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Recent advances in the development of CB_1R selective probes

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Cannabinoid subtype 1 receptors (CB_1Rs) are an important class of G protein-coupled receptors (GPCRs) belonging to the endocannabinoid system. CB_1Rs play a crucial modulatory role in the functioning of other neurotransmitter systems and are involved in a wide range of physiological functions and dysfunctions; thus, they are considered one of the most important targets for drug development, as well as diagnostic purposes. Despite this, only a few molecules targeting this receptor are available on the pharmaceutical market, thus emphasizing the need to gain a deeper understanding of the complex activation pathways of CB_1Rs and how they regulate diseases. As part of this review, we provide an overview of pharmacological and imaging tools useful for detecting CB_1Rs. Herein, we summarize the derivations of cannabinoids and terpenoids with fluorescent compounds, radiotracers, or photochromic motifs. CB_1Rs' molecular probes may be used *in vitro* and, in some cases, *in vivo* for investigating and exploring the roles of CB_1Rs together with the starting point for the development of CB_1R-targeted drugs.

KEYWORDS

cannabinoids, CB_1R, photoactivatable probes, fluorescent probes, PET imaging

1 Introduction

Since the dawn of civilization, *Cannabis sativa* L. has been used in a multitude of different ways for many purposes, from a recreational drug to medical uses, as well as for industrial goals including food, textile, paper, building, and energy industries, which have proven hemp to be an attractive solution for synthetic economies. (Abedi and Sahari, 2014; Alexander, 2016; Appendino, 2020; Finnan and Styles, 2013; Garcia-et al., 1998; Rehman et al., 2013). Recently, several studies have demonstrated that cannabis has positive effects on a wide range of health conditions (Campos et al., 2016). These outcomes are attributed to the active compounds found in the plant, thus prompting further research in the isolation and study of these secondary metabolites. Since the late 1960s, several compounds known to be present in cannabis have been isolated and characterized, including the psychoactive cannabinoid Δ^9-tetrahydrocannabinol (THC or Δ^9-THC, **1**, Figure 1) and the non-euphoric cannabidiol (CBD, **2**, Figure 1) (Iversen, 2018; Sholler et al., 2020). These compounds are now referred to as "major cannabinoids", including other important ones like cannabigerol (CBG), cannabichromene (CBC), and cannabinol (CBN) (Pollastro et al., 2018; Anokwuru et al., 2022; Maioli et al., 2022). The two major phytocannabinoids THC and CBD already reached the approval by FDA for

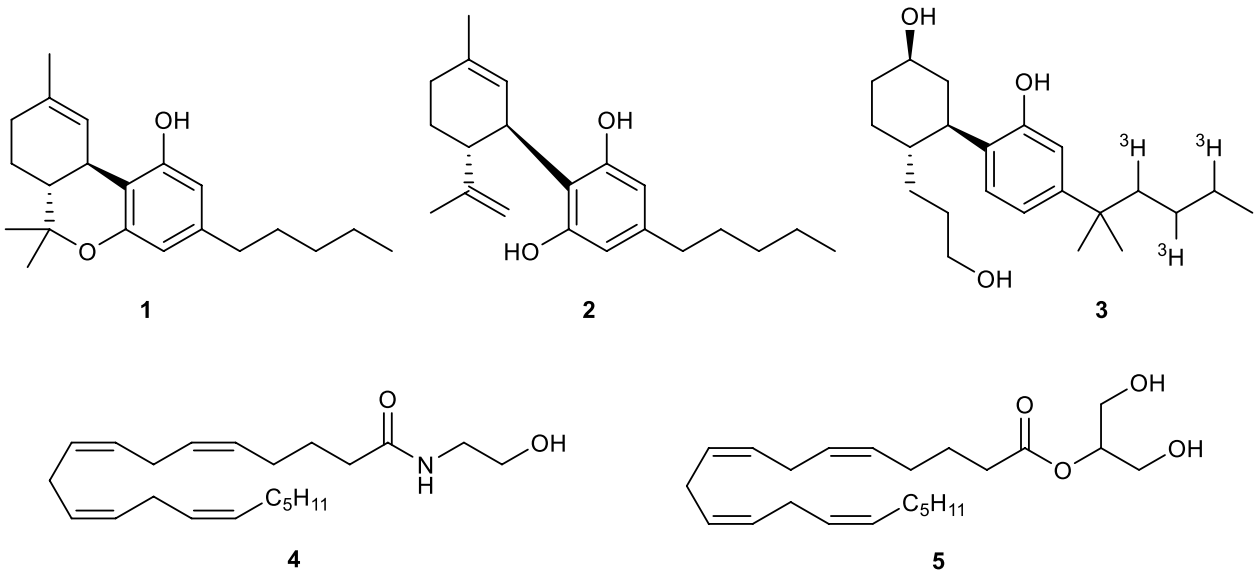

FIGURE 1
Structure of Δ^9-THC (**1**), CBD (**2**), tritiated CP 55,940 (**3**), AEA (**4**), and 2-AG (**5**).

commercialization in different forms: Sativex®, a combination of THC and CBD, is used to treat spasticity associated with multiple sclerosis, and CBD has also been developed as a single active pharmaceutical ingredient known as Epidiolex®, drug of choice for the treatment of certain rare genetic forms of epilepsy. Additionally, the main phytocannabinoids are still being studied for their potential in treating various pathologies (mainly inflammatory diseases) both *in vivo* and *in vitro* (Baratta et al., 2022), as well as some of their derivatives, most notably the aminocannabinoquinone VCE-004.8, which has been granted orphan drug status by the FDA and EMA for the treatment of scleroderma (Caprioglio et al., 2021). More recently, *Cannabis sativa* showed to have a neuroprotective effect, resultant from its anti-inflammatory and antioxidant properties (Viana et al., 2022), while phytocannabinoids have been demonstrated to have potential anti-cancer properties. They induce cell death, inhibit cell migration and proliferation, decrease angiogenesis, and inhibit the invasiveness of cancer cells of, e.g., the lung, prostate, skin, breast, or brain (Hinz and Ramer, 2019; Kovalchuk and Kovalchuk, 2020; Tomko et al., 2020). Despite the very large number of phytocannabinoids isolated from cannabis (more than 150 different compounds) (Hanuš et al., 2016), the main biological activities observed during the study of this plant are to be attributed to these compounds which occur in considerably greater quantities compared to the so-called minor cannabinoids, regardless of the chemotype of the plant (Caprioglio et al., 2022).

In humans, the biological pathway regulated by cannabinoids is called endocannabinoid system (ECS). ECS is composed of two types of receptors, the CB_1R and cannabinoid 2 receptor (CB_2R), as well as enzymes that break down and synthesize their endogenous ligands (referred to as endocannabinoids) (De Petrocellis and Di Marzo, 2009). The CB_1R was identified and characterized in the rat brain in 1988 and its location was confirmed using tritiated CP 55,940 (**3**, Figure 1) in 1990 (Herkenham et al., 1990). In 1992, the receptor was cloned and the DNA that encodes GPCRs was found (Matsuda et al., 1990). The CB_2 receptor was instead discovered in 1993 and was found to be predominantly expressed in macrophages in the spleen (Munro et al., 1993). Once CB_1R and CB_2R have been discovered, anandamide (AEA, **4**) and 2-arachidonoyl glycerol (2-AG, **5**) were found to be the endogenous ligands that interact with these receptors (Figure 1) (Di Marzo et al., 2004).

As a regulator of a wide range of physiological processes, the ECS plays an important role in many disorders (Bisogno and Di Marzo, 2010). To gain a broader view of the ECS, it would be extremely valuable to develop high-sensitivity and high-throughput analytical tools. The use of small molecule probes might provide information regarding the spatial and temporal dynamics of cannabinoid receptor expression, as it has been demonstrated in recent years for some specific classes of enzymes (Chang et al., 2009; 2012; Tully and Cravatt, 2010). As a consequence, the development of small molecule probes that can recognize cannabinoid receptors is currently in the spotlight, since they may complement, and in some cases eliminate, some of the limitations of available antibodies (Grimsey et al., 2008). The purpose of this review is to provide an overview of the recent development of CB_1R-specific probes: we will examine the various types of probes currently in use, describe how the chemistry of these molecules affects the effectiveness of these drugs, and examine where new probes and drugs may be developed in the future.

2 Covalent probes for CB_1R

Covalent chemical probes have found widespread use as research tools and clinical agents. Their use ranges from probes that are metabolically incorporated into proteins, to probes for photoaffinity labelling of targets and electrophilic probes for activity-based protein profiling. In the contest of the CB_1Rs,

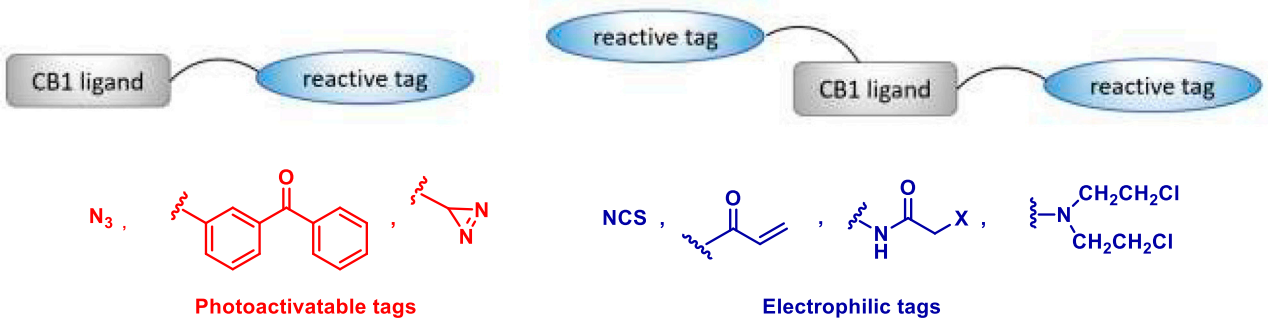

FIGURE 2
General structures for covalent probes of CB_1Rs.

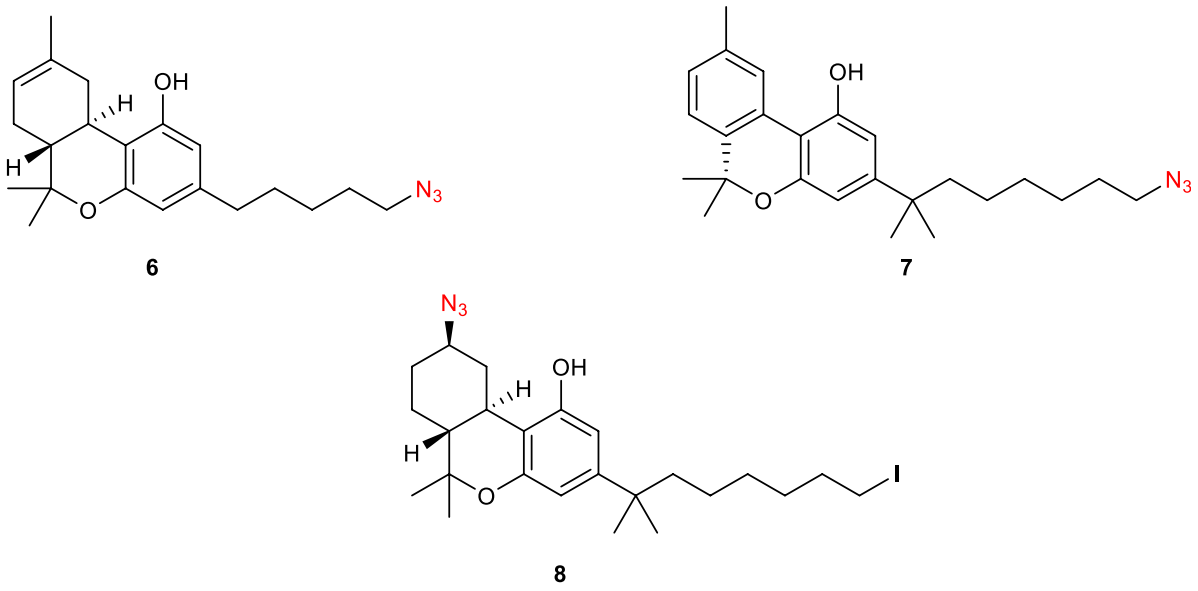

FIGURE 3
Structure of photoactivatable probes AM91 (**6**), 7′-Azido-1′,1′-dimethylheptyl-Δ^8-THC (**7**) and AM869 (**8**).

covalent probes have been designed by incorporating a reactive tag into a CB_1R ligand such as **1** or **4** (Figure 2). One type of reactive tag is a chemically inert group (*e.g.*, azides, benzophenones, or diazirine) which, upon UV irradiation, is converted into a highly reactive species able to react with any amino acid residue situated in its immediate environment. Alternatively, the reactive tag can be an electrophilic group (e.g., isothiocyanates, Michael acceptors, haloacetamides, or nitrogen mustards) able to target nucleophilic amino acids (e.g., lysine, histidine, and cysteine) situated at or near the binding site. When two reactive tags are attached to the ligands, then the corresponding bifunctionalized probes can potentially interact at two distinct sites within the CB_1R-binding domains. In this section, we reviewed the covalent probes for the CB_1Rs, classifying them as photoactivatable, electrophilic, and bifunctional probes.

2.1 Photoactivatable probes

Photoaffinity probes (PAPs) are composed of a target-specific ligand and a photoactivatable functional group. When bound to the corresponding target proteins and activated with wavelength-specific light, PAPs generate highly reactive chemical species that covalently cross-link proximal amino acid residues. This process is better known as PAL (photo-affinity labelling) and is widely employed to identify cellular targets of biologically active molecules (Sumranjit and Chung, 2013). Many photoactivatable probes have been developed for CB_1R, with 5′-Azido-Δ^8-THC or AM91 **6** (Figure 3), reported by Charalambous *et al.*, being the first example of this kind (Charalambous et al., 1992). This ligand, with an aliphatic azido group attached to the terminal carbon of the alkyl side chain, showed a higher affinity for rat CB_1R

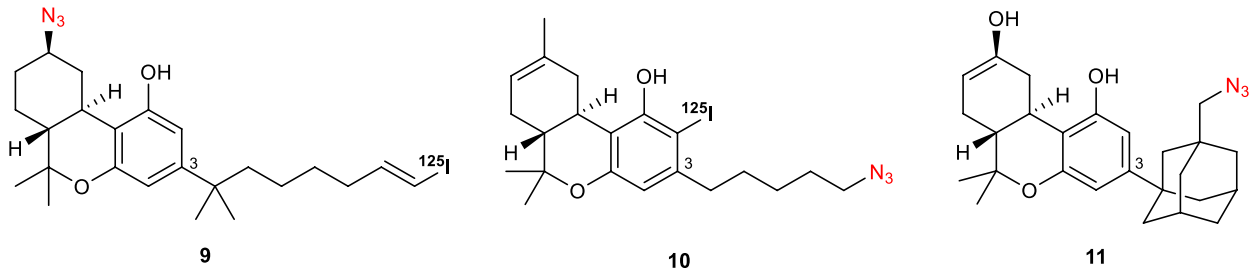

FIGURE 4
Structures of photoactivatable probes AM 1708 (**9**), **6**-^{125}I (**10**), and AM993 (**11**).

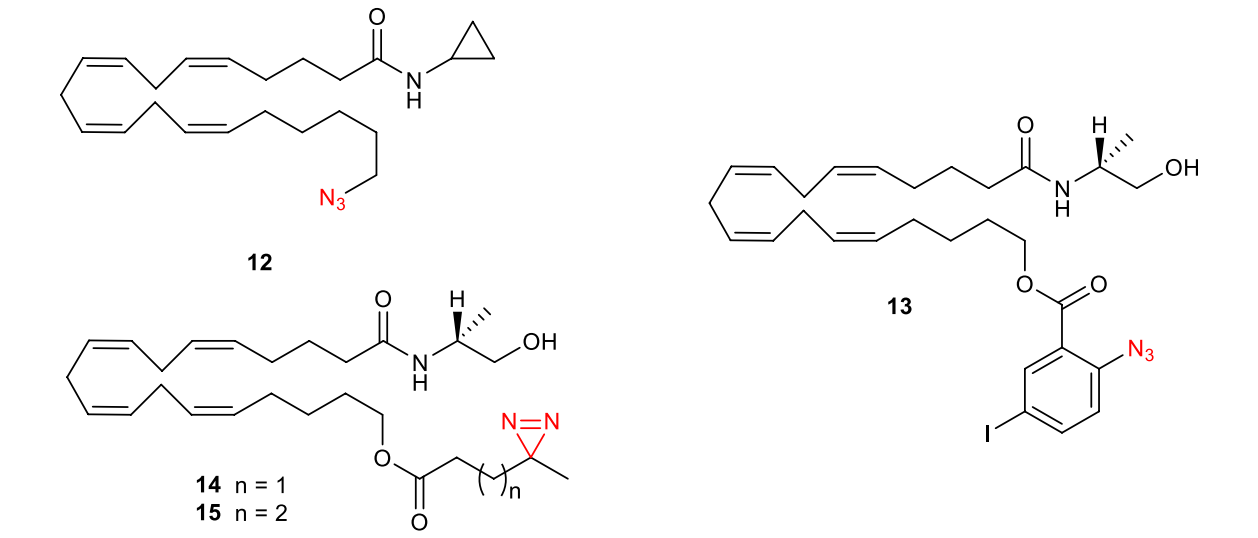

FIGURE 5
Structure of photoactivatable probes AM3661 (**12**) and the other anandamide-based photoaffinity probes **13-15**.

(rCB$_1$R, Ki = 19 nM) over its parent prototype (−)-Δ^8-THC (Ki = 35 nM). Moreover, equilibration of rat forebrain membranes with a 1 μM concentration of this compound, followed by UV-irradiation and washing to remove the unbound **6**, resulted in an 85% decrease in the number of CB$_1$R-binding sites of [^{3}H]-CP 55,940, the standard radiolabeled CB$_1$-agonist. Later on, the same group found that 7′-Azido-1′,1′-dimethylheptyl-Δ^8-THC **7** (Figure 3) has a markedly improved binding affinity (Ki rCB$_1$R = 0.4 nM) (Picone et al., 2002). Receptor binding studies revealed that **7** was effective at reducing the binding of [^{3}H]-CP 55,940 by ca. 75% at 1 nM ligand concentration. This improvement was attributed to the lengthening of the C-3 alkyl side chain as well as the addition of the geminal dimethyl group. It must be noted that the photoreactive tag could also be installed in another region of the ligand, as reported for AM869 **8** (Ki = 0.67 nM), although it showed limited selectivity between the two CBR-subtypes (Khanolkar et al., 2000).

With the successful incorporation of the azido group as the reactive tag, a series of photoactivable probes radiolabeled have been designed by decorating the scaffold with ^{125}I (Figure 4). Interestingly, while AM1708 **9** showed a similar affinity for CB$_1$R (K$_i$ = 0.72 nM) to that of **8**, compound **6**-^{125}I **(10)** displayed a high affinity for CB$_1$R sites in both brain (K$_d$ = 5.60 p.m.) and whole cell (K$_d$ = 9.38 p.m.) systems.

Continuing this modification at C-3 position of Δ^8-THC, compound AM993 **11** bearing an adamantyl group with a photoactivatable azido-group was synthesized (Figure 4) (Ogawa et al., 2015). This compound was found to act as an agonist of CB$_1$R (EC$_{50}$ = 2.4 nM) whereas showing a negligible response at CB$_2$R; moreover, it showed high affinity to CB$_1$R with 2-fold and 6-fold selectivity over human and mouse CB$_2$R (Ki CB$_1$R = 4.4 nM). Covalent labelling yielded a 67% decrease in CB$_1$R [^{3}H]-CP 55,940 binding.

Since anandamide (**4**) is an endogenous polyunsaturated long-chain fatty acid agonist of CB$_1$Rs, photolabeling probes based on this endocannabinoid have been designed (Li et al., 2005). Specifically, AM3661 **12** (Figure 5) showed to possess CB$_1$R-selectivity with a Ki value of 0.9 nM. Photolabeling experiments revealed a 68% reduction in [^{3}H]-CP 55,940 (**3**) binding and therefore indicate that this ligand could be a useful probe for CB$_1$R. Based on these results, Balas *et al.* synthesized a photoactivatable aryl azide probe **13**

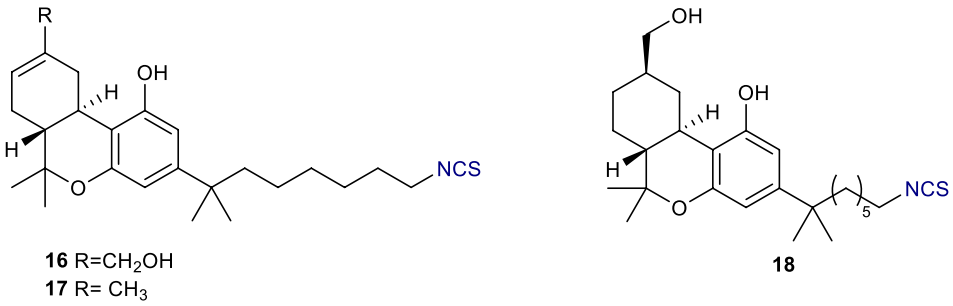

FIGURE 6
Structures of electrophilic probes AM 708 (**16**), **17,** and AM841 (**18**).

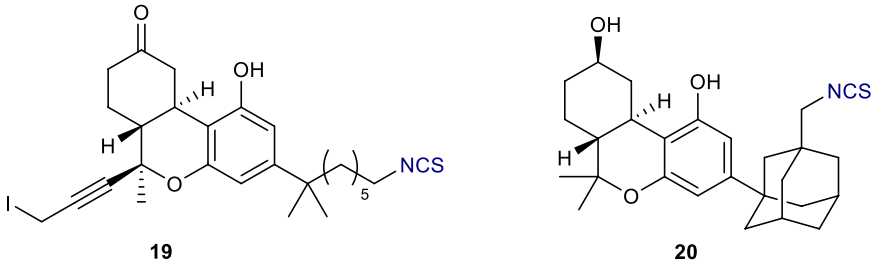

FIGURE 7
Structure of electrophilic probes AM960 (**19**) and AM 994 (**20**).

(Figure 5) (Balas et al., 2006). This compound showed a decreased affinity to human CB_1R (hCB_1R, K_i = 0.9 μM) when compared with the endogenous anandamide ligand (K_i = 0.07). These results suggest its potential use as a tool for the discovery of new potential endocannabinoid receptors. The same group further synthesized other anandamide-based photoaffinity probes (**14-15**, Figure 5) by replacing the 2-azido-5-idobenzoate group with short diazirine containing alkyl chains (Balas et al., 2009); however, no further studies on CB_1Rs have been conducted with these compounds.

2.2 Electrophilic probes

Electrophilic probes containing reactive tags are able to target nucleophilic amino acids present in the proximity of the binding site of the protein of interest. The targeted amino acids are usually cysteine, lysine, and histidine (all containing a nucleophile in the side chain), whereas the reactive tags are usually isothiocyanates or other electrophiles. These tags are often easy to install, thus many electrophilic probes for CB_1Rs have been developed in the last decades. The first elecrophilic probes, reported by the Makriyannis group, were based on THC derivatives (Morse et al., 1995). (−)-11-OH-7′-NCS-1,1′-dimethylheptyl-Δ^8-THC or AM708 **16** and the analogue **17** bearing a methyl group instead of the hydroxymethyl at position 11 (Figure 6) exhibited potent binding at CB_1R with similar IC_{50} values and comparable [^{3}H]-CP 55,940 (**3**) displacement at 83%. Notably, the analogue of **16** in which the alkene has been reduced (compound AM841, **18**) showed to behave as a highly potent hCB_1R agonist (Ki = 9.05nM; EC_{50} = 0.94 nM) (Picone et al., 2005). Moreover, based on ligand-assisted protein structure analysis (LAPS), they identify a cysteine residue, C6.47 (355), within the transmembrane helix 6 of CB_1R, as the key site for the covalent binding of **18**. The ligand-receptor interaction was abolished when either C6.47 (355) was mutated to weaker or non-nucleophilic amino acid residues or the electrophilic isothiocyanate group on **18** was exchanged with non-electrophilic substituents.

Another electrophilic probe based on a modification of THC has been reported by Chu *et al.* (Figure 7) (Chu et al., 2003). In this case, AM960 **19**, containing a propargyl iodide, displayed successful binding at rCB_1R by occupying 50% of sites at 25 nM. In a similar fashion to AM993 **11** (*vide supra*), the adamantyl C-3 derivative AM994 **20** (Figure 7) showed high affinity to rCB_1R (Ki = 3.0 nM) with 3- and 10-fold selectivity over human and mouse CB_2R respectively; moreover, it displayed a 63% decrease in [^{3}H]-CP 55,940 (**3**) binding at 30 nM.

In 1996, a series of (aminoalkyl)indole isothiocyanates were reported as potential electrophilic affinity ligands (Yamada et al., 1996). Among them, compound **21** (Figure 8) showed to be a potent rCB_1R agonist (EC_{50} = 1.1 μM); equilibration of rat brain membranes with 1 μM concentration of this compound resulted

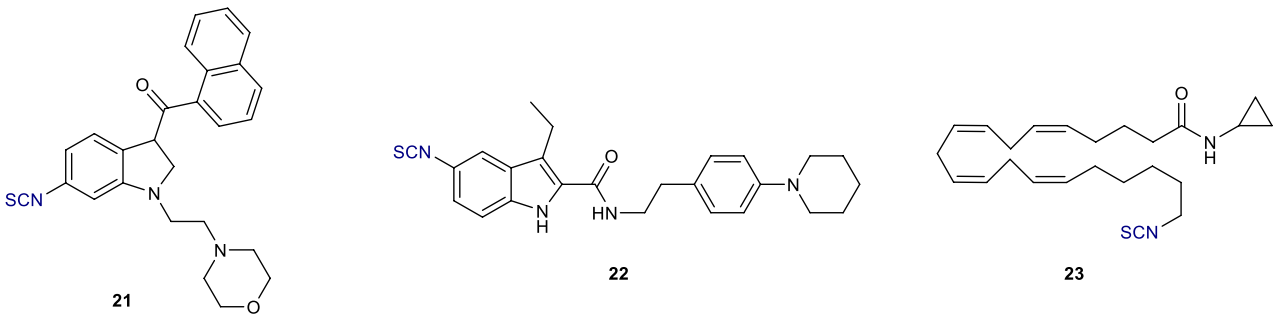

FIGURE 8
Structure of electrophilic probes **21**, GAT100 (**22**), and AM 3677 (**23**).

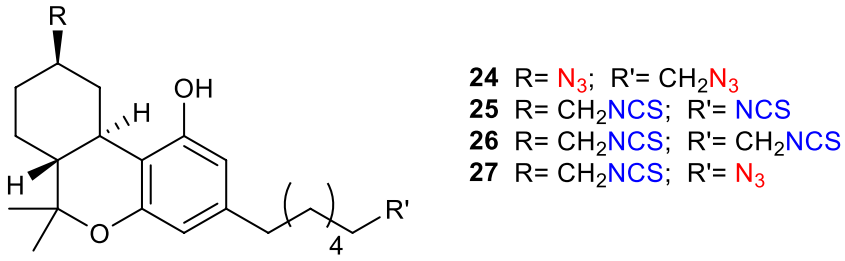

FIGURE 9
Structure of bifunctional probes AM859 (**24**), AM5823 (**25**), AM4099 (**26**) and AM5822 (**27**).

in a 70% loss of the specific binding of [^{3}H]CP-55,940. More recently, Kulkarni *et al.* described the synthesis of the first electrophilic ligands designed to bind irreversibly to the CB_1R allosteric site (Kulkarni et al., 2016). GAT100 **22** (Figure 8) emerged as the most potent negative allosteric modulator (NAM) without significant inverse agonist activity; preincubation of HEK-293 cells with 100 nM concentration of **22** increased the specific binding of [^{3}H]CP-55,940 at CB_1R by over 2-fold. This novel covalent probe can therefore serve as a useful tool to elucidate CB_1R allosteric ligand-binding motifs and to modulate the negative side effects of CB_1R activation.

Anandamide analogues with a reactive isothiocyanate functionality at the end of the hydrophobic tail were also reported as CB_1R electrophilic probes (Janero et al., 2015); between them, AM3677 **23** (Figure 8) exhibited high selectivity for rCB_1R with a Ki value of 1.3 nM. LAPS studies demonstrated that this compound reacts with a cysteine residue located in transmembrane helix 6 of h CB_1R, C6.47 (355), the same previously found in the ligand-binding profile of AM841 **18**. These data, therefore, confirmed the key role of this amino acid residue for receptor-ligand labelling in CB_1R.

2.3 Bifunctional probes

Bifunctional probes containing two electrophilic or two photoactivatable moieties (homobifunctional probes) or both of them (heterobifunctional probes) display the possibility to combine multiple imaging techniques creating a higher spatial resolution. AM859 **24** (Figure 9) features two azide functionalities and showed excellent affinity to CB_1R (Ki = 1.60 nM) despite being no selective between CB1- and CB2-receptors (Hamilton et al., 2021). Similarly, AM5823 **25** (Makriyannis, 2014) and AM4099 **26** (Zhou et al., 2017) are examples of homobifunctional probes containing two isothiocyanate groups, with the latter, reported by Zhou *et al.*, showing a high affinity for hCB_2R agonist, although no CB_1R data of this molecule have been reported. AM5822 **27** is instead a heterobifunctional ligand also containing an azide moiety for light activation (Makriyannis, 2014). Although most of these studies are preliminary, the potential of bifunctional probes to image the receptor at higher spatial resolution is becoming of growing interest.

3 Fluorescent probes

Fluorescently tagged small molecules have been widely used in the past decade as biological imaging tools as they offer the advantage to allow real-time monitoring of ligand-receptor interactions with high spatio-temporal precision (Briddon et al., 2011). Conjugation of a pharmacophore with a non-peptidic fluorescent tag can detrimentally change ligand-receptor affinity and therefore, a linker is generally required to separate the two entities (Stoddart et al., 2015). The composition and length of this linker (generally PEG or methylene chain), as well as the choice of the fluorophore, can also affect the physicochemical and

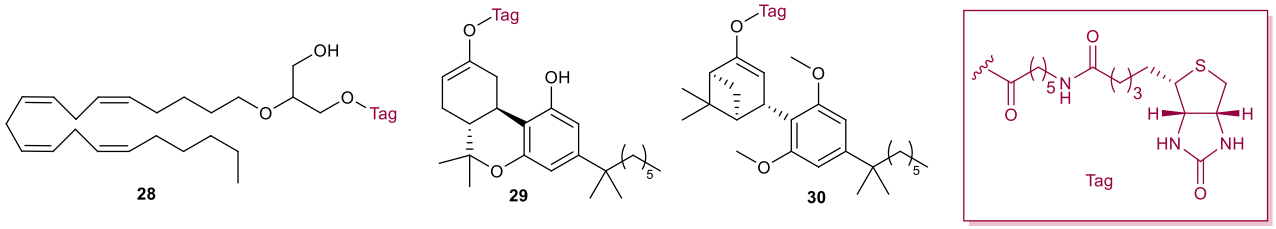

FIGURE 10
Structure of fluorescent probes **28-30**.

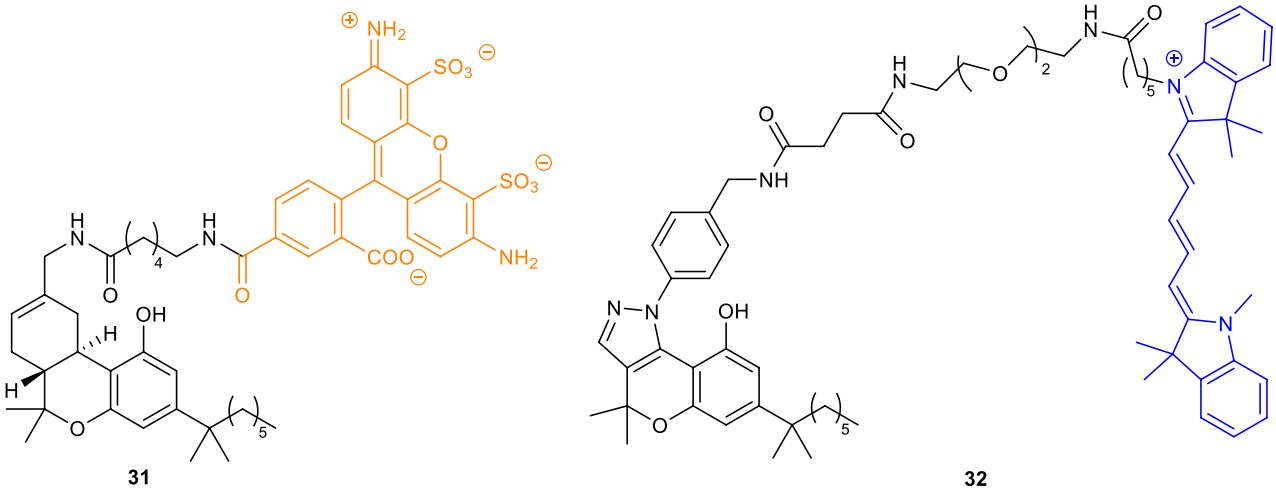

FIGURE 11
Structure of fluorescent probes **31-32**.

photophysical properties of the resulting fluorescent conjugate; therefore, the development of fluorescently labelled ligands is particularly challenging. Within recent years, a plethora of fluorescent ligands targeting CB_1R has been reported, mainly using biotin as target for the fluorophore. The addition of biotin to a ligand via a linker can, provide fluorescence, once the compound has docked with the target receptor, using fluorescent avidin conjugates. The biotinylated 2-AGE analogue **28** (Figure 10), showed moderate affinity to both human CB_1R and was selected for *in vitro* imaging of CB_1R (Martín-Couce et al., 2011). Biotinylated probes **29** and **30** (Figure 10), where the synthetic cannabinoid agonists HU210 and HU308, respectively, were conjugated to biotin via the free hydroxyl group, have also been successfully used for the visualization of CB_1R in neurons and in different immune cell (Martín-Couce et al., 2012). However, biotin probes require a two-step labelling process and additional steps to block endogenous biotin. This does not make them suitable for flow cytometry clinical routine or tissue staining.

To avoid these drawbacks, the cannabinoid agonist HU210 was coupled to the fluorescent tag Alexa Fluor 488 via a hexyl amide linker, generating the first fluorescent probe (**31**, Figure 11) with high affinity for CB_1R (Ki CB_1R = 27 nM) and selectivity over CB_2R (Martín-Fontecha et al., 2018). The use of this ligand as a chemical tool for the identification of functional CB_1R in human monocytes, T cells, and B cells was validated by multiplexed flow cytometry. This probe showed to be also suitable for the direct visualization of CB_1R in tonsil tissues, allowing the *in vivo* identification of tonsil CB_1R-expressing T and B cells.

Chromenopyrazole compounds, containing a similar THC structure, were also used as scaffolds for the conjugation with different fluorophores (BODIPY-630/650, BODIPY-FL, and Cy5) generating fluorescent ligands that have been shown to have high affinity to cannabinoid reports (Figure 11). However, these compounds (i.e., Cy-chromenylpyrazole **32**) showed higher affinity to CB_2R over CB_1R (pKi hCB_1R = 5.26; pKi hCB2R = 7.83) (Singh et al., 2019).

T117, a novel diarylpyrazole fluorescent ligand (**33**, Figure 12), was first reported by Daly *et al.* and derived by conjugation of the potent CB_1R inverse agonist AM251, with a fluorescent tetramethylrhodamine group (5-TAMA) (Davenport and Daly, 2010). In ligand binding studies, AM251 competed with [^{3}H]CP 55,940-labelled membranes to give a Ki of 0.8 nM. The addition of the 5-TAMA group significantly reduced the binding affinity of T117, providing only 10% displacement of [^{3}H]CP 55,940 at 1 μM.

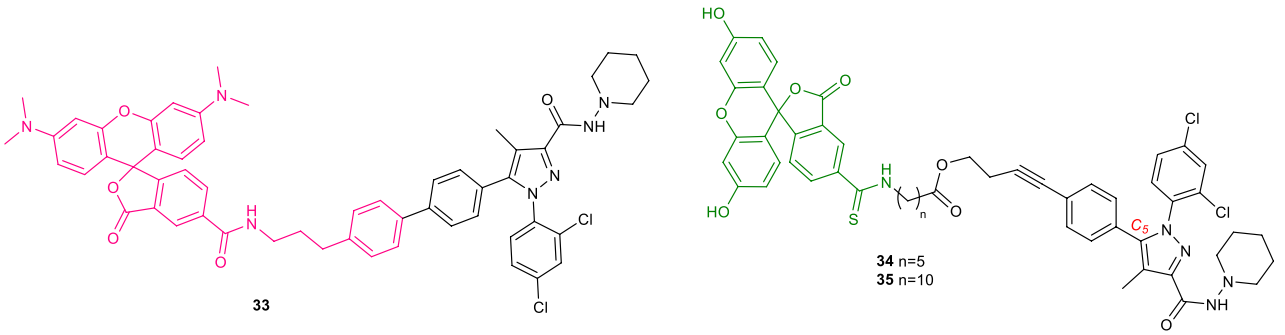

FIGURE 12
Structure of fluorescent probes **33-35**.

However, at a lower concentration (0.3 μM), binding of T1117 to wild-type (WT) mouse mesenteric artery was observed, using 543 nm excitation (590 nm emission). This ligand also displayed binding to cannabinoid-like (GPR55) receptors through Ca^{++} response in HEK-293 cells. Nevertheless, a conflicting study proved that T1117 binds endogenous and recombinant CB_1Rs with nanomolar affinity (Kd = 460 nM). Moreover, T1117 binding to CB_1R is sensitive to the allosteric ligand ORG27569 and thus it is applicable to the discovery of new allosteric drugs (Bruno et al., 2014). In 2009 a study carried out by Grant and co-workers focused on the CB_1R inverse agonist SR141716A, established the C5 position of its central pyrazole ring as the optimal site for fluorescent moiety linkage (Grant et al., 2019). Thus, CB_1R fluorescent probes (**34-35**, Figure 12) based on C5 conjugation of two SR141716A analogues with fluorescein isothiocyanate (FITC), were prepared. The affinity of the fluorescent probes **34** and **35** was then determined through radioligand competition binding assays at hCB_1R. The affinity of the 17-atom linker congener **35** (Ki = 2.1 μM) was modest; however, compound **34** bearing a 12-atom linker was found to display a useful level of affinity for CB_1R (K_i = 260 nM).

4 ^{18}F-labeled PET ligands

Positron emission tomography (PET) is a nuclear imaging technique that employs gamma rays to provide three-dimensional images that give information about the functioning of specific organs. PET is based on the detection of picomolar amounts of biological substances labeled with a short-lived positron-emitting radionuclide (tracer) sparing the biological system. This technique shows the advantage of being non-invasive, functional, and extremely sensitive (Li and Conti, 2010). Moreover, the PET probes have the same chemical structure as biomolecules and drugs, without altering their biological activity. ^{18}F has a short half-life (109.8 min), making it the ideal radionuclide for routine PET imaging (Damont et al., 2013). Thanks to its exceptional sensitivity, PET is well suitable to measure relatively low concentrations of enzymes and receptors also *in vivo* considering that most neuroreceptor populations in the human brain are expressed in a range between 10^{-8} and 10^{-12} M (Lopresti et al., 2023). The first selective CB_1R antagonist was rimonabant (**36**) (Rinaldi-Carmona et al., 1994), approved in Europe in 2006, to treat obesity but withdrawn from sale 2 years later by the European Medicines Agency (EMA) due to its manifest secondary effects and not being approved by the Food and Drug Administration (FDA). However, some of its analogs have been synthesized and labeled with [^{18}F] for PET imaging. Analogs of rimonabant showed activities on different and important biological targets, which makes them attractive for the development of new PET tracers (Gomes et al., 2020). In particular, two radiotracers [^{18}F]SR144385 (**37**) and [^{18}F]SR147963 (**38**, Figure 13) showed an appropriate regional brain distribution for cannabinoid receptors with a target ratio of 1.7 for [^{18}F]SR147963 and 2.5 for [^{18}F] SR144385 at 60 and 90 min post-injection, respectively (Mathews et al., 2000). Similar structures have been reported by Horti et al. in which the synthesis of two radiolabeled compounds named [^{18}F]NIDA-42033 (**39**) and its ethyl ester derivative **40** have been described (Figure 13) (Katoch-Rouse and Horti, 2003). The radiochemical yields were in the range of 1%–6% and sufficient quantities with specific radioactivity greater than 2,500 mCi = mmol and radiochemical purity >95%.

Another derivative named [^{18}F]-O1302 (**41**, Figure 14) having a short carbon chain at the para position of the phenyl group ending with a [^{18}F], showed high binding affinity (Ki 0.91 nM) and moderate lipophilicity after evaluation in mice (Mathews et al., 2000; Tobiishi et al., 2007).

The [^{18}F] isotope-labeled CB_1R inverse agonist DBPR211 (**42**, Figure 14) was synthesized, radiolabeled with halex exchange reaction, and analyzed for positron emission tomography scanning studies (Chang et al., 2019). After the purification, the compound was intravenously injected in mice showing a distribution percentage over 90-min scans among five regions of interest, including brain, heart, liver, thigh muscle, and kidney, lower than 1%, justifying itself as a peripherally CB_1R antagonist.

[^{18}F]MK-9470 (**43**, Figure 14) is a recent selective, high-affinity, inverse agonist (human IC_{50}, 0.7 nM) for the cannabinoid CB_1R developed for the imaging of the human brain (Burns et al., 2007). Autoradiographic studies in the rhesus monkey brain showed high specific binding in the cerebral cortex, cerebellum, caudate/putamen, hippocampus, substantia nigra, and globus pallidus. Positron emission tomography (PET) images in rhesus monkeys exhibited high brain uptake.

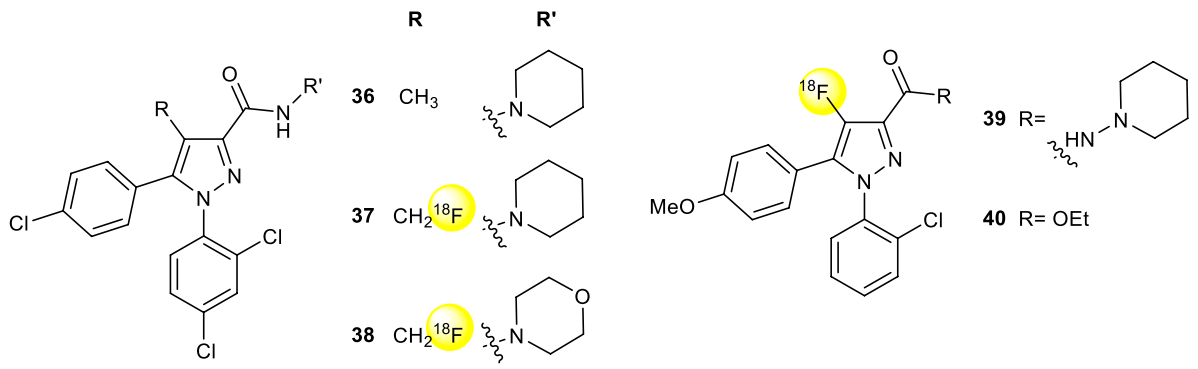

FIGURE 13
Structure of rimonabant (**36**) and ^{18}F radiolabeled pyrazole derivatives **37-40**.

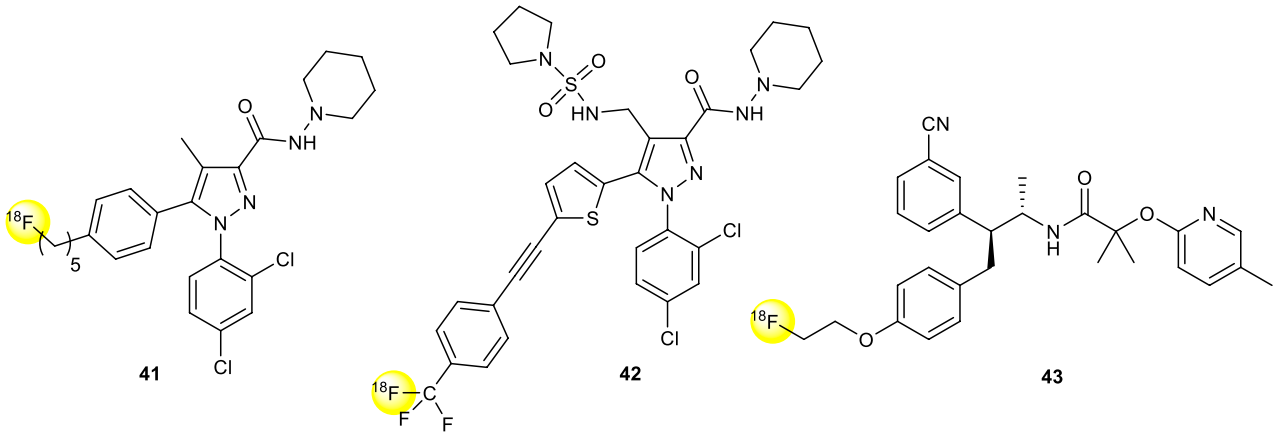

FIGURE 14
Structure of ^{18}F radiolabeled pyrazole derivatives **41-43**.

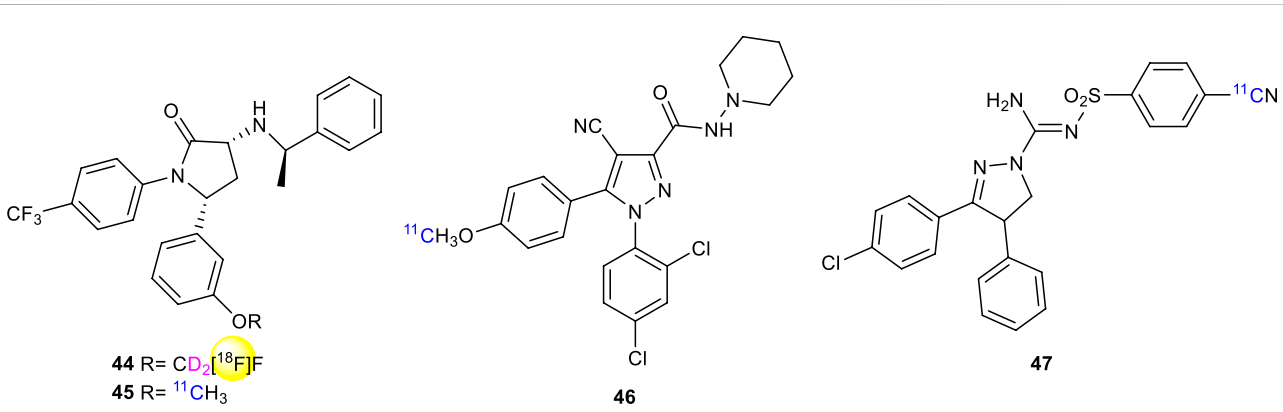

FIGURE 15
Structure of ^{18}F radiolabeled ligand **44** and ^{11}C radiolabeled ligands **45-47**.

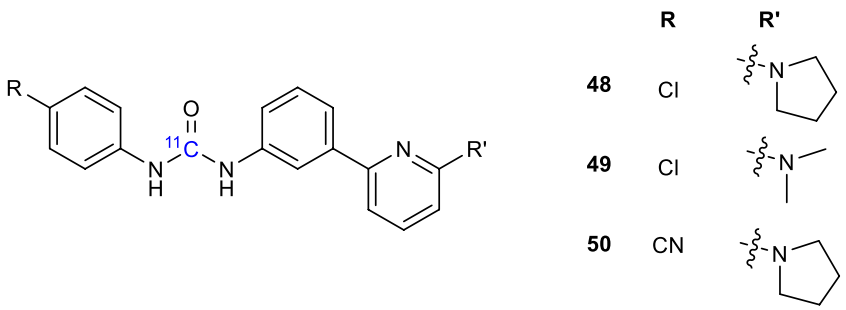

FIGURE 16
Structure of ^{11}C radiolabeled ligands **48-50**.

Donohue in 2008 (Donohue et al., 2008a) described the synthesis of [^{18}F]FMPEP-d_2 (**44**, Figure 15) having a superior performance of tracing compared with [^{11}C]MePPEP (**45**, Figure 15), due to greater precision and accuracy in detecting significant differences in CB_1R tracer uptake (Terry et al., 2010). **44** has been used to study abnormal levels of CB_1R binding in alcohol abuse (Hirvonen et al., 2013) or neurological disorders (e.g., schizophrenia) (Jenko et al., 2012). In preclinical and clinical studies [^{18}F]FMPEP-*d*2 has been used to image CB_1R expression in a mouse model of Alzheimer's disease (Takkinen et al., 2018).

5 ^{11}C-labeled PET ligands

^{11}C-labeled ligands offer certain advantages compared to ^{18}F-labeled compounds. The shorter half-life of ^{11}C enables more syntheses to be carried out in a shorter period of time, using the same hot-cell. Additionally, the lower radiation-absorbed doses permit more PET scans to be conducted on each subject. However, the shorter half-life of ^{11}C can create challenges in accurately quantifying radioligand kinetics or concentrations in plasma and the brain, especially when they are low. Today, ^{11}C-OMAR (**46**, Figure 15) represents the most studied and promising CB_1R radiolabeling agent for PET (Horti et al., 2006). In 2008 Donohue discovered and labeled 3,4-diarylpyrazoline derivatives as candidate radioligands for *in vivo* Imaging of Cannabinoid Subtype-1 using [^{11}C]cyanide ion as labeling agent and evaluated as PET radioligands in cynomolgus monkeys (Donohue et al., 2008b). Compound **47** ((−)-3-(4-chlorophenyl)-N′-[(4-cyanophenyl)sulfonyl]-4-phenyl-4,5-dihydro-1H-pyrazole-1-carboxamidine) ^{11}C-SD5024 (Figure 15) was found to get high-affinity and selectivity for binding to CB_1R. The same compound has been studied for the kinetics in humans and evaluated in seven healthy subjects with compartmental modeling (Tsujikawa et al., 2014). The compound showed a Ki = 0.47 nM at an intermediate level among the five CB_1R ligands and a lipophilicity of 3.79, which is appropriate for brain imaging together with a peak brain uptake of 1.5–3 standardized uptake value, slightly higher than that of ^{11}C-OMAR.

The synthesis of [^{11}C]MePPEP (**45**, Figure 15), a CB_1R mixed inverse agonist and antagonist, has been first reported in 2008 to ameliorate previous CB_1R ligands (Donohue et al., 2008b). The

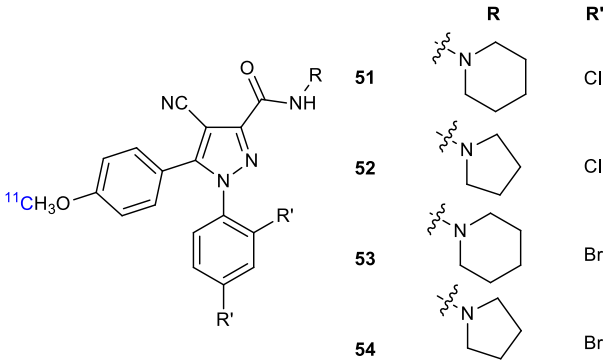

FIGURE 17
Structure of ^{11}C radiolabeled ligands **51-54**.

compound showed fairly high lipophilicity (LogD7.4 = 4.8) but still preserving high selectivity and affinity for the CB_1R. It readily entered the monkey brain within 20 min displaying stable measurements of distribution volume within 90 min (Yasuno et al., 2008).

PSNCBAM-1 (1-(4-Chlorophenyl)-3-(3-(6-(pyrrolidin-1-yl) pyridin-2-yl)phenyl)urea) (**48**, Figure 16) was developed as a potent allosteric antagonist for CB_1, able to reduce the appetite and body weight of rats. Other Several analogs were synthesized and radiolabeled using [^{11}C]$COCl_2$ and evaluated as PET ligands for CB_1R imaging using *in vitro* and *in vivo* techniques (**49-50**, Figure 16) (Yamasaki et al., 2017). In particular, compound **49** showed a strong binding affinity for peripheral CB_1R in an *in vitro* binding assay. PET imaging with showed considerable binding to peripheral CB_1R in the mouse brown adipose tissue (BAT), suggesting that **49** is a promising PET imaging agent for further evaluating pathophysiological and biological processes mediated by peripheral CB_1R.

Gao et al. reported an alternative synthetic route to PET CB_1R radioligands [^{11}C]OMAR analogs (**51-53**, Figure 17) that were prepared in high overall chemical yields (Gao et al., 2012). The radiosynthesis was employed at the oxygen position of the precursor the O-[^{11}C]methylation radiolabeling. Radiolabeling procedures

TABLE 1 Summary of the CB_1R binding of the probes reported in this review.

Ligand	Probe	CB_1R binding	Type
AM91 (**6**)	PA	Ki (rCB_1R) = 19 nM	pCB
(**7**)	PA	Ki (rCB_1R) = 0.4 nM	pCB
AM869 (**8**)	PA	Ki (rCB_1R) = 0.67 nM	pCB
AM1708 (**9**)	PA	Ki (rCB_1R) = 0.72 nM	pCB
(**10**)	PA	Kd (mCB_1R) = 5.60 P.m.; 9.38 p.m.	pCB
AM993 (**11**)	PA	Ki (rCB_1R) = 4.4 nM	pCB
AM3661 (**12**)	PA	Ki (rCB_1R) = 0.9 nM	eCB
(**13**)	PA	Ki (hCB_1R) = 0.9 μM	eCB
(**14**, **15**)	PA		eCB
AM708 (**16**)	EP	IC_{50} (rCB_1R) = 1.6 nM	pCB
(**17**)	EP	IC_{50} (rCB_1R) = 660 p.m.	pCB
AM841 (**18**)	EP	Ki (hCB_1R) = 9.05nM; EC_{50} = 0.94 nM	pCB
AM960 (**19**)	EP	IC_{50} (rCB_1R) = 25 nM	pCB
AM994 (**20**)	EP	Ki (rCB_1R) = 3.0 nM; EC_{50} = 0.8 nM	pCB
(**21**)	EP	EC_{50} (rCB_1R) = 1.1 μM	synth
GAT100 (**22**)	EP	EC_{50} (rCB_1R) = 2.09 nM	synth
AM3677 (**23**)	EP	Ki (hCB_1R) = 1.3 nM	eCB
AM859 (**24**)	BP	Ki (hCB_1R) = 1.60 nM	pCB
AM5823 (**25**)	BP		pCB
AM4099 (**26**)	BP	Ki (hCB_1R) = 12.6 nM	pCB
AM5822 (**27**)	BP		pCB
(**28**)	FP	Ki (hCB_1R) = 221 nM	eCB
(**29**)	FP	Ki (hCB_1R) = 2.4 nM	pCB
(**30**)	FP		pCB
(**31**)	FP	Ki (hCB_1R) = 27 nM	pCB
(**32**)	FP	Ki (hCB_1R) = 5.26 nM	pCB
T117 (**33**)	FP	Kd (hCB_1R) = 460 nM	pCB
(**34**)	FP	Ki (hCB_1R) = 260 nM	pCB
(**35**)	FP	Ki (hCB_1R) = 2.1 μM	pCB
(**36-38**)	PET	Ki (hCB_1R) = 5.6 nM; IC_{50} = 14 nM; IC_{50} = 120 nM	synth
(**41**)	PET	Ki (rCB_1R) = 0.9 nM	synth
(**43**)	PET	Ki (hCB_1R) = 2.2 nM; IC_{50} = 0.7 nM	synth
(**44, 45**)	PET	Kb (hCB_1R) = 0.574 nM	synth
(**46**)	PET	Ki (hCB_1R) = 11 nM	synth
(**47**)	PET	Ki (hCB_1R) = 0.47 nM	synth
(**48-50**)	PET	Ki (rCB_1R) = 0.7 μM; Ki (rCB_1R) = 14.4 μM	synth
(**51-53**)	PET	Ki (hCB_1R) = 11 nM; Ki (hCB_1R) = 2 nM; Ki (hCB_1R) = 4.7 nM	synth

PA, photoactivatable; EP, electrophilic; pCB, phytocannabinoids derivatives; eCB, endocannabinoids analogues; synth, synthetic cannabinoids derivatives; BP, bifunctional probes; FP, fluorescent probes; AG, 2-arachidonoylglycerol; PET, positron emission tomography.

incorporated efficiently [^{11}C]CH_3O with [^{11}C]CH_3OTf. The target tracers were isolated and purified in high radiochemical yields, short overall synthesis time, and high specific activity making them the potential preclinical and clinical PET agents in animals and humans.

6 Conclusion

In Table 1 are summarised the CB_1R binding of the probes reported in this review. As a result of the important repercussions in ECS modulation, targeting this system is currently one of the major trends in drug discovery. If we take in consideration CB_1R only, this receptor can be exploited to treat a variety of pathologies, including neurological disorders (i.e., Huntington's disease, multiple sclerosis and Alzheimer's disease) (McCaw et al., 2004; Pertwee, 2006; Pertwee, 2007; Liu et al., 2015), as well as peripheral disorders that involve energy metabolism, food intake, and obesity (DiPatrizio, 2021; Hijová, 2022). In addition to controlling liver and kidney function, it also controls bone remodeling, skeletal mass, and elongation under normal and pathophysiological conditions (Tam et al., 2018).

A significant amount of information has been accumulated in the literature concerning the *in vivo* and *in vitro* pharmacology of the CB_1R over the past decades, revealing new insights into pathways controlled and the roles of receptors, enzymes, and ligands. This knowledge has, however, made a complete transition into drug development in only a few cases (i.e., Sativex) (Namdar et al., 2020).

In order to develop novel therapeutic and diagnostic tools, it is necessary to understand the functions and molecular mechanisms associated with CB_1R modulation; to this aim, several molecular probes that utilize multiple interaction mechanisms (i.e., fluorescence, PET) have been developed in the past 30 years. These results demonstrate the great interest in this biological target: the development of new selective probes is therefore essential to obtaining new results that can lead to the introduction of new CB_1R-based drugs on the market.

Author contributions

Conceptualization, DC, VF, and DI; validation, DC, VF, and DI; formal analysis, AA; investigation, AA, VF, and DI; resources, AA, DC, VF, and DI; writing—original draft preparation, VF and DI; writing—review and editing, AA, DC, VF, and DI; supervision, AM, LP, and DP; project administration, DC, VF, and DI; funding acquisition, DC and AM. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors DC, DI, and VF declared that they were an editorial board member of Frontiers, at the time of submission. AM was employed by Plantachem SRL, at the time of submission. This had no impact on the peer review process and the final decision.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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