

# Imaging Cannabinoid Receptors: A Brief Collection of Covalent and Fluorescent Probes for CB<sub>1</sub> and CB<sub>2</sub> Receptors

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There has been an expanding public interest towards the notion that modulation of the sophisticated endocannabinoid system can lead to various therapeutic benefits that are yet to be fully explored. In recent years, the drug discovery paradigm in this field has been largely based on the development of selective CB<sub>2</sub> receptor agonists, avoiding the unwanted CB<sub>1</sub> receptor-mediated psychoactive side effects. Mechanistically, target engagement studies are crucial for confirming the ligand–receptor interaction and the subsequent biological cascades that lead to the observed therapeutic effects. Concurrently, imaging techniques for visualisation of cannabinoid receptors are increasingly reported in the literature. Small molecule imaging tools ranging from phytocannabinoids such as tetrahydrocannabinol (THC) and cannabidiol (CBD) to the endocannabinoids as well as the purely synthetic cannabimimetics, have been explored to date with varying degrees of success. This Review will cover currently known photoactivatable, electrophilic, and fluorescent ligands for both the CB<sub>1</sub> and CB<sub>2</sub> receptors. Structural insights from techniques such as ligand-assisted protein structure (LAPS) and the discovery of novel allosteric modulators are significant additions for better understanding of the endocannabinoid system. There has also been a plethora of fluorescent conjugates that have been assessed for their binding to cannabinoid receptors as well as their potential for cellular imaging. More recently, bifunctional probes containing either fluorophores or electrophilic tags are becoming more prevalent in the literature. Collectively, these molecular tools are invaluable in demonstrating target engagement within the human endocannabinoid system.

**Keywords:** imaging agents, cannabinoids, fluorescent probes, electrophilic probes, photoaffinity labelling, synthetic cannabinoids, cannabinoid receptor, ligand-assisted protein structure.

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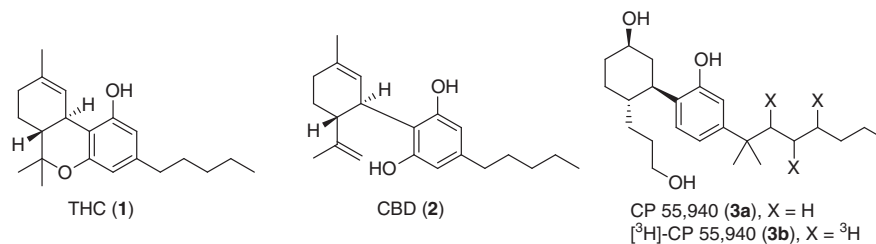
## Introduction

*Cannabis sativa* has been a widely used plant throughout civilisation for a multitude of different uses, namely as a recreational drug and for its medical purposes, as well as for industrial uses. Recent evidence has suggested *Cannabis* has beneficial therapeutic effects, which have been related to the active compounds located in the plant.<sup>[1–3]</sup> Several constituents known thus far in *Cannabis*, including the psychoactive (–)-*trans*- $\Delta^9$ -tetrahydrocannabinol (THC or  $\Delta^9$ -THC) (**1**) and the non-euphoric cannabidiol (CBD) (**2**), were isolated and characterised in the late 1960s, are now termed cannabinoids (Fig. 1).<sup>[4,5]</sup> Subsequently, the human biological pathway that these cannabinoids interact with, was discovered and titled the endocannabinoid system (ECS).

The ECS consists of two known receptors, cannabinoid type 1 receptor (CB<sub>1</sub>R) and cannabinoid type 2 receptor (CB<sub>2</sub>R), the endogenous ligands (endocannabinoids), and the associated enzymes that break down and synthesise these ligands. CB<sub>1</sub>R was identified and characterised in rat brain in 1988 and verified by receptor localisation in 1990 using tritiated CP 55,940 (**3a**, **3b**).<sup>[6,7]</sup> The receptor was cloned and the DNA that encodes the G protein-coupled receptor (GPCR) was found in the same

year.<sup>[8]</sup> CB<sub>2</sub>R was discovered and cloned in 1993 and was primarily found to be localised in macrophages from the spleen.<sup>[9]</sup> The identification of these two important receptors that mediate the effects of *Cannabis sativa* constituents eventually resulted in the discovery of anandamide (AEA) (**6**) and 2-arachidonoyl glycerol (2-AG) (**9**) as the endogenous ligands that activate these receptors (Fig. 2).

The eicosanoids, AEA and 2-AG, were first identified and characterised in 1992 and 1995, respectively.<sup>[10–12]</sup> AEA is a partial agonist at both mammalian CB<sub>1</sub>R and CB<sub>2</sub>R ( $K_i(\text{CB}_1\text{R}) = 61\text{--}543\text{ nM}$ ;  $K_i(\text{CB}_2\text{R}) = 279\text{--}1940\text{ nM}$ ), whereas 2-AG is a full agonist at CB<sub>1</sub>R and a partial agonist at CB<sub>2</sub>R ( $K_i(\text{CB}_1\text{R}) = 58.3\text{--}472\text{ nM}$ ;  $K_i(\text{CB}_2\text{R}) = 145\text{--}1400\text{ nM}$ ).<sup>[13]</sup> The enzymes related to the biosynthesis of these endocannabinoids have also been identified. *N*-Acylation of phosphatidylethanolamine (**4**) via acyltransferase generates *N*-arachidonoyl-phosphatidylethanolamine (NAPE) (**5**), which is further catalysed by NAPE-hydrolysing phospholipase D (NAPE-PLD) to produce AEA and several other *N*-acylethanolamines.<sup>[14]</sup> Fatty acid amide hydrolase (FAAH) hydrolyses AEA into arachidonic acid (AA) (**10**), as well as various other endogenous amides and *N*-acylethanolamines.<sup>[15]</sup> On the other



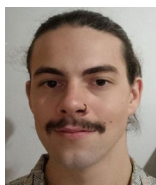
**Fig. 1.** Classical non-selective cannabinoids.

hand, 2-AG is synthesised from phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) (7) to diacylglycerol (DAG) (8) via *N*-arachidonoyl phosphatidyl ethanolamine-preferring phospholipase D (PLCβ). DAG is then hydrolysed by diacylglycerol lipase α and β (DAGLα/β) to yield 2-AG, which is further hydrolysed by monoacylglycerol lipase (MAGL) to AA.<sup>[16]</sup> Similar to NAPE-PLD, DAGLα/β and MAGL are not exclusive to the 2-AG biopathway and are responsible for enzymatic catalysis of several other monoacylglycerols.<sup>[17,18]</sup> Inhibition of MAGL and FAAH has been shown to negate inflammation associated with neurological disorders, inducing analgesia, or are potentially a safer alternative to CB<sub>1</sub>R agonists.<sup>[19–21]</sup> However, due to the multitude of biological pathways these enzymes contribute to, their inhibition can also lead to undesirable and unpredictable side effects.<sup>[22]</sup>

In recent years, the crystal structures of CB<sub>1</sub>R and CB<sub>2</sub>R have been reported, complexed with ligands AM6538 and AM10257, respectively.<sup>[23–25]</sup> Two orphan receptors, GPR55 and GPR119, have been identified to be activated by classical cannabinoids such as CP 55,490 and various endogenous acylethanolamides.<sup>[26,27]</sup> The THC and AEA derivative, *N*-arachidonoyl-glycine, acts as a full agonist at the putative cannabinoid

receptor GPR18, which is expressed in microglia suggesting a role in neuromodulation.<sup>[28–30]</sup> Transient receptor potential vanilloid 1 receptor (TRPV<sub>1</sub>) has also been shown to be activated by the two major endocannabinoids, AEA and 2-AG.<sup>[31]</sup> Interestingly, TRPV<sub>1</sub> is co-localised alongside CB<sub>1</sub>R and has been shown to be modulated by non-psychotropic phytocannabinoids.<sup>[32]</sup> Due to the complexity of the ECS and the multitude of potential ligand–protein interactions, different screening assays can produce varying results, indicating probable off target activity or a differing mode of action.<sup>[33]</sup>

THC is the primary psychoactive component of *Cannabis* and a partial agonist at CB<sub>1</sub>R and CB<sub>2</sub>R, yet over 100 unique phytocannabinoids have been isolated, many of which inducing no euphoric side effects.<sup>[34]</sup> CB<sub>1</sub>R is primarily located in the central nervous system whereas CB<sub>2</sub>R is predominantly expressed in the peripheral immune system, however, both receptors are located vice versa in a lesser abundance.<sup>[35]</sup> The hippocampus, cerebellum, cerebral cortex, basal ganglia, and amygdala have been identified to be highly populated with CB<sub>1</sub>R.<sup>[36,37]</sup> Generally, CB<sub>1</sub>R agonists have negative side effects such as a reduction in motor coordination, memory, and cognition, thus limiting the therapeutic potential. WIN



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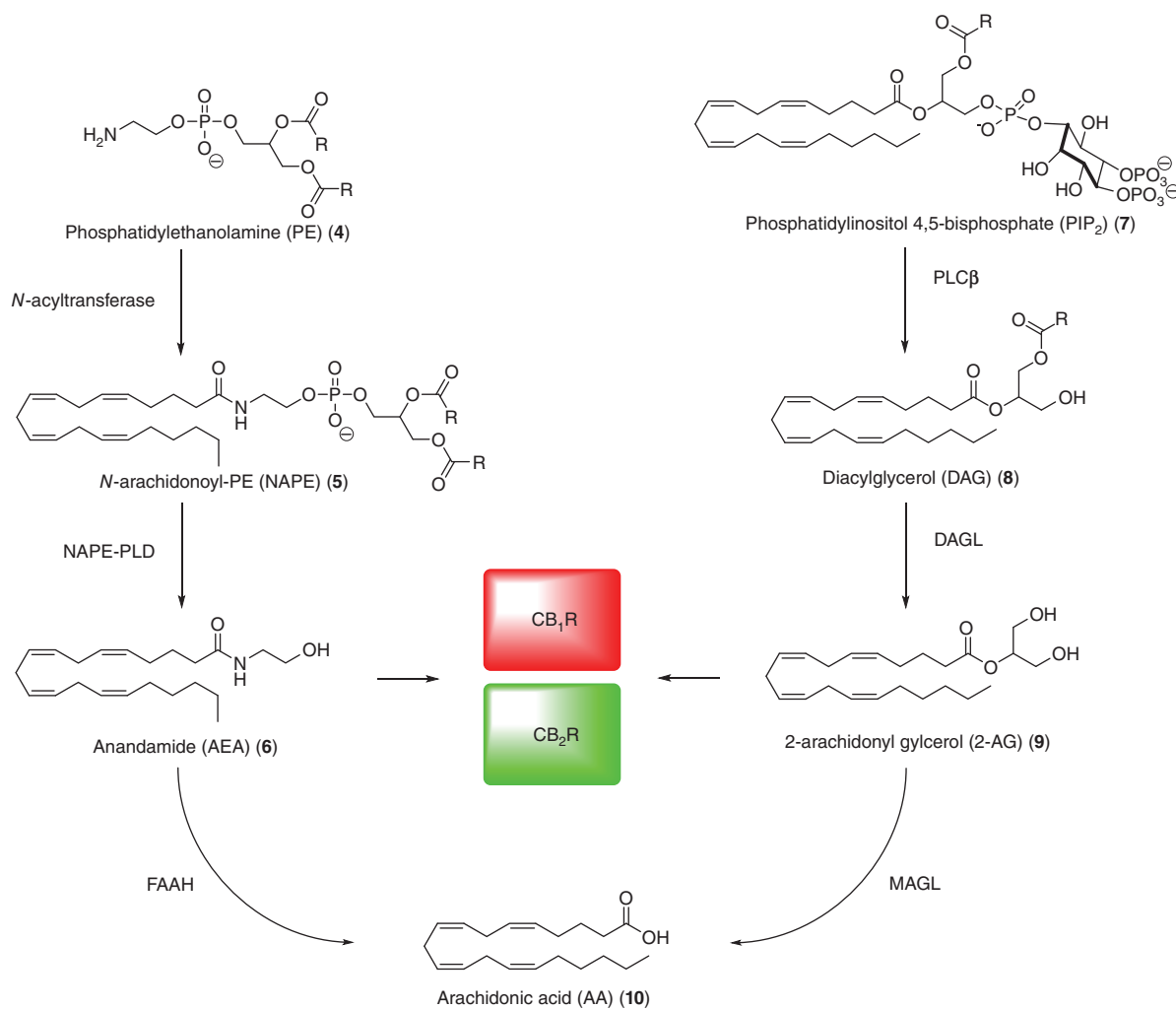
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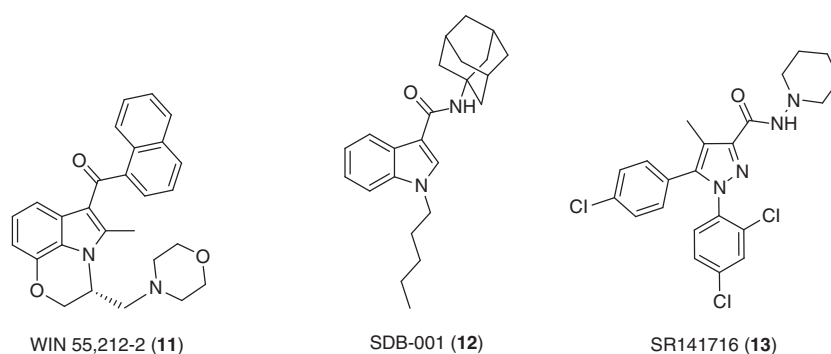
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**Fig. 2.** Simplified model of endocannabinoid synthesis. NAPE-PLD = *N*-arachidonoyl phosphatidylethanolamine-specific phospholipase D, FAAH = fatty acid amide hydrolase, PLCβ = *N*-arachidonoyl phosphatidyl ethanolamine-preferring phospholipase D, DAGL = diacylglycerol lipase, MAGL = monoacylglycerol lipase.



**Fig. 3.** Selected synthetic cannabinoids.

55,212-2 (11) and SDB-001 (12) (Fig. 3) are two known potent full agonists at both cannabinoid receptors, producing similar effects to THC.<sup>[38–40]</sup> Rimonabant (SR141716) (13) is a CB<sub>1</sub>R inverse agonist used as a treatment for obesity but was withdrawn due to extreme side effects.<sup>[41–44]</sup> However, several phytocannabinoids have been shown to have a wide range of medicinal uses for treatment and protection of several neuroimmune disorders.<sup>[45–47]</sup> Notably, CBD is of interest due to its

promising therapeutic potential—FDA approved as treatment for juvenile epilepsy—while containing no psychoactive properties.<sup>[48–50]</sup> Despite having low affinity to CB receptors, CBD has been shown to be a negative allosteric modulator at CB<sub>1</sub>R as well as being a partial agonist at CB<sub>2</sub>R.<sup>[51,52]</sup> While allosteric modulation of CB<sub>1</sub>R provides a promising therapeutic avenue, activating CB<sub>2</sub>R shows favourable treatment potential for a variety of neurological conditions.<sup>[53–55]</sup> CB<sub>2</sub>R is heavily

expressed in microglia cells, prominently involved in several neuroinflammatory diseases such as Alzheimer's, multiple sclerosis, and epilepsy. Blockage or inhibition of microglia has been shown to contribute to neuroprotection, by preventing production of proinflammatory mediators.<sup>[56]</sup> Several CB<sub>2</sub>R agonists, including phytocannabinoids, classical cannabinoids, and synthetic aminoalkylindoles, have displayed reductions in microglia activation in neurological diseases.<sup>[57–59]</sup>

The therapeutic potential of the endocannabinoid system and CB<sub>2</sub>R agonists have been well established, providing insights into potential selective drug design.<sup>[60,61]</sup> However, development of new drugs relies on understanding ligand–protein interactions or target engagement and the mechanism of action.<sup>[62]</sup> GPCRs are expressed in low levels in endogenous tissue, making target engagement difficult to identify, and is further accentuated by the adaptable nature of proteins when activated. Biomarkers are a useful tool for identifying target engagement *in vivo* by measuring the therapeutic effect, however this does not elucidate the compounds inability to interact with the receptor.<sup>[63]</sup> There are no biomarkers currently available for CB<sub>2</sub>R, increasing the demand for selective imaging methods and reducing off target interaction, although it is suggested changes in the ECS could be utilised as a biomarker for psychiatry.<sup>[33,64–66]</sup>

Small molecule probes serve as a comprehensive tool to image and identify information relating to ligand–receptor interactions and the applicability to disease treatment.<sup>[67]</sup> Positron emission tomography (PET) compounds have been widely utilised for verifying that the ligand binds with the target receptor via a radioisotope.<sup>[68]</sup> PET probes cannot only image the relevant proteins but are also capable of identifying biochemical processes at a molecular level before detectable anatomical variations. PET probes are utilised to determine progression or any alterations in a specific target's activity. PET probes have been widely used for medicinal imaging in a multitude of fields such as oncology, cardiology, and neurology, with continued use and further research.<sup>[69–72]</sup> Some major drawbacks of PET probes include requiring on site synthesis, exposure to radiation, short lived radionuclides, and overall cost to run. PET probes also require a lower nanomolar affinity to be applicable for imaging purposes, although sometimes yielding false-positive results. Cannabinoid PET tracers have been comprehensively covered in several review articles.<sup>[73,74]</sup> Other predominant methods used for small molecule–target engagement are fluorescence resonance energy transfer (FRET) and bioluminescence resonance energy transfer (BRET) which are also used extensively.<sup>[75]</sup> Both BRET and FRET have been used to image several GPCRs, displaying the applicability of the methods in ligand–protein visualisation.<sup>[76,77]</sup> However, both methods require modification of the protein to include a fluorescent donor, which can ultimately alter the protein's structure.<sup>[75]</sup> Several other imaging techniques have been applied to small cannabinoid molecules. This review will focus on the known cannabinoid receptor type 1 and type 2 probes utilising non-invasive methods, namely electrophilic and photoactivatable probes, as well as fluorescent tagged ligands.

### Photoactivatable and Electrophilic Probes

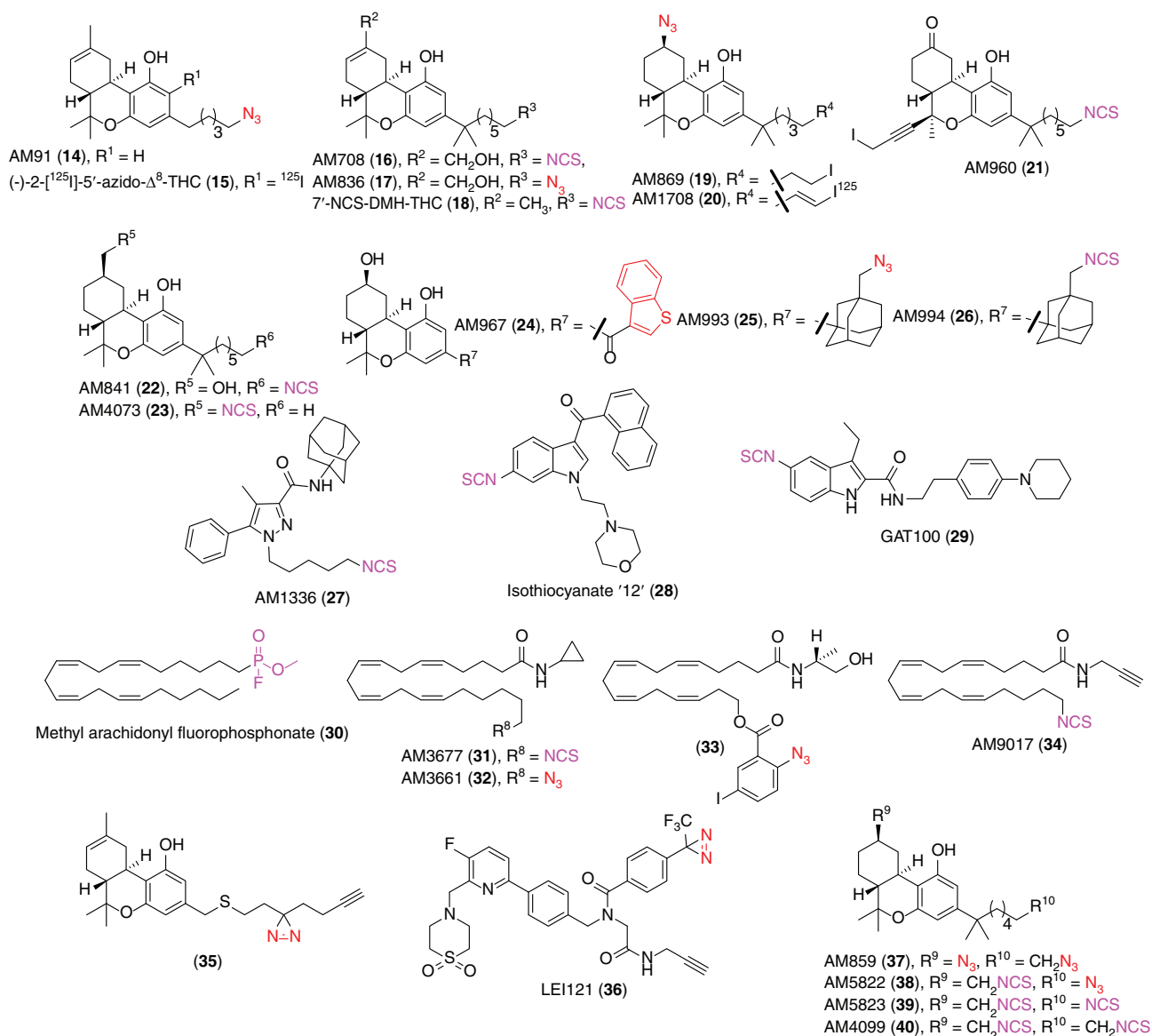
Small molecules that can covalently bind to specific amino acid side chains in certain receptors are a valuable tool in biological imaging. In GPCRs for example, the ligand first interacts with the target receptor through non-covalent binding, and ideally

with high affinity. Once engaged with the target protein, the activatable group covalently binds to an amino acid residue under specific conditions. Electrophilic ligands are modified to contain an electrophilic group, e.g. an isothiocyanate (NCS) group, which reacts with a nucleophilic amino acid, generally lysine, histidine, and cysteine, to form the covalent bond *in situ* without significant alteration of biological activity. Photoactivatable (or photoaffinity) based ligands have an inert group, generally azides, benzophenones, or diazirine moieties, which upon irradiation form a reactive species that covalently binds with nearby amino acids irreversibly.<sup>[78,79]</sup> Photoactivatable ligands have an advantage in comparison to electrophilic ligands due to the *in situ* reactive species of the latter binding to any nearby amino acids.<sup>[80]</sup> Monofunctional ligands containing one group have been primarily applied to receptor binding studies, however bifunctional ligands are becoming of more interest. Once the compound has irreversibly attached to the target protein, the difference in  $B_{\max}$  via a radioligand binding assay determines this degree of covalent interaction. Despite the advantageous use of studying ligand–protein interactions, a major drawback for the ligands is the lack of imaging potential. This can be circumvented by the addition of a second reactive group, utilising click chemistry to attach a photoprobe *in situ*, giving the imaging potential.<sup>[79,81]</sup> Since the early 1990s, several electrophilic and photoactivatable cannabinoids have been synthesised (Fig. 4, Table 1), some of which assist in determining crucial amino acid residues in ligand–receptor binding.

### Classical Cannabinoids

Several covalent cannabinoids containing similar scaffolds to endo and phytocannabinoids were initially synthesised in the early 1990s. A photoactivatable ligand, 5'-azido- $\Delta^8$ -THC or AM91 (**14**), showed a 2-fold increase in binding affinity when compared with its phytocannabinoid counterpart, (-)- $\Delta^8$ -THC ( $K_i(\text{rCB}_1\text{R}) = 35 \pm 11 \text{ nM}$ ) against [<sup>3</sup>H]-CP 55,940.<sup>[82]</sup> This ligand demonstrated the potential of photolabelling by labelling the receptor active site and the group would intend to develop further probes for identification of cannabinoid receptor subtypes. The radioiodinated equivalent, (-)-2-iodo-5'-azido- $\Delta^8$ -THC (**15**), when exposed to UV light was found to irreversibly bind to sites in both a mouse cerebral cortex ( $K_d = 5.60 \text{ pM}$ ) and S49 mouse lymphoma cells ( $K_d = 9.38 \text{ pM}$ ).<sup>[83]</sup> The Makriyannis group noted due to several bands obtained from the SDS–PAGE and autoradiography, that multiple cannabinoid receptors might be present in heterogenous cells, which was confirmed later.

The same group further explored this scaffold, utilising isothiocyanate for covalent binding, to yield (-)-11-OH-7'-NCS-1,1'-dimethylheptyl- $\Delta^8$ -THC (AM708) (**16**) as the first electrophilic cannabinoid ligand.<sup>[84]</sup> Pre-equilibration of rat forebrain membranes at 10  $\mu\text{M}$  with this compound resulted in an 80% decrease in [<sup>3</sup>H]-CP 55,940 binding at rCB<sub>1</sub>R and a 100% decrease at 100  $\mu\text{M}$ , with a nanomolar half maximal inhibitory concentration (IC<sub>50</sub>) value. An azido group at the terminus of the sidechain yielded AM839 (**17**), exhibiting potent binding, however no further studies have been conducted with this compound.<sup>[85]</sup> The results demonstrated that classical cannabinoids can be modified at the tail of the alkyl chain with electrophilic groups to irreversibly bind to the active site of the receptor. With this new insight, the Makriyannis group synthesised 7'-NCS-DMH- $\Delta^8$ -THC (**18**) removing the hydroxy at position 11.<sup>[86]</sup> The IC<sub>50</sub> value was comparable to the alkyl hydroxy derivative and similar [<sup>3</sup>H]-CP 55,940 displacement at 83%. The probe showed a 3-fold higher affinity than



**Fig. 4.** Available cannabinoid type 1 and type 2 receptor electrophilic and photoaffinity ligands. Electrophilic groups represented by pink and photoactivatable represented by red.

AM708, indicating that the aliphatic hydroxy does not affect affinity.

Several variations of THC have been synthesised and published incorporating an iodine into the scaffold. The aliphatic azido group was altered to be positioned at C-9 of the tricyclic ring for two novel photoaffinity probes, AM869 (19) and AM1708 (20).<sup>[87]</sup> Both compounds were shown to have high affinity to CB<sub>1</sub>R and CB<sub>2</sub>R, with AM1708 being radioiodinated at the terminal end of the aliphatic chain as well as an olefin. In 2003, an iodo and isothiocyanate probe AM960 (21) with structural similarity to Nabilone was synthesised by Chu et al.<sup>[88]</sup> Although very limited studies had been performed on the probe, it displayed successful irreversible covalent binding to rCB<sub>1</sub>R by occupying 50% of sites at 25 nM. The radioiodinated equivalent however has not been synthesised or tested.

AM841 (22) further explored the scaffold of THC with the continued trend of altering the terminal end of the DMH (dimethylheptane) chain with NCS for covalent binding.<sup>[89]</sup> The compound exhibited high affinity to CB<sub>1</sub>R expressing

CHO-K1 WT cells and displaced [<sup>3</sup>H]-CP 55,940 ( $K_d = 6.7 \pm 0.34$  nM) and [<sup>3</sup>H]-WIN 55,212-2 ( $K_d = 18.3 \pm 0.99$  nM). C6.47(355) is a cysteine residue located in transmembrane helix (TMH) 6 of CB<sub>1</sub>R that is of interest for ligand-receptor interaction. This study tested the binding potential of AM841 when the amino acid was mutated to less nucleophilic groups, serine, alanine, and leucine. Serine and alanine mutations showed little loss of affinity, whereas AM841 suffered a substantial loss of affinity with the mutated leucine residue. [<sup>3</sup>H]-CP 55,940 demonstrated a loss of affinity resulting from this mutation, while [<sup>3</sup>H]-WIN 55,212-2 remained unchanged. This data suggests that C6.47(355) plays a role in recognition of THC derived analogues but is not crucial for (aminoalkyl)indole compounds, indicating different binding motifs. This was supported by a reduction in [<sup>3</sup>H]-CP 55,940 binding to AM841 WT cells incubated for 1 h by 6-fold but was unobserved with [<sup>3</sup>H]-WIN 55,212-2.

Ligand-assisted protein structure (LAPS) is an analysis method in which high affinity probes covalently bind to amino



**Table 1. Binding and covalent labelling of electrophilic and photoactivatable cannabinoid probes**

Ligand	Binding data	Covalent labelling	Ref.
AM91 (14)	$K_i(\text{rCB}_1\text{R}) = 19 \pm 6 \text{ nM}$ , $\text{IC}_{50}(\text{rCB}_1\text{R}) = 31 \text{ nM}$	85 % at 1 $\mu\text{M}$	[82]
(-)-2-[ <sup>125</sup> I]-5'-azido- $\Delta^8$ -THC (15)	$K_d(\text{mCB}_1\text{R}) = 5.6 \text{ pM}$ , 9.38 pM		[83]
AM708 (16)	$\text{IC}_{50}(\text{rCB}_1\text{R}) = 1.6 \pm 0.3 \text{ nM}^{\text{A}}$	80 % at 10 nM	[84]
AM836 (17)	$\text{IC}_{50}(\text{rCB}_1\text{R}) = 0.16 \text{ nM}$		[84,85]
7'-NCS-DMH-THC (18)	$\text{IC}_{50}(\text{rCB}_1\text{R}) = 660 \text{ pM}^{\text{A}}$	83 % at 3.3 nM	[86]
AM869 (19)	$K_i(\text{CB}_1\text{R}) = 0.67 \text{ nM}$ $K_i(\text{CB}_2\text{R}) = 0.80 \text{ nM}$		[87]
AM1708 (20)	$K_i(\text{CB}_1\text{R}) = 0.72 \text{ nM}$ $K_i(\text{CB}_2\text{R}) = 0.85 \text{ nM}$		[87]
AM960 (21)	$\text{IC}_{50}(\text{rCB}_1\text{R}) = 25 \text{ nM}^{\text{A}}$	50 % at 25 nM	[88]
AM841 (22)	$K_i(\text{hCB}_1\text{R}) = 9.05 \pm 2.06 \text{ nM}^{\text{A}}$ , $\text{EC}_{50}(\text{hCB}_1\text{R}) = 0.94 \text{ nM}$ $K_i(\text{hCB}_2\text{R}) = 1.51 \text{ nM}^{\text{A}}$ , $\text{IC}_{50}(\text{hCB}_2\text{R}) = 0.079 \text{ nM}$		[89,90]
AM4073 (23)	$K_i(\text{hCB}_2\text{R}) = 3.3 \text{ nM}^{\text{A}}$ , $\text{IC}_{50}(\text{hCB}_2\text{R}) = 9.31 \text{ nM}$	60 % at 33 nM	[91]
AM967 (24)	$K_i(\text{rCB}_1\text{R}) = 1254 \text{ nM}$ $K_i(\text{mCB}_2\text{R}) = 34.2 \text{ nM}$ $K_i(\text{hCB}_2\text{R}) = 124.8 \text{ nM}$	67 % at 342 nM	[92]
AM993 (25)	$K_i(\text{rCB}_1\text{R}) = 4.4 \text{ nM}$ , $\text{EC}_{50}(\text{rCB}_1\text{R}) = 2.4 \text{ nM}$ $K_i(\text{mCB}_2\text{R}) = 26.4 \text{ nM}$ $K_i(\text{hCB}_2\text{R}) = 9.6 \text{ nM}$	67 % at 44 nM	[93]
AM994 (26)	$K_i(\text{rCB}_1\text{R}) = 3.0 \text{ nM}^{\text{A}}$ , $\text{EC}_{50}(\text{rCB}_1\text{R}) = 0.8 \text{ nM}$ $K_i(\text{mCB}_2\text{R}) = 34.6 \text{ nM}^{\text{A}}$ $K_i(\text{hCB}_2\text{R}) = 10.3 \text{ nM}^{\text{A}}$ , $\text{EC}_{50}(\text{hCB}_2\text{R}) > 400 \text{ nM}$	60 % at 96 nM 63 % at 30 nM 74 % at 103 nM	[93]
AM1336 (27)	$K_i(\text{hCB}_2\text{R}) = 0.54 \text{ nM}^{\text{A}}$ , $\text{EC}_{50}(\text{hCB}_2\text{R}) = 20.05 \text{ nM}$	60 % at 5.4 nM	[94]
Isothiocyanate 12 (28)	$\text{EC}_{50}(\text{rCB}_1\text{R}) = 1.1 \mu\text{M}^{\text{A}}$ , $\text{IC}_{50}(\text{rCB}_1\text{R}) = 160 \text{ nM}$	70 % at 1 $\mu\text{M}$	[95]
GAT100 (29)	$\text{EC}_{50}(\text{rCB}_1\text{R}) = 174 \text{ nM}$ (cAMP accumulation) $\text{EC}_{50}(\text{rCB}_1\text{R}) = 2.09 \text{ nM}$ ( $\beta$ -arrestin recruitment) $\text{EC}_{50}(\text{hCB}_1\text{R}) = 409.8 \text{ nM}$ (versus [ <sup>3</sup> H] SR141716A)		[96,97]
MAFP (30)	$\text{IC}_{50}(\text{rCB}_1\text{R}) = 20 \text{ nM}$		[98]
AM3677 (31)	$K_i(\text{rCB}_1\text{R}) = 1.3 \pm 0.2 \text{ nM}^{\text{A}}$ $K_i(\text{mCB}_2\text{R}) = 48.5 \pm 13 \text{ nM}^{\text{A}}$ $K_i(\text{hCB}_1\text{R}) = 1.7 \text{ nM}^{\text{A}}$ , $\text{IC}_{50}(\text{hCB}_1\text{R}) = 27.1 \pm 2.2 \text{ nM}$ , $\text{EC}_{50}(\text{hCB}_1\text{R}) = 0.05 \pm 0.2 \mu\text{M}$	58 % at 26 nM 43 % at 20 nM	[99,100]
AM3661 (32)	$K_i(\text{rCB}_1\text{R}) = 0.9 \pm 0.2 \text{ nM}$ $K_i(\text{mCB}_2\text{R}) = 57.6 \pm 19 \text{ nM}$ $K_i(\text{hCB}_1\text{R}) = 0.57 \pm 0.05 \mu\text{M}$ $K_i(\text{hCB}_2\text{R}) = 0.22 \pm 0.03 \mu\text{M}$	68 % at 18 nM	[99]
33			[101]
AM9017 (34)			[102]
35	$\text{p}K_i(\text{hCB}_1\text{R}) = 8.5 \pm 0.1$ $\text{p}K_i(\text{hCB}_2\text{R}) = 8.0 \pm 0.4$	Not active Not active	[103]
LEI121 (36)	$\text{p}K_i(\text{hCB}_1\text{R}) < 5$ $\text{p}K_i(\text{hCB}_2\text{R}) = 7.2 \pm 0.4$  $\text{pEC}_{50}(\text{hCB}_2\text{R}) = 7.03 \pm 0.3$ ( $\beta$ -arrestin recruitment) $\text{pEC}_{50}(\text{hCB}_2\text{R}) = 6.6 \pm 0.2$ (GTP $\gamma$ S binding)	~50 % ( $\lambda = 350 \text{ nm}$ )	[104]
AM859 (37)	$K_i(\text{CB}_1\text{R}) = 1.60 \text{ nM}$ $K_i(\text{CB}_2\text{R}) = 2.65 \text{ nM}$		[87]
AM5822 (38)			[102]
AM5823 (39)			[102]
AM4099 (40)	$K_i(\text{hCB}_2\text{R}) = 12.6 \text{ nM}^{\text{A}}$ , $\text{IC}_{50}(\text{hCB}_2\text{R}) = 22.88 \text{ nM}$	60 % at 126 nM	[91]

<sup>A</sup>Apparent  $K_i$  due to potential non-specific covalent binding at cysteine residues.

acids in proximity to the target protein.<sup>[105]</sup> Specific amino acids are then mutated, to govern the effect of the binding ability of the probes with these alterations, ultimately determining important residues in binding. Once achieved, *in silico* molecular modelling of the protein–ligand interaction is developed to further validate the experimental findings. Identification of the binding site can then be supported by liquid chromatography and/or mass spectrometry proteomics via an attachment of the covalent ligand to the protein. Supported by the LAPS approach, a cysteine residue in TMH6, C6.47(257), was found to be an essential amino acid in CB<sub>2</sub>R binding with AM841.<sup>[90]</sup> It was revealed that C6.47(257) of hCB<sub>2</sub>R is homologous to C6.47(355) of hCB<sub>1</sub>R as per covalent binding of AM841. Altering C6.47(257) to serine and alanine in HEK-293 WT-hCB<sub>2</sub>R cells found similar displacement of [<sup>3</sup>H]-CP 55,940 and a 2-fold increase, respectively. The serine and alanine mutated receptors also found AM841 displaced [<sup>3</sup>H]-WIN 55,212-2 with a 3 and 6-fold greater affinity, respectively. AM841 binds covalently to the WT hCB<sub>2</sub>R, reducing the B<sub>max</sub> of [<sup>3</sup>H]-CP 55,940 by 80%, whereas the C6.47(257)A and C6.47(257)S displayed no sign of irreversible binding. Mutation of two close proximity cysteine residues, C7.38(284) and C7.42(288), to serine did not affect the affinity of AM841 and hence it was concluded that C6.47(257) is the residue in which covalent attachment occurs. Interestingly, mutation of K3.28<sup>[109]</sup> in hCB<sub>2</sub>R resulted in a similar K<sub>i</sub> to the WT and no saturable binding was noted via mutation of K3.28(192) in hCB<sub>1</sub>R, indicating hCB<sub>1</sub>R and hCB<sub>2</sub>R have unique binding regions.

Validation of C6.47(257) and the covalent interaction between AM841 was supported by multiple reaction mass spectrometry monitoring (MRM-MS).<sup>[106]</sup> The seven TMHs of hCB<sub>2</sub>R were first separated and exposed which confirmed AM841 binding to TMH6. Then, a competitive binding assay against [<sup>3</sup>H]-CP 55,940 in hCB<sub>2</sub>R overexpressed in *Spodoptera frugiperda* (*Sf*-21) cells as *N*-terminal FLAG-tagged/*C*-terminal 6His-tagged (FLAG-hCB<sub>2</sub>R-6His) yielded a similar B<sub>max</sub> decrease at ~75% upon prior incubation with AM841. MRM-MS allowed visualisation of two discrete TMH6 peaks and incubation of AM841 in purified FLAG-hCB<sub>2</sub>R-6His displayed an ~75% decrease of the TMH6 peak relative to that without AM841. Further high-resolution MS demonstrated that AM841 is selective to covalently modifying hCB<sub>2</sub>R at C6.47(257). AM841 has also been shown to have anti-inflammatory properties in central and peripheral mCB<sub>1</sub>R and mCB<sub>2</sub>R, in an *in vitro* and *in vivo* study of mouse models of colitis.<sup>[107]</sup> Another study demonstrated that AM841 is a peripheral restricted ligand, slowing GI motility in normal mice conditions and normalising accelerated motility in stressed mice.<sup>[108]</sup> Further studies of covalent ligands as a potential treatment of disease is an interesting route, however consideration should be taken due to the reactive and irreversible nature of the probes.

AM4703 (**23**) was first reported in 2017 as a derivative of AM841.<sup>[91]</sup> The NCS group is relocated to C-11, as opposed to the terminal end of the alkyl chain, to further expand upon cannabinoid binding. AM4703 displayed a high affinity to WT hCB<sub>2</sub>R HEK-293 cells against [<sup>3</sup>H]-CP 55,940 and was observed to be a potent irreversible agonist, although weaker than AM841. Covalent binding was found to be ~60% at 33 nM in which similar results were obtained to mutated cysteine residues in TMH1, 6, and 7. However, [<sup>3</sup>H]-CP 55,940 specifically bound to C2.59(89)S was completely displaced indicating covalent interaction with this residue in TMH2 and a potent binding domain for classical cannabinoids. In confirmation with

experimental data, molecular modelling of AM4703 with hCB<sub>2</sub>R suggests that classical orthosteric agonists bind to TMH2 and 3 as guided by the location of NCS. The study is the first to involve C2.59<sup>[89]</sup> in hCB<sub>2</sub>R ligand–receptor binding, providing new insights into classical cannabinoid motifs and binding regions.

Previous studies had shown that an arylphenone at C-3 of Δ<sup>8</sup>-THC displayed high affinity to CB<sub>2</sub>R. Focusing on modifying the C-3 alkyl chain to different arylphenone substituents, the Makriyannis group found that 3-benzothiophenyl (AM967) (**24**) had moderate affinity to mCB<sub>2</sub>R and hCB<sub>2</sub>R.<sup>[92]</sup> The photolabelling of this compound was performed on HEK-293 cells expressing mCB<sub>2</sub>R, exhibiting a 67% photolabel to the receptor. The study revealed that 3-ketoaryl compounds have moderate selectivity to CB<sub>2</sub>R in mouse models and have the potential to photolabel in relatively high yields. Continuing this modification of the C-3, adamantyl groups with varying photoactivatable and electrophilic functionality at tertiary carbons were synthesised and tested. The two most potent compounds, AM993 (**25**) and AM994 (**26**), differed by an isothiocyanato and azide group through a methylene, respectively.<sup>[93]</sup> AM994 showed high affinity to CB<sub>1</sub>R with 3-fold and 10-fold selectivity over human and mouse CB<sub>2</sub>R, respectively. Pretreatment of the cells with AM994 reduced [<sup>3</sup>H]-CP 55,940 ability to bind by 63 and 74% at CB<sub>1</sub>R and CB<sub>2</sub>R with 10-fold the compound's K<sub>i</sub> value. Similar to AM994, AM993 showed high affinity to CB<sub>1</sub>R with 2-fold and 6-fold selectivity over human and mouse CB<sub>2</sub>R. Covalent labelling via irradiation of the compound at 10-fold the K<sub>i</sub>, yielded a reduction in [<sup>3</sup>H]-CP 55,940 binding by 67% at CB<sub>1</sub>R and 60% at CB<sub>2</sub>R. AM993 was tested before irradiation for covalent binding, observing no reduction in specific binding of [<sup>3</sup>H]-CP 55,940. Both AM994 and AM993 were found to be agonists at CB<sub>1</sub>R but showed negligible response at CB<sub>2</sub>R. These compounds were suggested to be used to obtain information about the structure of CB<sub>1</sub>R and were being explored for X-ray crystallography to determine ligand–receptor complexes.

#### Arylpyrazole Cannabinoids

With C6.47(355) being an essential acceptor for receptor–ligand labelling in CB<sub>1</sub>R, a selective covalent ligand, AM1336 (**27**), was designed to target cysteine residues in hCB<sub>2</sub>R.<sup>[94]</sup> Modifying the biarylpyrazole CB<sub>2</sub>R antagonist, SR144528, the first CB<sub>2</sub>R selective electrophilic probe, AM1336 was synthesised. AM1336 was found to bind irreversibly to hCB<sub>2</sub>R as 60% of [<sup>3</sup>H]-CP 55,940 could not interact with the occupied receptor after pre-incubation and washing. AM1336 displayed a noticeable drop in covalent labelling to the mutated TMH7 residues, C7.38(284) and C7.42(288), indicating these amino acids are important for inverse agonist interactions. The binding competency of hCB<sub>2</sub>R overexpressed in *Sf*-21 cells (FlaghCB2his6) was tested by labelling with AM1336.<sup>[109]</sup> AM1336 (50 nM) showed 73% labelling of the receptor, decreasing the B<sub>max</sub> of [<sup>3</sup>H]-CP 55,940. Mass spectrometry analysis of hCB<sub>2</sub>R complexed with AM3661 again identified C7.38(284) of TMH7 as important for inverse agonist binding in LAPS analysis. C6.47(257) was also noted to be of importance in the same study for agonist binding using AM841.

#### Indole Cannabinoids

In 1996, a range of electrophilic (aminoalkyl)indole compounds were synthesised based on the agonist Pravadoline.<sup>[95]</sup> From the compounds, isothiocyanate '12' (**28**) was the most potent with a half maximal effective concentration (EC<sub>50</sub>) of 1.1 μM, with most other compounds being inactive at < 10 μM. rCB<sub>1</sub>R membranes

incubated with 1  $\mu\text{M}$  of isothiocyanate '12' reduced [ $^3\text{H}$ ]-CP 55,940 binding by 70 %, demonstrating irreversible binding. At 100  $\mu\text{M}$ , no further loss of [ $^3\text{H}$ ]-CP 55,940 binding was observed. Allosteric modulation of GPCRs can regulate pharmacological responses by altering the receptor conformation such that orthosteric ligand efficacy is modified.<sup>[110,111]</sup> The effect of an allosteric ligand binding can either give positive or negative responses, known as positive allosteric modulators (PAM) or negative allosteric modulators (NAM), respectively. Expanding on information about controlling the undesirable psychoactive effects of CB<sub>1</sub>R, allosteric modulation can help utilise the therapeutic potential of the ECS. Org27569 is a NAM with a moderate potency at CB<sub>1</sub>R ( $\text{pEC}_{50} = 8.64 \pm 0.11$  at 100 nM).<sup>[112]</sup> Looking for cysteine residues assisted by LAPS, GAT100 (**29**) was found to be the most effective of four compounds based off Org27569. The electrophilic ligand contains an isothiocyanate at C-5 of the indole, demonstrating higher potency than Org27569, and did not possess any inverse agonist activity.<sup>[96]</sup> Preincubation of HEK-293 cells with GAT100 at 100 nM increased the specific binding of [ $^3\text{H}$ ]-CP 55,940 at CB<sub>1</sub>R by over 2-fold, exhibiting covalent binding and negative allosteric modulation, whereas Org27569 had no effect. Ligand-docking and *in silico* analysis of GAT100 in complex with hCB<sub>1</sub>R determined C7.32(382) as the residue with which GAT100 feasibly covalently interacts.<sup>[97]</sup> The same study also found that GAT100 is an effective NAM in conjunction with AEA and 2-AG when evaluating its orthosteric-probe dependence. Electrophilic NAM cannabinoids could potentially elucidate new binding pockets in which allosteric modulation occurs, which could theoretically help future modulation of negative side effects of CB<sub>1</sub>R activation.

### Endocannabinoids

Methyl arachidonyl fluorophosphonate (MAFP) (**30**) was found to be an irreversible CB<sub>1</sub>R antagonist reducing binding of classic cannabinoid agonists, WIN 55,212-2 and CP 55,940, while not affecting the binding abilities of non-cannabinoids. MAFP was also found to generate irreversible inhibition of phospholipase A<sub>2</sub> and FAAH.<sup>[98,113]</sup> The Makriyannis group designed and characterised two high affinity AEA probes, AM3677 (**31**) and AM3661 (**32**), containing the electrophilic thioisocyanate and photoactive N<sub>3</sub> at the terminal C-20 alkyl chain, respectively. Both compounds when compared with ACPA ( $K_i(\text{rCB}_1\text{R}) = 2.7 \pm 0.4$  nM;  $K_i(\text{rCB}_2\text{R}) = 157 \pm 37$  nM), showed an increase in affinity to both CB receptors. [ $^3\text{H}$ ]-CP 55,940 binding was lowered by 58 % in rat forebrain membranes when equilibrated with 26 nM of AM3677. The covalent binding was determined to reach maximum concentration as uptake plateaued at 30 min.<sup>[99]</sup> AM3677 was also found to inhibit forskolin-activated cAMP formation with an IC<sub>50</sub> of  $27.1 \pm 2.2$  nM in Flp-In-293 cells overexpressing CB<sub>1</sub>R.<sup>[100]</sup> One-hour preincubation of AM3677 displayed a 43 % irreversible binding to hCB<sub>1</sub>R, where [ $^3\text{H}$ ]-CP 55,940 was unable to interact with the same receptor. The same study found AM3677 binds covalently to hCB<sub>1</sub>R at C6.47(355), in which AM841 binds covalently as well, indicating this residue is critical to CB<sub>1</sub>R binding.<sup>[100]</sup> AM3661 was equilibrated at 3-fold the  $K_i$ , then irradiated with UV light (254 nm) which resulted in 23 % irreversible labelling of rCB<sub>1</sub>R.<sup>[99]</sup> When pre-incubated with AM3661, a reduction of 68 % to [ $^3\text{H}$ ]-CP 55,940 binding was exhibited at 18 nM. The results indicated that AM3661 could be a useful probe for rCB<sub>1</sub>R imaging, in elucidating the binding pocket, helping assist with future probe design. With evidence suggesting AEA novel receptors exist, Balas et al. produced a

photoactivatable probe derived from endocannabinoids (**33**). The compound featured a 2-azido-5-iodobenzoate ester group, due to the advantage of wavelength activation higher than protein UV absorbance. The compound showed some affinity to both receptors in recombinant human receptors, with a decrease of affinity to hCB<sub>1</sub>R when compared with AEA. The compound was suggested to be a tool for discovery of potential new endocannabinoid receptors.<sup>[101]</sup> Balas et al. further synthesised AEA probes, based on the preliminary data. A replacement of the arylazide to 2-*tert*-butyl-2-methyl-1,3-benzodioxole-4-carboxylate (TBMB), removed all notable affinity to both receptors ( $K_i(\text{hCB}_1\text{R}) = 2.80 \pm 0.05$   $\mu\text{M}$ ;  $K_i(\text{hCB}_2\text{R}) = 1.02 \pm 0.03$   $\mu\text{M}$ ).<sup>[114]</sup> AM9017 (**34**) is an AEA derived covalent probe with the ability to potentially perform click reactions *in situ*.<sup>[102]</sup>

### Bifunctional Cannabinoids

Bifunctional probes bare the ability to utilise a multitude of different imaging techniques to create a higher spatial resolution. A novel  $\Delta^8$ -THC analogue **35** utilises a diazirine as the photoactive handle and an alkyne for *in situ* click chemistry via the alkyl chain at C-3.<sup>[103]</sup> The compound displaced [ $^3\text{H}$ ]-CP 55,940 in CB<sub>1</sub>R and CB<sub>2</sub>R in overexpressing CHO cells with a high affinity to both receptors. The probe using a two-step photoaffinity labelling assay did not label either CB receptor at 2  $\mu\text{M}$ , which suggested that changing the position of the photoreactive group to the rigid tricyclic core might permit covalent interaction with the receptor. The same group modified LEI101 to contain a terminal alkyne and a trifluoromethyl-diazirine-benzoyl, yielding LEI121 (**36**) as a CB<sub>2</sub>R photoaffinity compound.<sup>[104]</sup> The presence of the alkyne couples the fluorophore(s) by a click reaction to give the resulting triazole linked probe. The ligand without any linked probes was tested via a radio ligand binding competition assay using [ $^3\text{H}$ ]-CP 55,940 in hCB<sub>2</sub>R-expressing CHO cells. LEI101 ( $\text{p}K_i(\text{CB}_2\text{R}) < 7.5 \pm 0.1$ ) had a similar affinity to the novel probe, LEI121 ( $\text{p}K_i(\text{CB}_2\text{R}) < 7.2 \pm 0.4$ ), which underwent a slight loss of CB<sub>2</sub>R affinity while showing little CB<sub>1</sub>R affinity. After UV-irradiation of LEI121 bound to CB<sub>2</sub>R, specific binding of [ $^3\text{H}$ ]-CP 55,940 was substantially lowered, while the binding remained unchanged without light activation, indicating covalent binding to the protein. While LEI101 is reported to be a partial agonist, LEI121 was found to be an inverse agonist through two functional assays.

AM859 (**37**) is a unique compound that acts similarly to AM869, except functioning as a bifunctional covalent probe due to the terminal azide as opposed to an iodine. The probe displayed excellent affinity to both receptors with the hope of elucidating more information about ligand and receptor interaction. Several unpublished probes have been synthesised and reported by the Makriyannis group.<sup>[102]</sup> These probes are notable due to the bifunctional ability, either homo or hetero covalent ligands. AM5822 (**38**) and AM5823 (**39**) are both bifunctional probes with altering functional groups at the terminal end of the DMH chain. AM5823 offers the ability to covalently bind twice due to multiple isothiocyanate groups, whereas AM5822 contains an azide group for light activation. Although most probes are preliminary, the potential of bifunctional ligands to image the receptor at higher spatial resolution is becoming of more interest.

In the same study as AM4073, the bifunctional ligand AM4099 (**40**) contains an isothiocyanate at the terminus of the C-3 sidechain and at C-11 of the cyclic scaffold.<sup>[91]</sup> Interestingly, the probe does not bind to hCB<sub>2</sub>R C6.47(257) as AM841



does, instead binding to C2.59<sup>[89]</sup> like AM4073. Due to the similar binding affinities of AM4073 and AM4099, it is postulated that C6.47(257) orients AM841 within the hCB<sub>2</sub>R binding pocket to obtain ‘megagonist’ potency. This is heavily supported by the NCS lacking AM841 analogue, AM4056, yielding similar potency to AM4073 and AM4099.<sup>[90]</sup> While it is evident that the position of the electrophilic and photoactivatable group affects the binding and potency of the compound, further development of classical, endo, and synthetic cannabinoid motifs can be explored and aided by techniques, such as LAPS, for a more comprehensive understanding of ligand–protein binding.

### Fluorescent Probes

Small molecules that contain a fluorescent moiety have been widely developed and applied to biological imaging. These compounds offer a safer and more accessible method of imaging when compared with radioligands, as they can be applied to in vivo studies in native systems and even act as a competing ligand in binding assays.<sup>[115,116]</sup> The difficulty of selective non-peptide fluorophore development has been well established, and a direct addition of a fluorescent scaffold can detrimentally affect affinity.<sup>[117]</sup> However, these compounds are designed with a linker, generally polyethylene glycol (PEG) or methylene chain, to ensure affinity of the parent compound remains.<sup>[118]</sup> Tissue penetration is another issue with fluorescent ligands; however, near-infrared (NIR) dyes can be imaged, minimising problematic auto-fluorescence within the biological system.<sup>[119,120]</sup> These fluorescent moieties must absorb and emit above ~600 nm, as well as having good chemical and photostability, for the dye to be applicable for receptor imaging. Quantum yields in aqueous and biological systems must also be considered when fluorescent probes are to be used for in vivo imaging, due to probable issues with detection. Fluorescent probes also show potential capabilities of direct examination of allosteric and orthosteric ligand–receptor interactions.<sup>[121]</sup> Within recent years, several known potent cannabinoids have been modified to contain fluorescent compounds (Fig. 5, Table 2), with the hopes of elucidating cannabinoid pharmacology.

### Biotinylated Cannabinoids

Biotinylation is the addition of biotin to a ligand via a linker. Once the compound has docked with the target receptor, fluorescent avidin conjugates are used due to the specificity to interact with biotin, subsequently providing fluorescence to the protein.<sup>[143]</sup> Endogenous biotin can be blocked by an avidin conjugate to view wild-type and endogenous systems without undesirable interaction. A biotinylated equivalent of AEA (linked via a PEG chain), had similar lipophilicity to the endogenous ligand and was used to show the distribution of AEA, without interfering or interacting with other endogenous systems.<sup>[144]</sup> In 2011, several endocannabinoids were modified via biotinylation or an alkyne addition for in situ click reactions.<sup>[122]</sup> The structures synthesised structurally resemble AEA, 2-AG, and 2-arachidonyl glyceryl ether (2-AGE) in which all compounds are partial agonists. The most potent of the endocannabinoid derivatives belonged to 2-AG and 2-AGE, whereas AEA and methanandamide compounds showed little affinity. The potent 2-AG and 2-AGE derivatives were screened against [<sup>3</sup>H]-CP 55,940 in human CB receptor transfected HEK-293-EBNA cells. Both compounds showed an increase in CB<sub>2</sub>R affinity over 2-fold, and a decrease towards CB<sub>1</sub>R over 8-fold. The biotinylated 2-AG analogue was described to be a

selective CB<sub>2</sub>R probe having little CB<sub>1</sub>R affinity ( $K_i(\text{CB}_1\text{R}) > 5000 \text{ nM}$ ;  $K_i(\text{CB}_2\text{R}) = 379 \text{ nM}$ ), whereas 2-AGE-3b (**41**) had retained moderate affinity to both receptors. 2-AGE-3b was selected for in vitro imaging of CB<sub>1</sub>R due to its affinity and was incubated in CB<sub>1</sub>R transfected mouse hippocampal cell line HT-22. Streptavidin-Alexa488 was used successfully to image the probe, visualising the receptor. To verify CB<sub>1</sub>R binding, the compound was tested in non-CB<sub>1</sub>R transfected cells against an excess of HU-210, to show little staining with the streptavidin. Non-specific binding was dismissed by co-labelling the compound with an anti-CB<sub>1</sub> antibody and anti-rabbit Alexa633, in which both fluorescent compounds overlapped.

The research group continued by modifying potent synthetic cannabinoids with the biotin moiety.<sup>[123]</sup> HU-210 is a non-selective potent agonist at both CB<sub>1</sub>R and CB<sub>2</sub>R originally synthesised in 1988 by Mechoulam, mimicking the effects and structure of THC ( $K_i(\text{CB}_1\text{R}) = 0.061 \text{ nM}$ ;  $K_i(\text{CB}_2\text{R}) = 0.52 \text{ nM}$ ).<sup>[145]</sup> The Mechoulam group further explored this model and synthesised HU-308 in 1999, with more selectivity towards CB<sub>2</sub>R ( $K_i(\text{CB}_1\text{R}) > 10000 \text{ nM}$ ;  $K_i(\text{CB}_2\text{R}) = 22.7 \text{ nM}$ ).<sup>[146]</sup> The compounds synthesised were conjugated via the free hydroxy, either at C-1 or C-11, to biotin through a linker, in efforts for higher affinity probes.<sup>[123]</sup> These compounds were screened against [<sup>3</sup>H]-CP 55,940 in human CB receptor transfected HEK-293-EBNA cells. Out of the two biotinylated HU-210 derivatives, HU210-1 (**42**), had the highest affinity to both receptors with the C-1 biotin linked compound exhibiting an equally high affinity ( $K_i(\text{CB}_1\text{R}) = 11 \pm 2 \text{ nM}$ ;  $K_i(\text{CB}_2\text{R}) = 3 \pm 1 \text{ nM}$ ). The HU-308 linked with biotin, HU308-3 (**43**) maintained its selectivity to CB<sub>2</sub>R while only enduring a minor loss of affinity. Probes HU210-1 and HU308-3 were selected for in vitro imaging in native systems, labelling neurons in CB<sub>1</sub>R and microglia in CB<sub>2</sub>R, respectively.<sup>[123]</sup> Compound **42** labelled neurons in CB<sub>1</sub>R, detected by streptavidin-AlexaFluor488, and when co-labelled with anti-microtubule-associated protein 2 antibody plus AlexaFluor594, fluorescent overlapping was observed. Both **42** and **43** were successfully visualised at CB<sub>2</sub>R in rat microglial cells detected by streptavidin-AlexaFluor488. Additionally, an excess of HU-210 with either compound gave no fluorescent signal. The results indicated the first suitable probes for imaging both CB receptors with moderate affinities and specific binding.

### Fluorescent Classical Cannabinoid Derivatives

The major drawbacks of a biotin probe, being two-step labelling and blocking endogenous biotin, results in the biotinylated cannabinoids not being suitable for tissue staining. Recently, AlexaFluor 488, with a hexyl linker was selected as the probe for HU-210 at the allylic hydroxyl (**44**).<sup>[124]</sup> A competitive radio ligand binding assay with [<sup>3</sup>H]-CP 55,940 using human CB<sub>1</sub> and CB<sub>2</sub> transfected HEK-293-EBNA cell membranes gave CB<sub>1</sub>R selectivity with moderate CB<sub>2</sub>R affinity ( $K_i(\text{CB}_1\text{R}) = 27 \pm 4 \text{ nM}$ ;  $K_i(\text{CB}_2\text{R}) = 0.8 \pm 0.2 \mu\text{M}$ ). The fluorophore showed in vivo visualisation of human tonsil CB<sub>1</sub>-expressing cells using confocal microscopy. When compared with the non-selective binding properties of HU-210, the CB<sub>1</sub> selectivity was obtained upon the addition of the AlexaFluor488 linker. This insight could lead to selective CB<sub>1</sub>R and CB<sub>2</sub>R fluorescent probes derived exclusively from HU-210.

Chromenopyrazole compounds, containing a similar structural design as THC and HU compounds, have been shown to have high affinity to cannabinoid receptors.<sup>[147]</sup> It was reported

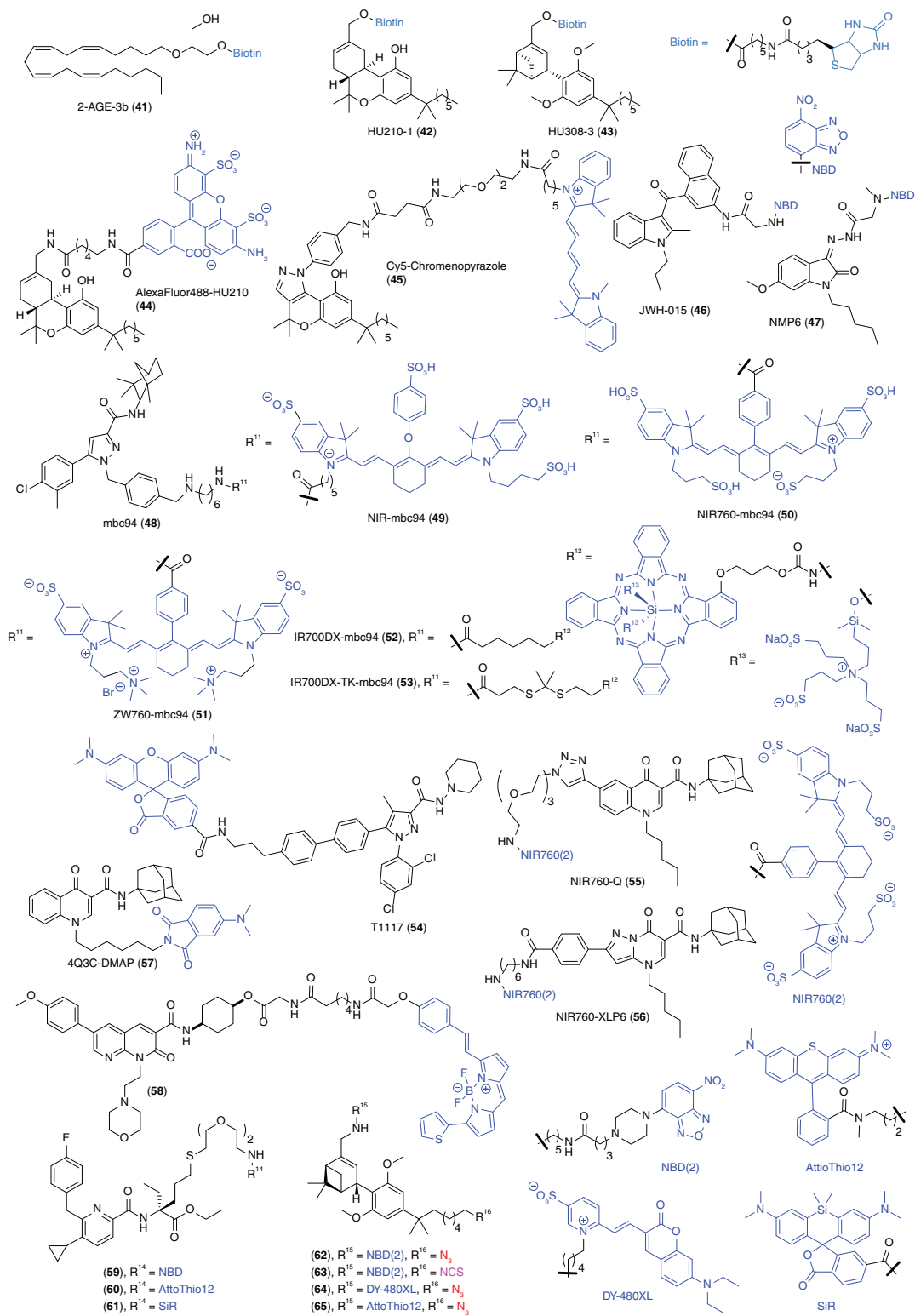


Fig. 5. Available cannabinoid type 1 and type 2 receptor fluorescent ligands. Fluorophore tags represented by blue.

that phenolic alkylation of the pyrazole ring resulted in higher CB<sub>2</sub>R affinity, making this suitable for a linker position.<sup>[148]</sup> Coupled with three different fluorophores, BODIPY-630/650, BODIPY-FL, and Cy5, the phenolic chromenopyrazole scaffold was utilised for potential CB fluorescent ligands.<sup>[125]</sup> The C-4 position of the phenyl was found to have better linker tolerance and higher affinity to CB<sub>2</sub>R. Using BODIPY-630/650 as the

fluorophore, the binding affinity ( $pK_i(\text{CB}_2\text{R}) = 5.80 \pm 0.12$ ) was deemed too low to be useful for imaging studies. Extending the PEG linker from 2 to 5 showed no improvement to the fluorophore's imaging potential or selectivity for BODIPY-630/650. The BODIPY-FL attached linker exhibited an increase by ~10 fold to CB<sub>2</sub>R selectivity ( $pK_i(\text{CB}_2\text{R}) = 6.84 \pm 0.04$ ), suggesting a smaller fluorophore is more ideal. The highest CB<sub>2</sub>R

**Table 2. Binding data and fluorescent emission associated with fluorescent cannabinoid ligands**

Ligand	Binding data	Emission [nm]	Ref.
2-AGE-3b (41)	$K_i(\text{hCB}_1\text{R}) = 221 \pm 8 \text{ nM}$ $K_i(\text{hCB}_2\text{R}) = 450 \pm 11 \text{ nM}$		[122]
HU210-1 (42)	$K_i(\text{hCB}_1\text{R}) = 2.4 \pm 0.4 \text{ nM}$ $K_i(\text{hCB}_2\text{R}) = 1.6 \pm 0.4 \text{ nM}$		[123]
HU303-3 (43)	$K_i(\text{hCB}_1\text{R}) > 5000 \text{ nM}$ $K_i(\text{hCB}_2\text{R}) = 44 \pm 4 \text{ nM}$		[123]
AlexaFluor488-HU210 (44)	$K_i(\text{hCB}_1\text{R}) = 27 \pm 4 \text{ nM}$ $K_i(\text{hCB}_2\text{R}) = 0.8 \pm 0.2 \mu\text{M}$		[124]
Cy5-Chromenopyrazole (45)	$pK_i(\text{hCB}_1\text{R}) = 5.26 \pm 0.11$ $pK_i(\text{hCB}_2\text{R}) = 7.83 \pm 0.05$ $pIC_{50}(\text{hCB}_2\text{R}) = 6.93 \pm 0.04$		[125]
NBD-JWH-015 (46)	$K_i(\text{hCB}_1\text{R}) = 25 \% \text{ displacement at } 10 \mu\text{M}$		[126]
NMP6 (47)	$K_i(\text{hCB}_1\text{R}) < 40 \% \text{ at } 10 \mu\text{M}$ $K_i(\text{hCB}_2\text{R}) = 387 \text{ nM}$	~530 (MeCN)	[127]
NIR-mbc94 (49)	$K_i(\text{mCB}_2\text{R}) = 260 \text{ nM}$	797 (H <sub>2</sub> O)	[128]
NIR760-mbc94 (50)	$K_d(\text{mCB}_2\text{R}) = 26.9 \pm 3.7 \text{ nM}$	785 (H <sub>2</sub> O)	[129]
ZW760-mbc94 (51)	$K_d(\text{mCB}_2\text{R}) = 53.9 \pm 13.0 \text{ nM}$	781 (H <sub>2</sub> O)	[130]
IR700DX-mbc94 (52)	$K_d(\text{mCB}_2\text{R}) = 42.0 \pm 19.6 \text{ nM}$ $K_i(\text{hCB}_2\text{R}) = 1.86 \pm 1.38 \text{ nM}$	690 (MeOH)	[131,132]
IR700DX-TK-mbc94 (53)	$K_i(\text{hCB}_2\text{R}) = 2.67 \pm 1.78 \text{ nM}$		[132]
T1117 (54)	$K_i(\text{mCB}_2\text{R}) = 10 \% \text{ displacement at } 1 \mu\text{M}$ $K_d(\text{hCB}_2\text{R}) = 460 \pm 80 \text{ nM}$		[133,134]
NIR760-Q (55)	$K_d(\text{hCB}_2\text{R}) = 75.51 \pm 27.97 \text{ nM}$	787 (H <sub>2</sub> O)	[135]
NIR760-XLP6 (56)	$K_d(\text{mCB}_1\text{R}) > 10000 \text{ nM}$ $K_d(\text{mCB}_2\text{R}) = 169.1 \pm 66.09 \text{ nM}$	766 (H <sub>2</sub> O)	[136]
4Q3C-DMAP (57)	$K_i(\text{hCB}_1\text{R}) = 14 \% \text{ displacement at } 1 \mu\text{M}$ $K_i(\text{hCB}_2\text{R}) = 130 \pm 7 \text{ nM}$	504 (CHCl <sub>3</sub> ) 520 (PBS) 534 (DMSO) 544 (EtOH)	[137]
58	$pK_i(\text{hCB}_1\text{R}) < 5$ $pK_i(\text{hCB}_2\text{R}) = 6.33 \pm 0.02$ $pIC_{50}(\text{hCB}_2\text{R}) = 6.72 \pm 0.18$		[138]
59	$K_i(\text{hCB}_1\text{R}) = 617 \text{ nM}$ , $EC_{50}(\text{hCB}_1\text{R}) > 10 \mu\text{M}$ $K_i(\text{hCB}_2\text{R}) = 9.1 \text{ nM}$ , $EC_{50}(\text{hCB}_2\text{R}) = 2.2 \text{ nM}$ $K_i(\text{mCB}_2\text{R}) = 33 \text{ nM}$ , $EC_{50}(\text{mCB}_2\text{R}) = 21 \text{ nM}$	550 (DPBS)	[139]
60	$K_i(\text{hCB}_1\text{R}) = 63 \text{ nM}$ $K_i(\text{hCB}_2\text{R}) = 3.2 \text{ nM}$ $K_i(\text{mCB}_2\text{R}) = 4.6 \text{ nM}$	610 (DPBS)	[139]
61	$K_i(\text{hCB}_1\text{R}) = 114 \text{ nM}$ , $EC_{50}(\text{hCB}_1\text{R}) > 10 \mu\text{M}$ $K_i(\text{hCB}_2\text{R}) = 62 \text{ nM}$ , $EC_{50}(\text{hCB}_2\text{R}) = 67 \text{ nM}$ $K_i(\text{mCB}_2\text{R}) = 117 \text{ nM}$ , $EC_{50}(\text{mCB}_2\text{R}) = 66 \text{ nM}$	674 (derived from literature)	[139]
62	$K_i(\text{hCB}_1\text{R}) > 10 \mu\text{M}$ $K_i(\text{hCB}_2\text{R}) = 4.2 \text{ nM}$		[140]
63	$K_i(\text{hCB}_1\text{R}) > 10 \mu\text{M}$ , $EC_{50}(\text{hCB}_1\text{R}) > 10 \mu\text{M}$ $K_i(\text{hCB}_2\text{R}) = 4.7 \text{ nM}$ , $EC_{50}(\text{hCB}_2\text{R}) = 0.5 \text{ nM}$ $K_i(\text{mCB}_2\text{R}) = 78 \text{ nM}$ , $EC_{50}(\text{mCB}_2\text{R}) = 2.6 \text{ nM}$	530 (DCM & EtOAc) 540 (MeCN) 546 (DMSO & MeOH) 558 (PBS)	[140]
64	$K_i(\text{hCB}_1\text{R}) = 2378 \text{ nM}$ , $EC_{50}(\text{hCB}_1\text{R}) > 10 \mu\text{M}$ $K_i(\text{hCB}_2\text{R}) = 21 \text{ nM}$ , $EC_{50}(\text{hCB}_2\text{R}) = 171 \text{ nM}$ $K_i(\text{mCB}_2\text{R}) = 1459 \text{ nM}$ , $EC_{50}(\text{mCB}_2\text{R}) = 118 \text{ nM}$	578 (EtOAc) 594 (DCM) 616 (MeCN) 626 (DMSO) 628 (MeOH) 684 (DPBS)	[141,142]
65	$K_i(\text{hCB}_1\text{R}) = 1075 \text{ nM}$ , $EC_{50}(\text{hCB}_1\text{R}) > 10 \mu\text{M}$ $K_i(\text{hCB}_2\text{R}) = 4.7 \text{ nM}$ , $EC_{50}(\text{hCB}_2\text{R}) = 17 \text{ nM}$ $K_i(\text{mCB}_2\text{R}) = 1.1 \text{ nM}$ , $EC_{50}(\text{mCB}_2\text{R}) = 5.6 \text{ nM}$	596 (DCM & EtOAc) 600 (DPBS, MeCN & MeOH) 610 (DMSO)	[141,142]

affinity however came from the commercially available Cy5 dye with little CB<sub>1</sub>R interaction, showing promising potential for the scaffold **45** ( $pK_i(\text{CB}_1\text{R}) = 5.22 \pm 0.11$ ;  $pK_i(\text{CB}_2\text{R}) = 7.38 \pm 0.05$ ). The Cy5 compound was analysed via a cAMP BRET assay, revealing the compound to act as an inverse agonist, with higher potency than SR144528. Incubation of CB<sub>2</sub>R expressing HEK-293 cells with the Cy5 fluorophore demonstrated specific binding from a decrease in fluorescence when SR144528 is competing. Due to the potential issues of ligand with cell permeability, the probe is suggested to be beneficial for in vitro and ex vivo imaging, but no testing has been published thus far.

#### Fluorescent Indole Derivatives

JWH-015 is a potent cannabinoid agonist, containing a naphthylindole scaffold, with roughly 28-fold the affinity to CB<sub>2</sub>R over CB<sub>1</sub>R. Due to the high selectivity of compounds at the time, one of the first fluorophores for CB imaging was designed and synthesised to contain the fluorescent dye, nitrobenzofurazan (NBD).<sup>[126]</sup> The NBD fluorophore **46** was linked through the naphthalene via a short amide link to prevent a non-fluorescent, PET active compound being produced.<sup>[149]</sup> CB<sub>2</sub>R affinity was determined by a displacement assay against [<sup>3</sup>H]-CP 55,940 in CHO cells expressing CB<sub>2</sub>R. The new NBD JWH-015 resulted in only a 25% displacement at 10 μM. When visualised under a confocal microscope, most of the fluorescence was localised in the cytoplasm due to the lipophilicity of the compound and hence rapid cellular uptake. The precursors of the compound were found to still contain CB<sub>2</sub>R activity, however this avenue was not explored and in recent years indole compounds were found to primarily tolerate fluorophores at C-7 despite being antagonists.<sup>[150]</sup>

With this loss of affinity, employment of a potent CB<sub>2</sub>R antagonist scaffold, 6-methoxy-*N*-pentyl isatin acylhydrazide, was conjugated with the NBD dye.<sup>[127]</sup> NMP6 (**47**) was synthesised over two steps, utilising NBD to bind to the large hydrophobic pocket for potential CB<sub>2</sub>R affinity. NMP6 showed a  $K_i$  value of 387 nM in Chinese hamster ovary (CHO-K1) cells, when screened against [<sup>3</sup>H]-CP 55,940 in a competitive binding assay. The excitation and emission of NMP6 in acetonitrile were 470 and ~530 nm, respectively. Visualisation of the ligand was achieved by staining mouse T-cells with PE-conjugated anti-CD4 antibody, in which 5 μM of NMP6 was required to stain CD4<sup>+</sup> T-cells. Pre-incubating the cells with GW842166X, a CB<sub>2</sub>R agonist, shows a decrease in cell staining due to a reduction in binding. Despite the scaffold showing reliable CB<sub>2</sub>R affinity, further analogues containing the isatin scaffold have not been tested as an avenue for CB imaging.

#### Fluorescent Pyrazole Derivatives

SR144528 was one of the first synthetic cannabinoids to act as an inverse agonist selectively at CB<sub>2</sub>R ( $K_i(\text{hCB}_1\text{R}) = 400 \text{ nM}$ ;  $K_i(\text{hCB}_2\text{R}) = 0.6 \text{ nM}$ ).<sup>[151]</sup> Despite having a high affinity and selectivity to CB<sub>2</sub>R, SR144528 was never used as an imaging agent, due to a lack of a conjugatable group. In 2008, mbc94 (**48**) was first synthesised with a benzyl bromide group in place of a methyl for coupling capabilities.<sup>[152]</sup> The near-infrared (NIR) dye, IRDye 800CW NHS ester, was coupled to mbc94 to give NIRmbc94 (**49**), the first reported CB<sub>2</sub>R selective probe. The probe itself has promising photophysical properties with a maximum absorbance of 779 nm and an emission of 797 nm in water. NIRmbc94 labelled CB<sub>2</sub>-expressing DBT cells from a mouse astrocytoma cell line, whereas the NIR dye incubated in

the cells showed no signal. The fluorescent signal was reduced when non-CB<sub>2</sub>R expressing cells were incubated with the same concentration.

The mbc94 scaffold was further tested to investigate its affinity to CB<sub>2</sub>R. In 2011, a competitive binding assay against [<sup>3</sup>H]-CP 55,940 showed NIRmbc94 having a  $K_i$  of 260 nM in CB<sub>2</sub> mid DBT cells, whereas the non-conjugated mbc94 had a  $K_i$  of 15 nM.<sup>[128]</sup> The same study found that NIRmbc94 binds specifically to CB<sub>2</sub>-mid DBT cells and does not bind to DBT cells without endogenous CB<sub>2</sub>R. These results allowed further competition curves against potent CB<sub>2</sub>R compounds, SR144528 and WIN 55,212-2. Both compounds competing against NIRmbc94 for CB<sub>2</sub>R binding gave  $K_i$  values of 4.7 and 3 nM, respectively. The NIRmbc94 signal was found to remain undisturbed with non-CB<sub>2</sub>R active compounds present and active in BV-2 cells with a  $K_i$  of 3 nM against WIN 55,212-2, showing binding to endogenous CB<sub>2</sub>R expressed by the mouse microglia cells.

Further expanding on NIRmbc94, the same group synthesised NIR760-mbc94 (**50**), with the same pharmacophore but a different fluorophore.<sup>[129]</sup> The new fluorophore, NIR760, has similar properties to IRDye 800CW with a different linker position to the pharmacophore. The absorbance for the new fluorescent probe is 766 nm and a fluorescence emission of 785 nm with a quantum yield of 15.2%. In a saturated binding assay with SR144528, the NIR760-mbc94 binds to the CB<sub>2</sub>R with a  $K_d$  of 26.9 nM. NIR760-mbc94 incubated in CB<sub>2</sub>-mid DBT cells exhibited a 4-fold higher fluorescent signal than that of cells incubated with NIR760. In the presence of SR144528, the NIR760-mbc94 incubated cells showed 40% lower fluorescence intensity. In vivo testing found NIR760-mbc94 increased the tumour-to-normal ratio by 3.7-fold at 72 h post-injection. The selective binding to CB<sub>2</sub>R, both in vitro and in vivo, suggests NIR760-mbc94 to be a promising imaging agent for CB<sub>2</sub>R-positive cancers.

With new zwitterionic NIR fluorescent probes being used for imaging, the mbc94 pharmacophore group looked at using a novel NIR probe, ZW760-mbc94 (**51**),<sup>[130]</sup> which contained a similar scaffold to NIR760, whereby the alkyl sulfonic acid groups were substituted with trimethyl amine cations. When compared with other ZW800-1, it contains a carbon-carbon linkage at the *meso* position as opposed to an enol ether.<sup>[153]</sup> The dye itself exhibited a fluorescence emission of 805 nm in DMSO, 786 nm in methanol, and 775 nm in H<sub>2</sub>O. When in the presence of a control ligand/blocking agent, 4-quinolone-3-carboxamide (4Q3C), the uptake of ZW760-mbc94 was lowered in CB<sub>2</sub>-mid DBT cells, with roughly ~50% blockage. In WT DBT cells (non-CB<sub>2</sub>R expressing cells) the uptake of ZW760-mbc94 is ~40% lower than of the CB<sub>2</sub>-mid DBT cells, demonstrating its specificity to CB<sub>2</sub>R. An in vitro fluorescence saturation binding study against 4Q3C gave a  $K_d = 53.9 \text{ nM}$  when binding to CB<sub>2</sub>R. When compared with the previous NIR760-mbc94, the new ZW760 showed an improved binding specificity in vitro and ex vivo.

The same group developed IR700DX-mbc94 (**52**), utilising the NIR probe, IR700DX, as a potential phototherapeutic option for tumour cells overexpressing CB<sub>2</sub>R.<sup>[131]</sup> The compound displayed an absorbance and emission of 682 and 690 nm in methanol, respectively. IR700DX-mbc94 was determined to be non-toxic with the lack of irradiation, in both CHO-K1/CB<sub>2</sub> and CB<sub>2</sub>-mid DBT cells. The compound displayed a  $K_d$  of  $42.0 \pm 19.6 \text{ nM}$  and exhibited an ability to kill and inhibit CB<sub>2</sub><sup>+</sup> cells when irradiated, although requiring binding to CB<sub>2</sub>R. An in vivo targeted phototherapy study using IR700DX-mbc94 highlighted that <sup>1</sup>O<sub>2</sub> and free radicals were



produced upon irradiation, producing a necrosis-like cell death.<sup>[154]</sup> IR700DX-mbc94 shows potential to be a selective phototherapy agent for cancer cells; it bares little to no cytotoxicity before irradiation and displays selectivity towards CB<sub>2</sub>+ cells. IR700DX-mbc94 was further modified to contain a dimethyl thioketal within the linker. The new probe, IR700DX-TK-mbc94 (**53**), was found to displace [<sup>3</sup>H]-CP 55,940 from hCB<sub>2</sub>R with nanomolar affinity, as did IR700DX-mbc94.<sup>[132]</sup> In CB<sub>2</sub>R+ mouse delayed brain tumour CB<sub>2</sub>-mid DBT cells, IR700DX-TK-mbc94 showed 59% cell death upon irradiation whereas IR700DX-mbc94 showed negligible amounts. The higher percentage of cell death when treated with IR700DX-TK-mbc94 was consistent across varying the time and concentration of the incubation. HEK-293 cells exhibited much less cell death than CB<sub>2</sub>-mid DBT cells, indicating the potential of selective phototherapy agents for tumours expressing CB<sub>2</sub>R.

T1117 (**54**) was first reported in 2010 and derived from the CB<sub>1</sub>R inverse agonist, AM251, tagged with fluorescent moiety, 5-carboxytetramethylrhodamine (5-TAMRA).<sup>[133]</sup> AM251 displaced CP 55,940 from CB<sub>1</sub>R WT mouse brain membranes with a  $K_i$  of 0.8 nM, whereas T1117 only displayed 10% displacement at 1  $\mu$ M. T1117 also displayed binding to GPR55 through Ca<sup>+</sup> response in HEK-293 cells overexpressing recombinant GPR55 and showed fluorescence in WT mouse mesenteric artery (MMA). A conflicting study in 2014 demonstrated T1117 binds to CB<sub>1</sub>R showcasing a  $K_d$  of 460  $\pm$  80 nM.<sup>[134]</sup> In their fluorescence quenching study, T1117 displayed a decrease in fluorescence when incubated in mouse membranes when compared with the PBS control and an increase when AM251 was added. It was validated that fluorescence of T1117 was quenched upon binding to CB<sub>1</sub>R in overexpressing HEK-293 cells. T1117 binding to CB<sub>1</sub>R was shown to be sensitive to the NAM Org27569 and this fluorescence-quenching method has been proposed to be suitable for rapid assessment of novel orthosteric and allosteric modulators. Despite this, the ligand was opted to be not suitable for in situ CB<sub>1</sub>R identification due to non-specific membrane permeability. A study in 2019 focusing on linker attachments of SR141716A suggested that C-5 of the pharmacophore was a suitable position for fluorescent moiety linkage.<sup>[155]</sup> A fluorescein fluorophore showed affinity to hCB<sub>1</sub>R at 260  $\pm$  20 nM with a linker length of 12 atoms, suggesting the C-5 position can be utilised for high affinity CB<sub>1</sub>R probes.

#### Fluorescent Oxoquinoline Derivatives

As one of the first fluorophores to be used for CB<sub>2</sub>R in vivo imaging, NIR760 dye was further coupled to another selective CB<sub>2</sub>R ligand. The quinoline scaffold had been shown to be a highly selective CB<sub>2</sub>R ligand with selectivity up to 0.2 nM.<sup>[156]</sup> The 4-quinoline-3-carboxamide with a pentyl at *N*-1 was the chosen scaffold for NIR760 coupling. NIR760-Q (**55**) was synthesised through reported steps to form 4Q3C and linked via a click reaction to form a 1,2,3-triazole with an ether bridge.<sup>[135]</sup> The novel probe, NIR760-Q, has an absorbance and emission of 768 and 787 nm, respectively, and a quantum yield of 16.5% in water. Having similar absorbance and emission as NIRmbc94, the compound's binding affinity was tested in Jurkat cells. These cells overexpress CB<sub>2</sub>R, whereas testing previously was done on CB<sub>2</sub>-mid DBT cells. NIR760 showed a  $K_d$  of 75.51 nM within an excess of 4Q3C to saturate binding sites. Cells with both 4Q3C and NIR760-Q had lower fluorescence than that of just the probe, indicating specific CB<sub>2</sub>R binding. The Jurkat cells

incubated with NIR760-Q showed a  $\times 2.8$  increase over those just treated with NIR760. However, partial inhibition of the compound indicated non-specific binding, which was accounted for by the overall net charge of the compound.

A study proposed a new scaffold, pyrazolo[1,5-*a*]pyrimidine, for CB<sub>2</sub>R selective compounds boasting a similar structure to 4Q3C. With the large hydrophobic adamantane amide and the *N*-1 alkyl pentane, the *para*-substituted phenyl derivatives had high affinities to CB<sub>2</sub>R.<sup>[157]</sup> The substitution at the C-2 position has very little effect on the binding affinity with these compounds. Based on this, XLP4 was initially synthesised, containing a bromide handle at C-2 for further chemistry.<sup>[136]</sup> This compound underwent Suzuki cross coupling with 4-carboxyphenylboronic acid, followed by a 1,6-diaminohexane addition to form XLP6, which was coupled to NIR760 dye to yield the CB<sub>2</sub>R selective probe, NIR760-XLP6 (**56**). The probe has an absorbance and emission of 766 and 785 nm, respectively, and a quantum yield of 11.6% in water, boasting similar photophysical properties to NIR760-Q. Saturated binding with XLP4 in DBT-CB<sub>2</sub> cells, showed NIR760-XLP6 binding to CB<sub>2</sub> with a  $K_d$  of 169.1 nM. Higher fluorescence was observed in the DBT-CB<sub>2</sub> cells as opposed to the DBT-CB<sub>1</sub> cells, confirming CB<sub>2</sub>R binding. The compound was found to have selectivity towards CB<sub>2</sub>R both in vitro and in vivo. Further tests on the CB<sub>2</sub> overexpressing pancreatic duct adenocarcinoma cells (PDAC) found NIR760-XLP6 binds to CB<sub>2</sub>R in PNA-1 with high imaging contrast in vivo after 9 h post injection and ex vivo up to 48 h with certain lymph nodes.<sup>[158]</sup>

The mbc94 and NIR760 probes (Q and XLP6) had proven to show good fluorescence properties in biological systems while maintaining moderate affinity to CB<sub>2</sub>R. Similar to NIR760-Q, the *N*-adamantyl-4-oxo-1,4-dihydroquinoline-3-carboxamide scaffold was selected due to CB<sub>2</sub>R affinity and its tolerance to bulky, aromatic groups at *N*-1.<sup>[159]</sup> Large fluorophores, 4-dimethylaminophthalimide (4-DMAP), NBD, and fluorescein-thiourea (FTU), were selected as the fluorescent moieties with the selective quinoline.<sup>[137]</sup> The linker for this study was a varying methylene chain from six to twelve carbons, in which a 6 chain-DMAP probe (**57**) was found have the highest affinity ( $K_i$ (hCB<sub>1</sub>R) > 10000 nM;  $K_i$ (hCB<sub>2</sub>R) = 130 nM). The emission of the DMAP fluorophore varied between 504 and 544 nm in various solvents, combined with ideal pharmacological properties, the probe was chosen for fluorescent-activated cell sorting (FACS). The results obtained from a fluoro-binding assay against WIN 55,212-2 and GW405833 matched literature values.<sup>[160]</sup> The compound displayed low cytotoxicity in numerous different cell lines within 24 h of incubation. A saturation binding assay in CB<sub>2</sub>R overexpressing HEK-293 cells displaced GW405833 with a  $K_d$  of 44 nM, while an absence of fluorescence was observed in WT HEK-293 cells. Several CB<sub>2</sub>R expressing cancer cell lines were tested with the fluorescent ligand and GW405833 in saturation binding assays, in which the results were consistent with the HEK-293 CB<sub>2</sub>R cell model. Fluorescence microscopy demonstrated the ideal concentration for imaging of the fluorescent ligand was 15  $\mu$ M, with higher concentrations exhibiting off-site binding. The IC<sub>50</sub> of GW405833 (IC<sub>50</sub> = 3.94 nM) was obtained using the model and verified by comparison to the radioligand binding ( $K_i$  = 3.92 nM), validating the compounds ability to be applied as an option to conventional radioligand assays with CB<sub>2</sub>R specificity.

Similarly to the quinoline analogues, 1,8-naphthyridine compounds have shown to be highly selective CB<sub>2</sub>R compounds, while maintaining lower lipophilicity. Variations on the *N*-1, C-3,

and C-6 positions have highlighted a tolerance for bulky groups at *N*-1, whereas substituents at C-6 affect functional activity, potentially reducing CB<sub>2</sub>R affinity. The receptor binding affinity of the carboxamide at C-3 can be altered using 4-methylcyclohexyl, with *cis* derivatives giving higher affinity than *trans*. In 2018, Cooper et al. modified the scaffold at *N*-1 with PEG and methylene linkers bound to a BODIPY630/650-X fluorophore.<sup>[138]</sup> These two compounds were screened by a radioligand competition binding assay displacing [<sup>3</sup>H]-CP 55,940. The previously reported *N*-ethyl morpholino analogue, used as a comparison, had an affinity similar to that of SR144528 ( $pK_i(\text{CB}_1\text{R}) < 5$ ;  $pK_i(\text{CB}_2\text{R}) = 7.26 \pm 0.04$ ). The attachment of both linkers and BODIPY630/650-X resulted in a loss in CB<sub>2</sub>R affinity as well as inverse agonism due to substitution at C-6 ( $pK_i(\text{CB}_1\text{R}) < 5$ ;  $pK_i(\text{CB}_2\text{R}) < 5$ ). Due to a lack of affinity with the C-6 substitution, modifying the cyclohexyl group at C-3 with the fluorophore was performed. The only fluorescent ligand to have moderate CB<sub>2</sub>R affinity was BODIPY, in the *cis* conformer **58**. This compound showed a similar  $pIC_{50}$  to that of SR144528 despite having a weaker affinity ( $IC_{50}(\text{CB}_2\text{R}) = 6.72 \pm 0.18$ ). Unfortunately, this compound lacked any beneficial fluorescent properties from cellular imaging and cells pre-incubated with SR144528 did not affect the labelling, displaying non-CB<sub>2</sub>R fluorescence.

#### Fluorescent Pyrazine/Pyridine Cannabinoids

The Carreira group focussed on modifying pyrazine and pyridine scaffolds, in which no previous cannabinoid fluorescent probes have been published.<sup>[139]</sup> With parent compounds RO6839251 ( $K_i(\text{hCB}_2\text{R}) = 0.2 \text{ nM}$ ) and RO6852763 ( $K_i(\text{hCB}_2\text{R}) = 0.2 \text{ nM}$ ), displaying good affinity and selectivity to CB<sub>2</sub>R, fluorescent moieties NBD, AttoThio12, Silicon Rhodamine (SiR), and Cy5.5 were linked via variations on PEG or C-6 alkyl chains. NBD (**59**) and AttoThio12 (**60**) linked compounds displayed the highest affinity to CB<sub>2</sub>R but maintained moderate affinity to CB<sub>1</sub>R. In flow cytometry experiments, AttoThio12 and SiR (**61**) bound compounds labelled mouse and human CB<sub>2</sub>R, CHO cells overexpressing cannabinoid receptors, as well as WT CHO cells. Specificity was further validated by displacing agonist, JWH133 and an inverse agonist, RO6851228. Utilising time-resolved FRET (TR-FRET), the values obtained in competitive binding against HU-308 and SR144528 with **61** supported the use of this methodology for CB<sub>2</sub>R high-throughput screening. Lastly, SiR was able to label hCB<sub>2</sub>R-overexpressing CHO cells, displaying no membrane labelling indicating CB<sub>2</sub>R specificity. This ultimately signifies a range of fluorescent probes that potentially can assist in elucidation of the CB<sub>2</sub>R mechanism of action.

#### Bifunctional Fluorescent Probes

A range of bifunctional CB compounds based of a hybrid between HU-308 and AM841 were synthesised by the Carreira group, containing a fluorescent or photoaffinity moiety and an electrophilic group.<sup>[140]</sup> The compounds were tested within the membrane of CHO cells overexpressing human cannabinoid receptors via a radioligand assay against [<sup>3</sup>H]-CP 55,940. Selectivity at hCB<sub>2</sub>R of these compounds ranged from 1 to > 2381, with NBD compounds having the highest potency. Amongst the NBD compounds, both the isothiocyano (**62**) and azido (**63**) had the highest hCB<sub>2</sub>R selectivity. It was noted that **63** did not covalently bind to hCB<sub>2</sub>R overexpressing CHO cells as per a polyacrylamide gel electrophoresis assay. Further establishing upon the scaffold, the fluorophore was altered to

DY-480XL, Alexa647, and AttoThio12, while the terminal azido remained consistent.<sup>[141,142]</sup> From these compounds, the DY-480XL (**64**) and AttoThio12 (**65**) dyes demonstrated high affinity and selectivity to CB<sub>2</sub>R. Both probes were suggested to be alternatives to radioligands as per a TR-FRET assay. Notably, **65** could be detected in 5xFAD mice, expressing mCB<sub>2</sub>R AD microglia, and labelled endogenous hCB<sub>2</sub>R MDA-MB-231 breast cancer cells by flow cytometry. Finally, real-time confocal fluorescence microscopy displayed that **65** labelled mCB<sub>2</sub>R splenocytes and hCB<sub>2</sub>R macrophages with minimal internalisation, suggesting the probe can be used for endogenous CB<sub>2</sub>R in murine and human models.

#### Conclusions and Future Perspectives

Modulation of the endocannabinoid system is an ideal therapeutic target for a plethora of neurological diseases and anti-inflammatory effects, primarily focusing on CB<sub>2</sub>R selectivity. Modern drug discovery ultimately necessitates the capability to image the receptor and the demonstration of target engagement, to understand the mechanism of action in a complicated system, such as the ECS. With a multitude of screening assays and several potential off-target interactions, a more holistic and comprehensive screening approach can give a better view of the true activity spectrum.<sup>[33]</sup> Small molecular probes are a necessity for further drug development, in which the current trend in cannabinoid small molecule probes is shifted towards CB<sub>2</sub>R selective compounds with a fluorescent moiety. Within recent years, bifunctional probes applying the ability to covalently bind to the receptor, via an electrophile or photoactivatable group, have become more prominent. To determine and further elucidate CB<sub>2</sub>R pharmacology, selective scaffolds can be highly utilised for attachment of imaging moieties, specifically noting quinolone, indole, and thiophene derivatives, containing a large hydrophobic carboxamide.<sup>[161–163]</sup> For a consistent analysis of CB imaging compounds, altering the probe and/or position exclusively within the same scaffold could clarify binding affinity and efficacy, however this ultimately could prove to be a large task.

Ultimately, new small molecular probes can be designed for in vivo imaging, with the possibility of elucidating biological pathways and highlighting diseases for treatment and diagnosis within these pathways. Several issues have the potential to arise with in vivo testing, namely lipophilicity and specificity of the ligand once the fluorophore is linked. While most PET probes have minimal issues with blood–brain barrier permeation, the addition of a fluorophore tag could have negative effects on the physiochemical properties and possibly render neurological imaging difficult. Another issue is competitive clearance pathways, in which net-neutral charged fluorophores are suggested to avoid this rapid clearance issue.<sup>[164]</sup> Finally, the probe needs to have a high affinity to the receptor, to overcome non-selective binding, which is affected upon fluorophore coupling. Non-invasive probes utilising near-infrared and BODIPY dyes have the potential for in vivo imaging due to a negation of bioluminescence, although membrane permeability could still prove to be problematic.<sup>[165,166]</sup> With recent successes in cannabinoid imaging, the probes mentioned in this review can potentially further guide drug discovery and help uncover the sophisticated cannabinoid pharmacology.

#### Conflicts of Interest

The authors declare no conflicts of interest.

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