used for ABPP studies. Biotinylated FP probes were used for streptavidin affinity enrichment of **BIA 10-2474** targets. The serine hydrolase (off)-targets of **BIA 10-2474** could then be identified by chemoproteomics. This analysis demonstrated that **BIA 10-2474** and its primary metabolite presented multiple off-targets within the tested drug concentration range. Although the FP-biotinylated probe is a broadband probe for serine hydrolases and not a reverse-design probe (as is the focus of this article), this example showcases the fundamental contribution of labeled chemical probes to a successful and safe drug development. Moreover, the fatality of this case confirms the need to gain fast access to selective, high-quality probes that can accurately characterize drug candidates at the preclinical stage.

2.3 *Endocannabinoid-generating Enzymes (NAPE-PLD and DAGL)*

Phospholipase NAPE-PLD is a key enzyme regulating the biosynthesis of AEA and further important signaling lipids in the brain. Using a high throughput screen and subsequent iterative optimization cycles, the van der Stelt group was able to identify **LEI-401** as a potent and selective NAPE-PLD inhibitor (Scheme 5). The ligand exhibits good pharmacokinetic properties including high brain exposure, target engagement, and it triggered target-mediated pharmacodynamic effects.[34b]

LEI-401 and the SAR associated with this ligand guided the design and synthesis of a bifunctional photoaffinity probe **7**. A spacer bearing a trifluoromethyl-diazirine was introduced for photocrosslinking. In addition, the cyclopropylcarbinyl group was exchanged by a propargylic ligation handle to introduce reporter groups *via* CuAAC chemistry. After confirmation that the probe **7** binds to NAPE-PLD, target engagement studies in NAPE-PLD expressing cell lines were conducted. The cells were treated with the NAPE-PLD probe, irradiated at 350 nm to trigger photoactivation, expulsion of nitrogen, carbene formation, and crosslinking to NAPE-PLD to form adduct **8**. Subsequent lysis, conjugation with a Cy5-N_3 fluorophore to arrive at structure **9** and SDS-polyacrylamide gel electrophoresis demonstrated successful protein labeling by in-gel fluorescence scanning. Upon co-incubation with the inhibitor **LEI-401** fluorescence intensity of the NAPE-PLD band was reduced in a dose-dependent manner thus proving target engagement of **LEI-401** in living cells. Expansion of such ABPP studies to tissues, disease models or even human samples, can provide highly relevant target engagement data to support drug discovery programs.

Production of the second most relevant endocannabinoid 2-AG is regulated by the serine hydrolases DAGLα and DAGLβ which share the same catalytic Ser-His-Asp triad. To monitor DAGL activity in living systems an activity-based probe was designed by merging structural elements of optimized DAGL inhibitor **DH376**[57] and fluorescent DAGL probe **HT-01**,[58] leading to the discovery of the bifunctional probe **DH379**[34a] (Scheme 6). Its electrophilic 1,2,3-triazole urea guarantees covalent and irreversible binding to the active site of the lipase *via* the formation of adduct **10**. The norbornene serves as a biorthogonal handle for introduction of reporters *via* inverse electron-demand Diels–Alder (IEDDA) reactions. For example, tetrazines attached to fluorophores offer a rapid, metal-free alternative to CuAAC. Generally, such two-step approaches avoid reporter-induced impairment of probe affinity, selectivity, cell permeability, metabolic stability, protein binding, oral bioavailability, brain penetration and toxicity. DH379 was shown to be a potent inhibitor of DAGLα and DAGLβ with pIC_{50}s of 8.3 and 8.6, respectively, and it demonstrated good cell membrane permeability. Importantly, the probe was successfully applied for live cell labeling in DAGLα overexpressing U2OS cells by *in situ* treatment with BODIPY-tetrazine **11**, leading to the formation of Diels-Alder adduct **12**. Although biorthogonal handles with faster reaction rates and fluorogenic tetrazines with red-shifted wavelengths would be required for studying endogenous DAGL activity, these initial studies showcase the overall potential of the two-step ABPP approach.

3. Conclusions and Outlook

In this short review we have described the concept of reverse-design for the generation of labeled chemical probes. These consist of a target recognition element and a reporter group optionally being interconnected by a linker. We highlight the importance of such probes for drug discovery by discussing representative examples from selected ECS targets. Chemical probes can provide answers to many important questions which are asked in the course

of drug discovery programs: Where is the target expressed and under which circumstances? Which assay can be applied for hit identification and lead optimization? What is the mechanism that drives the pharmacodynamic effect? Is there a target engagement and/or occupancy assay available? What biomarkers are available to support pharmacological studies and human trials? Working with suboptimal tools can lead to wrong conclusions and might put development programs and patient lives at risk. Accordingly, chemical probes need to be of excellent quality with regards to overall physicochemical profile, on-target potency, off-target selectivity and ADMET properties required for specific applications.

The design and validation of optimized chemical probes can be as challenging as the generation of small molecule-derived, development candidates. Once suitable target recognition elements have been identified, attempts to introduce a reporter can commence. Ideally, structure-based design supports this effort and helps to maintain high on-target potency without compromising selectivity and/or ADMET properties. The size and influence of the reporter unit can vary broadly. Some probes may contain only small reporters such as radionuclides. In other cases, large tags might even dominate the overall properties of the chemical entity as seen with certain fluorescent probes. In the latter case, linkers are often required which position the reporter unit towards a spatial area where the binding affinity is not affected. Some applications of reverse design include ABPP, which profit from the covalent attachment of a chemical probe to the target. The reverse-design approach leverages generation of chemical probes from leads already optimized by medicinal chemists and, hence, maximizes the chances for successful outcomes.

The endocannabinoid lipid signaling network, playing an important role in many human health and disease states, has been selected as a system for discussion of reverse design.[6] Cell-permeable fluorescent probes allowed labeling of CBRs in living endogenously expressing cell populations and even animals illustrating the high translational value.[12] Both probes hold potential for studying target occupancy in animal studies or even human trials.[9,48,49]

Our focus on the most relevant six ECS protein targets merely scratches the surface regarding the vast possibilities of generating and applying tailor-made reverse design-based chemical probes and illustrating their critical importance for drug discovery. Ideally, chemical probe work commences immediately once validated lead structures or high-quality pharmacological tools have been identified. Nevertheless, the generation of chemical probes is often neglected in pharmaceutical companies because it is tedious, not always in focus and because timelines might not match with tight schedules for generating development candidates. This gap offers great opportunities for collaborations between academia and industry as successfully demonstrated in several of the reported case studies. A key element for success is that industrial partners provide access to high-quality starting points as well as profiling capabilities, which enable academic groups to generate excellent reverse-design based probes with all sorts of flavors. Following validation and publication of results, the probes ought to be made broadly accessible, a process which has already been successfully implemented for pharmacological tools by both academic and industrial institutions.[59] Overall, such an approach will support high quality research leading to lower attrition rates and faster access to novel drugs, thereby helping to address unmet medical needs.

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