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Chapter 2

Chemical Tools and Strategies to Study the Cannabinoid Receptor Type 2
2.1 Introduction

The cannabinoid receptor type 2 (CB2R) is associated with a variety of diseases, such as cardiovascular, liver, kidney and neurodegenerative diseases, neuropathic pain, cancer, osteoporosis and inflammatory diseases, such as rheumatoid arthritis, Crohn’s disease and multiple sclerosis, and is therefore considered an important therapeutic target. However, despite compelling evidence of efficacy in preclinical settings, several CB2R-selective drug candidates failed in phase II clinical trials due to a lack in vivo efficacy (see Chapter 1). Although the reasons for this non-translatability are yet unclear, possible explanations may be 1) a lack of translational animal models for therapeutic efficacy, possibly due to interspecies signaling differences, 2) no proof of target engagement, or 3) the role of CB2R was not properly validated for the disease studied. It is essential that tools (both chemical and biological) used for CB2R target validation, target engagement or modulation have high selectivity and a well-defined mode of action, to enable translation of information from preclinical studies on non-human species to the patient.

The cellular expression profile of CB2R has been widely investigated with qRT-PCR (e.g. in brain or bladder) or antibodies, using immunohistochemical analysis. However, mRNA levels do not correlate necessarily with functional protein and the specificity of CB2R antibodies is under major debate, which limits the use of standard biochemical methods to study CB2R. In recombinant systems it is possible to express a tagged CB2R, e.g. with green fluorescent protein (GFP) or a peptide tag (e.g. Rho), to enable visualization or purification of CB2R, respectively. In native systems, however, it is a challenge to study the cellular expression profile of CB2R, because it is, like many GPCRs, expressed at very low levels in native cells and tissues. In addition, the continuous conformational changes of CB2R from inactive to active conformations and the internalization and desensitization upon prolonged activation may lead to variable surface expression of the receptor. Many research efforts have therefore focused on the development of chemical tools (or “probes”) and strategies that can be applied to study CB2R distribution, expression level, occupancy, internalization and pharmacology, both in vitro and in vivo. Chemical tools can be either radioligands, PET tracers, fluorescent or biotinylated small molecules or covalent probes, such as electrophilic or photo-activatable probes. This chapter provides an extensive summary of chemical tools reported for the CB2R, along with their structures and CBR affinity values (if reported).

2.2 Radioligands and PET tracers

Radioligands and positron emission tomography (PET) tracers are small molecules that contain radioisotopes, such as $^3$H, $^{11}$C, $^{14}$C, $^{18}$F, $^{35}$S, or $^{125}$I, which are detected and quantified by measuring their radioactive decay (emission of $\beta$-particles, positrons, or $\gamma$ radiation).
Radioligands are mostly used in vitro to determine the molecular pharmacology of unlabeled ligands, whereas some radioligands, but mostly PET tracers, are used in vivo to determine receptor expression in tissues and/or the distribution and receptor occupancy of drug candidates in patients. In case of CB₂R PET tracers, special interest is taken into the development of brain-penetrable PET tracers to study the role or expression level of CB₂R in neuroinflammation or neurological disorders in which the CB₂R is upregulated, such as Parkinson’s or Alzheimer’s disease, amyloid lateral sclerosis (ALS), and Multiple sclerosis.

2.2.1 CBR Radioligands
The first CBR radioligands were THC-based, carrying the tricyclic classical cannabinoid scaffold. These molecules were developed to determine elimination kinetics of THC in mice and to detect THC protein targets. Nowadays, these THC-based radioligands are not commonly used due to high nonspecific binding. In 1988, the development and application of the synthetic agonist [³H]CP55940 (Figure 1, Table 1), a ‘mixed’ (i.e. has equal affinity and activity for both CBRs) bicyclic non-classical cannabinoid, led to the discovery of the CB₁R, followed by the CB₂R in 1993. Currently, [³H]CP55940 is still the most common radioligand for CB₂R, together with [³H]WIN55212-2 (2), and is predominantly used in vitro to determine receptor expression (Bₘₐₓ), and the molecular pharmacology of novel ligands, such as equilibrium binding affinity, binding kinetics or receptor internalization. Two CB₂R-selective radioligands have been reported, [³⁵S]SCH225336 (3) and [³H]RO6957022 (4), both inverse agonists. Biodistribution of [³⁵S]SCH225336 was evaluated in healthy mice, indicating it may be a good candidate for in vivo imaging of CB₂R. In addition, [³⁵S]SCH225336 showed CB₂R-selective binding in human spleen sections.

Labeled endocannabinoids have been mostly used to study receptor and endogenous ligand trafficking, to image fatty acid amid hydrolase (FAAH), or to image endocannabinoid metabolism. These ligands are metabolically very unstable and have not been used to study CBR expression, distribution or function.

2.2.2 ‘Mixed’ CBR PET tracers
The first CBR PET tracer described was a THC-based, classic cannabinoid, [¹⁸F]-Δ⁸-THC (5) (Figure 2, Table 1).
This molecule was unable to show specific binding \textit{in vivo} and probably lacks receptor subtype selectivity, although this was not tested because this molecule was reported before the discovery of CB$_2$R.\textsuperscript{51} More recently, PET tracer $[^{11}\text{C}]$AZD1940 (6) was used as part of the preclinical evaluation of the mixed CBR agonist AZD1940 (a drug candidate for treatment of neuropathic pain) in monkeys and showed that AZD1940 was mostly peripherally restricted.\textsuperscript{59} However, AZD1940 failed to show efficacy in a capsaicin-induced pain model in healthy human volunteers.\textsuperscript{60}

2.2.3 CB$_2$R-selective PET tracers

The first CB$_2$R-selective PET tracer reported was the triaryl bis-sulfon $[^{11}\text{C}]$SCH225336 (7) (Figure 3, Table 1 (page 36)) by Leuven University,\textsuperscript{54} followed by the thiazole derivative $[^{11}\text{C}]$A-836339 (8) by the Horti research group.\textsuperscript{61, 62} However, these ligands lacked specificity for CB$_2$R,\textsuperscript{63} had poor \textit{in vivo} stability due to fast metabolization,\textsuperscript{54, 61, 62, 64, 65} and/or high generation of brain permeable radiometabolites.\textsuperscript{66} Attempts by the same research group to increase the specificity and metabolic stability of $[^{11}\text{C}]$A-836339 were only partly successful.\textsuperscript{67-69} Simultaneously, the more metabolically stable 2-oxoquinoline $[^{11}\text{C}]$NE40 tracer (9) was reported by Leuven University, and additionally used in healthy human subjects for \textit{in vivo} PET imaging of the CB$_2$R.\textsuperscript{70-73} This PET tracer was predominantly taken up by lymphoid tissue, which is in agreement with known expression of the CB$_2$R. It was hypothesized that $[^{11}\text{C}]$NE40 may be an important tool for studying CB$_2$R expression in pathophysiological conditions. However, when tested \textit{in vivo}, in patients suffering from Alzheimer’s disease (AD), no increased CB$_2$R expression was observed in AD patients compared to the healthy controls, which was in contrast to preclinical findings,\textsuperscript{74} thereby making CB$_2$R less promising as a biomarker in AD. $[^{11}\text{C}]$NE40 was also used in a rat photothrombotic stroke model,\textsuperscript{75, 76} in one study together with the ‘mixed’ CBR PET tracer $[^{18}\text{F}]$MK-9470 (10) (Figure 2, Table 1 (page 36)).\textsuperscript{76, 77} This study indicated a connection between CB$_1$R upregulation in stroke, but no role of CB$_2$R could be identified in this model. In contrast, another study did conclude that $[^{11}\text{C}]$NE40 is a suitable tool to detect CB$_2$R expression in early stages of brain disorders.\textsuperscript{75} The Leuven University group also developed PET tracers $[^{11}\text{C}]$MA2 (11) and $[^{18}\text{F}]$MA3 (12), which showed favorable biodistribution in healthy mice.\textsuperscript{78}
This molecule was unable to show specific binding in vivo and probably lacks receptor subtype selectivity, although this was not tested because this molecule was reported before the discovery of CB$_2$R.

More recently, PET tracer $[^{11}C]$AZD1940 (6) was used as part of the preclinical evaluation of the mixed CBR agonist AZD1940 (a drug candidate for treatment of neuropathic pain) in monkeys and showed that AZD1940 was mostly peripherally restricted.

However, AZD1940 failed to show efficacy in a capsaicin-induced pain model in healthy human volunteers.

Figure 2. Structures of 'mixed' CBR PET tracers.

### 2.2.3 CB$_2$R-selective PET tracers

The first CB$_2$R-selective PET tracer reported was the triaryl bis-sulfon $[^{11}C]$SCH225336 (7) (Figure 3, Table 1) by Leuven University, followed by the thiazole derivative $[^{11}C]$A-83639 (8) by the Horti research group. However, these ligands lacked specificity for CB$_2$R, had poor in vivo stability due to fast metabolization, and/or high generation of brain permeable radiometabolites.

Attempts by the same research group to increase the specificity and metabolic stability of $[^{11}C]$A-83639 were only partly successful.

Simultaneously, the more metabolically stable 2-oxoquinoline $[^{11}C]$NE40 tracer (9) was reported by Leuven University, and additionally used in healthy human subjects for in vivo PET imaging of the CB$_2$R.

This PET tracer was predominantly taken up by lymphoid tissue, which is in agreement with known expression of the CB$_2$R. It was hypothesized that $[^{11}C]$NE40 may be an important tool for studying CB$_2$R expression in pathophysiological conditions. However, when tested in vivo, in patients suffering from Alzheimer’s disease (AD), no increased CB$_2$R expression was observed in AD patients compared to the healthy controls, which was in contrast to preclinical findings, thereby making CB$_2$R less promising as a biomarker in AD. $[^{11}C]$NE40 was also used in a rat photothrombotic stroke model, in one study together with the 'mixed' CBR PET tracer $[^{18}F]$MK-9470 (10) (Figure 2, Table 1). This study indicated a connection between CB$_1$R upregulation in stroke, but no role of CB$_2$R could be identified in this model.

In contrast, another study did conclude that $[^{11}C]$NE40 is a suitable tool to detect CB$_2$R expression in early stages of brain disorders. The Leuven University group also developed PET tracers $[^{11}C]$MA2 (11): R = $[^{11}$CH$_3$ $[^{11}F]$MA3 (12): R = CH$_3$CH$_2$F $[^{11}C]$GW405833 (24): R = CH$_3$CH$_2$F $[^{11}C]$GW405833 (25): R = $[^{11}$CH$_3$ GW405833 (26): R = CH$_3$.

### Uncharacterized


$[^{11}C]$JL-015 (21): R$^1$, R$^2$ = Cyclohexane


### Inverse agonists

$[^{11}C]$Sch225336 (7) $[^{18}F]$CB91 (22): cis and trans

Figure 3. Structures of CB$_2$R-selective ligands and PET tracers. CB$_2$R-selective agonists, functionally unclassified ligands and inverse agonists.
PET tracers \[^{11}C\]KD2 (13) (Figure 3, Table 1 (page 36)) and \[^{18}F\]LV58 (structure not published) were used for CB\(_2\)R imaging in the neurological disorder Amyotrophic Lateral Sclerosis (ALS), in post-mortem spinal cord slices of ALS patients\(^{79}\) or animal models.\(^{44}\) Triazine-based radioligand \[^{18}F\]d2-3 (14) was used for PET detection of CB\(_2\)R expression in rhesus monkey and baboon models of neuroinflammation,\(^{80}\) whereas another triazine-based radioligand, \[^{18}F\]S (15), was developed to study in vivo distribution of a series of triazine-based CB\(_2\)R agonists.\(^{81}\) High CB\(_2\)R binding of pyridine- or oxoquinoline-based PET tracers \[^{11}C\]KD2 (13),\(^{82, 83}\) \[^{11}C\]RSR-056 (16),\(^{84, 85}\) \[^{11}C\]RS-016 (17),\(^{86, 87}\) \[^{18}F\]RS-126 (18),\(^{88, 89}\) and \[^{11}C\]KP23 (19),\(^{90}\) all from the Zürich University research group, was observed in the spleen of rodents. Furthermore, these PET tracers all exhibited similar patterns of biodistribution, low brain uptake, but high nonspecific binding to liver and small intestine.

Interestingly, in the case of \[^{11}C\]RSR-056 (16) and \[^{11}C\]RS-016 (17), an increase in brain radioligand uptake was observed in a neuroinflammatory mice model,\(^{84, 86}\) compared with healthy mice, indicating a higher CB\(_2\)R expression level in the brain induced by lipopolysaccharide (LPS, applied to induce neuroinflammation in this model). However, it should be noted that this may also be a result of increased brain permeability due to inflammation-induced disruption of the blood-brain barrier (BBB). \[^{11}C\]RS-016 (17), the result of structural optimization efforts from \[^{11}C\]KD2 (15), had higher specific CB\(_2\)R binding in post-mortem ALS patient spinal cord tissues.\(^{84}\) Recently, \[^{11}C\]RS-016 (17) showed increased uptake in atherosclerotic plaques in an atherosclerotic mouse model, indicating in vivo CB\(_2\)R targeting, but could not distinguish between a stable or an unstable vulnerable plaque. It is therefore unlikely to be a suitable PET tracer for diagnostic imaging unstable atherosclerotic plaques and thereby prevent sudden major problems such as a heart attack or stroke. Therefore, the authors hypothesized that \[^{11}C\]RS-016 (17) may be more suitable for CB\(_2\)R imaging in other CB\(_2\)R associated pathologies, e.g. neuroinflammation.\(^{91}\) In contrast, the same researchers reported thiophene-based PET tracers \[^{11}C\]AAT-015 (20) and \[^{11}C\]AAT-778 (21), which did not show any specific binding in spleen and were therefore not evaluated further.\(^{92}\)

Another series of oxoquinoline derived PET tracers was designed to be more metabolically stable, but suffered from poor uptake, low specificity and low solubility.\(^{93, 94}\) Both cis and trans isomers of a naphthyridin-based radioligand, \[^{18}F\]CB91 (22),\(^{95}\) showed biodistribution in spleen, gut, kidneys, pancreas and brown adipose tissue and accumulation in the liver, indicating high nonspecific binding and a rapid clearance by the hepatobiliary route.\(^{96}\) A series of triaryl CB\(_2\)R-selective PET tracers showed substantial nonspecific binding, possibly due to its high lipophilicity.\(^{97}\) A carbazole-based PET tracer, \[^{18}F\]1a (23), had high selectivity towards CB\(_2\)R over CB\(_1\)R, but suffered from rapid metabolism into a possibly toxic metabolite.\(^{66, 98}\) Recently, analogs of this PET tracer were synthesized to optimize the pharmacokinetics of this scaffold, leading to the discovery of a more metabolically stable analog, which may be synthesized as PET tracer in the future.\(^{99}\)
PET tracers $[^{18}F]FE$-GW405833 (24) and $[^{11}C]GW405833$ (25) (Figure 3, Table 1 (page 36)), were developed for brain CB$_2$R imaging, but are unlikely candidates for further imaging in neuroinflammatory conditions due to their relatively low binding affinity, slow washout and high nonspecific binding. A $[^{11}C]$-labeled analog (structure not published) of former clinical trial candidate GW842166X (27) was used for evaluation of the biodistribution of GW842166X in the brain, but no results have been published. The Leuven University researchers mention the synthesis of PET tracers, based on the tricyclic pyrazole Gp-1a (28), and that these were not further developed due to low binding affinity, but these results were never published.

Finally, synthesis of PET radioligands based on aminoalkyl indoles, propionamides, pyrazoles, and benzenesulfonamides have been reported, although without any biological data.

2.2.4 Summary of radioligands and PET tracers

Most of the reported CB$_2$R radioligands and PET tracers have the limitation that they show high nonspecific binding due to their high lipophilicity. In addition, most PET tracers are susceptible to metabolism or lack CB$_2$R selectivity, whereas high stability and receptor subtype selectivity are essential to avoid toxicity and radiation hazards. In addition, general limitations of radioligands are that they require specialized equipment and facilities to meet all the legal and safety requirements corresponding to radioactive handling, disposal, synthesis, and/or storage. Therefore, chemical tools with other detection elements, such as fluorophores or biotinylated molecules are developed to avoid the use of radioactive isotopes.

2.3 Fluorescent tools and strategies

Fluorescent small molecules have been widely used to study GPCR pharmacology, expression level and localization, for example using fluorescence confocal microscopy, flow cytometry or fluorescence correlation spectroscopy. In most cases, a fluorophore is attached to a known CBR scaffold with a linker. Fluorophores that exert some biological activity by themselves have been reported for other GPCRs, such as adenosine receptors, but not for CB$_2$R.

2.3.1 Mixed CBR fluorescent small molecules

The first cannabinoid-based fluorescent small molecules were dansyl derivatives of THC, described almost 50 years ago, used for analytical tracing of cannabinoids. However, these were not explicitly used to study CBRs, because they were developed and applied before their discovery (CB$_1$R in 1988 and CB$_2$R in 1993).
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Shortly after the discovery of CB₂R, synthesis of the endocannabinoid anandamide, linked with a benzo[dia]zole-based fluorophore, was reported,¹²⁰ as well as a total synthesis of a fluorescent endocannabinoid-based probe,¹²¹ albeit without biological evaluation. Nowadays, there are multiple fluorescently labeled endocannabinoids commercially available, although these compounds do not show CBR subtype selectivity and suffer from high nonspecific fluorescent background due to the high lipophilicity of endocannabinoids.

2.3.2 CB₂R-selective fluorescent small molecules and FRET

The need for CB₂R selectivity and improved physicochemical properties led to the rationale design of a fluorescent naphthoyl indole CB₂R-selective agonist based on JWH015 (29), NBD-JWH015 (30) (Figure 4, Table 1 (page 36)), using computational studies.¹²² However, this compound showed a significant loss of CB₂R affinity, as well as cytosolic accumulation and nonspecific binding. N-alkyl isatin acylhydrazone derivatives, discovered in 2008 to be highly potent CB₂R-selective agonists,¹²³ were synthetized with a nitro-benzo[dia]zole (NBD) fluorophore, leading to the development of NMP6 (31).¹²⁴ Confocal microscopy and flow cytometry studies using NMP6 showed specific CB₂R binding of NMP6 on CD4⁺ T cells and B lymphocytes, respectively, which could be prevented by the CB₂R-selective agonist GW842166X. However, no further studies with this compound have been reported. Time-resolved fluorescence resonance energy transfer (FRET) with a CB₂R-selective agonist indicated the presence of a second binding site in CB₂R and heteromerization of the receptor with GPR55.¹²⁵

![Figure 4. Structures of CB₂R-selective fluorescent agonists.](image)

2.3.3 CB₂R-selective near-infrared probes for in vivo imaging

For in vivo imaging, longer wavelength fluorophores such as those in the near-infrared (NIR) region (700–900 nm) and the NIR-II region (1000–1700 nm) may be better suited.¹²⁶ A decade ago, a SR144528 (32) derivative with a linker was reported, mbc94 (33) (Figure 5, Table 1 (page 36)), which did not display a loss in receptor subtype selectivity and CB₂R affinity.¹²⁷ The same research group reported several mbc94 derivatives in the following years, conjugated with a IRDye 800CW (“NIR-mbc94” (34)),¹²⁸ a NIR760 fluorophore (“NIR760-mbc94” (35)),¹²⁹ and a IR700DX fluorophore (“IR700DX-mbc94” (36)).¹³⁰
In addition, they reported a zwitterionic derivative of mbc94 (“ZW760-mbc94” (37)), a quinolone-based ligand (“NIR760-Q” (38)) and a pyrazolopyrimidine-based ligand (“NIR760-XLP6” (39)), all conjugated with a NIR760 fluorophore. These fluorescent tools were all designed for diagnostic imaging in tumor cells, to study the role of CB2R in cancer. NIR-mbc94 (34) successfully detected CB2R in intact CB2R-transfected mouse delayed brain tumor cells (CB2R-mid DBT cells), but displayed a high level of nonspecific binding in primary microglia cells.128 NIR760-mbc94 (35) was developed to enable in vivo application of these ligands, but also showed high nonspecific binding in CB2R-mid DBT cells and in a complete Freund’s adjuvant-induced inflammation mouse model.136 Because high nonspecific binding in Jurkat cells was also observed with NIR760-Q (38), the researchers hypothesized this was caused by the net negative charge of NIR760. To test this hypothesis, ZW760-mbc94 (37) was developed, which showed indeed a moderate improvement in CB2R-mid DBT cells, but still 50% nonspecific binding.131 Pyrazolopyrimidine derivative NIR760-XLP6 (39) showed less nonspecific binding in CB2R-mid DBT cells and selectivity over CB1R-mid DBT cells and may therefore be a suitable tool for in vivo imaging.133, 134 Finally, IR700DX-mbc94 (36), developed as a phototherapy agent, was reported to be CB2R-specific and effective only in CB2R-positive tumors, both in vitro and in vivo.130, 137 No further studies with NIR760-XLP6 (39) or IR700DX-mbc94 (36) have been reported to date, but this research group filed a patent on these molecules in 2013.138

**Near-infrared probes**

Figure 5. Structures of CB2R-selective near-infrared fluorescent probes.
2.3.4 Fluorescent visualization of CB₂R with ‘clickable’ or biotinylated probes

Indirect use of fluorescence has been applied to visualize or study CB₂R, using biotinylated 2-arachidonoylglycerol (“2-AGE-biotin” (40)) or 2-AG carrying a ‘clickable’ bioorthogonal ligation handle, such as an alkyne (“2-AGE-alkyne” (41)) (Figure 6, Table 1 (page 36)). These chemical tools have been developed to visualize CB₂R in a two-step process, by conjugation with a streptavidin-fluorophore or click reaction with an azide-fluorophore, respectively.¹³⁹ These molecules, however, lacked receptor subtype selectivity, were metabolically instable and showed high nonspecific binding, possibly due to their high lipophilicity. A biotinylated anandamide (AEA) (42) was not used to study CBRs, but instead used to study AEA accumulation.¹⁴⁰ Biotinylated tools were reported with the scaffold of the nonselective, THC-based HU210 (43) (“HU210-biotin” (44)) and CB₂R-selective, bicyclic HU308 (45) (“HU308-biotin” (46)).¹⁴¹ By conjugation with streptavidin-AlexaFluor488, followed by analysis with confocal microscopy, these probes were successfully applied to visualize endogenous CB₁R and CB₂R in neurons and microglia, respectively.¹⁴¹ More recently, HU210-biotin (44) was used to visualize CB₁R expression in immune cells from donors with allergic rhinitis, atopic dermatitis, or food allergies. In these experiments, endogenously biotinylated proteins and CB₂R were blocked using a biotin-blocking kit from Invitrogen and an excess of HU308 (45), respectively.¹⁴² To date, no further studies using these molecules have been reported.

Non-selective agonists

Endocannabinoids

2-AGE-biotin (40): R¹ = A
2-AGE-alkyne (41): R¹ = B
2-AEA-biotin (42): R² = A

Classical cannabinoids

HU210 (43): R³ = H
HU210-biotin (44): R³ = C
HU308 (45): R³ = H
HU308-biotin (46): R³ = C

CB₂R-selective agonists

Figure 6. Structures of ‘clickable’ or biotinylated CBR probes.

2.3.5 Summary of fluorescent tools and strategies

In general, fluorescent and biotinylated chemical tools have shown to interfere with receptor affinity and/or selectivity,¹²², ¹²⁸, ¹³⁹, ¹⁴¹ may increase nonspecific binding¹²², ¹²⁸, ¹²⁹, ¹³² and/or lead to a decrease in metabolic stability.¹³⁹ However, nonspecific membrane binding is probe specific and not a general feature of fluorescent ligands, as demonstrated by fluorescent ligands for other class A GPCRs that can be used to study GPCRs in native cell environments.¹¹³
Another drawback is that fluorescent and biotinylated probes rely on non-covalent interactions with the receptor, which can be easily disrupted by various experimental conditions. The latter can be avoided by using chemical tools that form a covalent bond with the receptor.

### 2.4 Covalent chemical tools

Chemical tools that form a covalent bond with GPCRs can be either photoactivatable or electrophilic, which ideally react specifically with one or more amino acid side chains located at or near the ligand binding site. Photoactivatable ligands, also called photoaffinity probes, possess a photoreactive moiety, such as an azide, diazirine or benzophenone. Upon irradiation with a specific wavelength, these moieties form a reactive nitrone, carbene or biradical, respectively, which can form a covalent bond with a nearby amino acid. Electrophilic ligands, also called covalent probes, usually contain a reactive electrophilic functional group that can react with a nucleophilic amino acid side chain to form a covalent bond. Although covalent chemical tools for GPCRs are mostly used for receptor binding site mapping, some covalent tools have aided in the isolation and purification of several class A GPCR subtypes and/or improved receptor stability for GPCR X-ray crystal structure determination. In case of CB2R, the most photoaffinity or covalent probes have been developed by the Makriyannis group (chemical tools starting with “AM”), to study the ligand binding site. In addition to probes with an electrophilic or a photoactivatable moiety, probes that contain two electrophilic or photoactivatable moieties, or one of both, have also been reported, which are referred to as bifunctional probes in literature.

#### 2.4.1 Electrophilic probes

The reactive moieties used in covalent probes mostly include isothiocyanates, halomethylketones, reactive thiols, Michael acceptors, and nitrogen mustards, although the isothiocyanate (NCS) is the most popular choice because it is easily synthesized from primary amines, is stable in water, but reactive with several nucleophilic amino acid residues. The first electrophilic chemical tools reported by the Makriyannis group were THC derivatives with an NCS moiety: AM708 and AM960 (Figure 7, Table 1). Although these molecules do not show selectivity between CBR subtypes, they were only evaluated at the CB1R and no data on CB2R has been reported, which is also the case for methyl arachidonoyl fluorophosphanate (MAFP), a covalent endocannabinoid, and an electrophilic aminoalkylindole derivative based on JWH200 ("JWH200-NCS" (151)). Other covalent tools, based on the endocannabinoid arachidonoyl anandamide, AM3661 and AM3677, were reported to be CB2R-selective and therefore not used for CB2R studies, despite their moderate CB2R affinity of ~50 nM.
More recently, electrophilic THC derivatives AM841 (54) and AM994 (55) were developed.\textsuperscript{162, 163} Although AM841 lacked receptor subtype selectivity, it has been used to map the CB\textsubscript{2}R binding site in combination with site-directed mutagenesis,\textsuperscript{164, 165} to investigate the (patho)physiological role of CB\textsubscript{2}R in inflammatory bowel disease in a mouse model,\textsuperscript{166} or to examine gastrointestinal motility in healthy and stressed mice.\textsuperscript{167} Further exploration of AM841 (54) derivatives led to the design of AM4073 (56), which carries the electrophilic NCS group at the tricyclic ring and also showed irreversible binding to CB\textsubscript{2}R’s active site. Although AM4073 (56) is based on AM841 (54), and therefore probably nonselective, no CB\textsubscript{1}R data of this molecule has been reported.\textsuperscript{150} A CB\textsubscript{2}R-selective covalent chemical tool, the diarylpyrazole AM1336 (57), was successfully used for CB\textsubscript{2}R binding site mapping, combined with site-directed mutagenesis.\textsuperscript{168, 169} In vivo use of covalent chemical tools has not been widely reported, but can be risky due to irreversible binding and/or (increased) off-target interactions due to high reactivity of the electrophile.

**Mixed CBR agonists**

**Classical cannabinoids**

![Chemical structures of AM708 (47), AM960 (48), AM841 (54), AM994 (55), and AM4073 (56).](image)

**Endocannabinoids**

![Chemical structures of MAFP (49), AM3661 (52): R\textsuperscript{1} = N\textsubscript{3}, AM3677 (53): R\textsuperscript{1} = NCS, JWH200 (50): R\textsuperscript{1} = H, JWH200-NCS (51): R\textsuperscript{1} = NCS.](image)

**Aminoalkylindoles**

**CB\textsubscript{2}R-selective inverse agonists**

**Diarylpyrazoles**

![Chemical structure of AM1336 (57).](image)

*Figure 7. Structures of electrophilic CBR probes*
2.4.2 Photoaffinity probes

Similar as to the first electrophilic chemical tools developed, the first photoaffinity probes were THC-based, which carried an azide as the photoactivatable moiety (“AM91” (58), Figure 8, Table 1 (page 36)). The radiolabeled derivative (59) was used to detect the CB1R in mouse brain preparations. AM91 was not used to study CB2R, because it was developed before its discovery, although it did provide the first evidence of a second cannabinoid receptor, which was later confirmed to be CB2R. More recently, the THC-based photoaffinity probe AM993 (60) was reported, in the same article as AM994 (55) (electrophilic probe mentioned above). Both probes underwent a covalent interaction with both CBRs, but no further applications of these molecules have been reported. In addition, several other CBR nonselective photoaffinity probes have been reported without biological data, such as an analog of AM91 with improved affinity (“Dimethylheptyl-AM91” (61)), a photoaffinity analog of the electrophilic chemical tool AM708 (“AM836” (62)), anandamide-based photoaffinity probes (63-64), and a iodinated (“AM869” (65)) and radioiodinated probe (“AM1708” (66)).

The first CB2R-selective photoaffinity probes, with a THC-based scaffold and a benzophenone as the photoreactive moiety, were reported in 2012. From this series, AM967 (67) showed the highest CB2R labeling efficiency, as established using radioligand displacement assays with AM967-pretreated membranes.

Mixed CBR agonists

![Mixed CBR agonists](image)

CB2R-selective agonists

![CB2R-selective agonists](image)

Figure 8. Structures of CBR photoaffinity probes. Mixed CBR agonists (top) and CB2R-selective agonists (bottom).
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2.4.3 ‘Bifunctional’ probes

Bifunctional probes that contain two electrophilic or photoactivatable moieties (‘homobifunctional’ probes) are AM4099 (68) (reported together with AM4073 (56), discussed above150) or AM859 (69) (two azide groups).151 The only ‘heterobifunctional’ CB2R probe reported is AM5822 (70), which contains both a photoactivatable group and a electrophilic moiety (NCS and azide group) (Figure 9, Table 1 (page 36)).152 From these molecules, only AM4099 (68) was shown to irreversibly bind to CB2R’s active site.

Although AM4099 (68) is based on AM841 (55), and therefore probably nonselective, no CB1R data of this molecule has been reported.150 However, because these probes do not possess the functionality for direct use in imaging studies, it can be argued that the term “bifunctional probe” may be better suited for chemical tools that are able to report on two different biological processes,174 or combine the covalent addition to the protein’s active site with detection.

Mixed CBR agonists

Classical cannabinoids

AM4099 (68): R1 = CH2NCS, R2 = NCS
AM859 (69): R1 = N2, R2 = N3
AM5822 (70): R1 = CH2NCS, R2 = N3

Figure 9. Structure of ‘bifunctional’ probes.

2.4.4 Summary of electrophilic and photoaffinity probes

In summary, covalent small molecules such as electrophilic and photoactivatable probes can be powerful tools to map the CB2R binding site and specific amino acid residues that interact with cannabinoid ligands of interest, when used in conjunction with mutation studies.163, 173 The covalent tools discussed here have been predominantly used in in vitro studies, because in vivo studies are risky due to irreversible binding or off-target interactions due to high reactivity of the electrophile.175 A drawback from these covalent tools is that these do not possess a detection element, such as a fluorophore or biotin tag. However, fluorescent and biotinylated tools have shown to suffer from low receptor affinity and/or selectivity, or high nonspecific binding, as discussed above. These problems can be avoided by electrophilic or photoaffinity probes with a biorthogonal ligation handle, to introduce the detection element after covalent addition of the molecule to the protein’s active site. This technique is called two-step (photo)affinity-based protein profiling (AfpBPP and pAfpBPP, respectively).


2.5 Two-step (photo)affinity-based protein profiling

In two-step (photo)affinity labeling, the detection element, such as a fluorophore or biotin tag, is introduced after the covalent addition of the probe to a protein,\textsuperscript{144} using a biorthogonal ligation handle (e.g. alkyne or azide). This technique is widely used to visualize small molecule-protein interactions in living systems.\textsuperscript{15, 135, 176, 177} The advantage of this technique, in contrast to chemical tools with a covalent moiety but without a detection element, is that such probes can be used to study CB\textsubscript{2}R expression and ligand engagement independent from the use of peptide sequencing or antibodies, which are generally very nonspecific for CB\textsubscript{2}R. No photoaffinity probes with a ligation handle have been reported for CB\textsubscript{2}R to date, but a two-step electrophilic probe with an alkyne moiety (“AM9017” (71), Figure 10, Table 1 (page 36)) was mentioned in a perspective,\textsuperscript{152} albeit without any biological data.

![AM9017](image)

**Figure 10. Structure of an electrophilic probe with ligation handle**

An advantage of photoaffinity probes in contrast to electrophilic covalent tools is that photoaffinity probes do not rely on a nucleophilic residue nearby, which enables this technique to not only be used to validate target engagement of lead compounds with known (or hypothesized) protein targets, but also to identify unknown protein targets (i.e. target identification) of biologically active compounds.\textsuperscript{176-178} In case of photoaffinity probes, this strategy is termed two-step photoaffinity-based protein profiling (pA\textsubscript{f}BPP, Figure 11).
Figure 11. Two-step photoaffinity labeling for target engagement and target identification. Proteomes or live cells are incubated with the compound of interest or vehicle and subsequently with the photoaffinity probe. UV irradiation then results in crosslinking the probe to target proteins, which, after ligation with an affinity reporter tag (e.g. biotin), can be isolated and identified using LC/MS and proteomics. Ligation of proteomes or whole cells with a fluorophore reporter tag enables visualization using SDS-PAGE and FACS analysis, respectively.

<table>
<thead>
<tr>
<th>Nr.</th>
<th>Name</th>
<th>CB1R</th>
<th>CB2R</th>
<th>Functionality</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>[3H]CP55940</td>
<td>Kᵦ = 0.133 nM (r)⁵⁵</td>
<td>Kᵦ = 2.55 ± 0.19 nM (h)⁹</td>
<td>Agonist</td>
</tr>
<tr>
<td>2</td>
<td>[3H]WIN55212-2</td>
<td>Kᵦ = 0.54 ± 0.07 nM (h)⁷⁹</td>
<td>Kᵦ = 2.1 ± 0.2 nM (h)⁴⁵</td>
<td>Agonist</td>
</tr>
<tr>
<td>3</td>
<td>[3H]SC225336</td>
<td>Kᵦ = 15.0 ± 4.12 nM (r)⁹¹</td>
<td>Kᵦ = 11.9 ± 1.9 nM (h)⁴²</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>[3H]RO657022</td>
<td>NR</td>
<td>NR</td>
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</table>

**PET tracers**

<table>
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<th>CB2R</th>
<th>Functionality</th>
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</thead>
<tbody>
<tr>
<td>5</td>
<td>[18F]D-THC³⁴</td>
<td>NR</td>
<td>NR</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>[11C]AZD1940</td>
<td>Kᵦ = 11.75 nM (h)⁶⁵</td>
<td>Kᵦ = 0.87 nM (h)⁵⁹</td>
<td>Agonist</td>
</tr>
<tr>
<td>7</td>
<td>[11C]SC225336</td>
<td>Kᵦ = 78.5 ± 10.4 nM (h)⁴</td>
<td>Kᵦ = 4.54 ± 0.48 nM (h)⁴⁴</td>
<td>Inverse agonist</td>
</tr>
<tr>
<td>8</td>
<td>[11C]JA-83633⁴</td>
<td>NR</td>
<td>NR</td>
<td></td>
</tr>
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<td>9</td>
<td>[11C]NE40</td>
<td>Kᵦ &gt;1000 nM (h)⁷¹</td>
<td>Kᵦ = 9.6 nM (h)⁴¹</td>
<td>Inverse agonist</td>
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<tr>
<td>10</td>
<td>[18F]MK-9470</td>
<td>IC₅₀ = 0.7 nM (h)⁷⁷</td>
<td>IC₅₀ = 44 nM (h)⁷⁷</td>
<td>Inverse agonist</td>
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<tr>
<td>11</td>
<td>[11C]MA2</td>
<td>Kᵦ = 1611 nM (h)⁷⁴</td>
<td>Kᵦ = 87 nM (h)⁷⁶</td>
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<td>12</td>
<td>[18F]MA3</td>
<td>Kᵦ = 102 nM (h)⁷⁶</td>
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<td>13</td>
<td>[11C]K202</td>
<td>Kᵦ &gt;10000 nM (h)⁸⁵</td>
<td>Kᵦ = 1.7 ± 2.0 nM (h)⁸²</td>
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<tr>
<td>14</td>
<td>[18F]J2-3</td>
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<td>IC₅₀ = 1.4 nM (h)⁸⁰</td>
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<td>15</td>
<td>[11C]J5</td>
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<tr>
<td>16</td>
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<tr>
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<td>[11C]RS-016</td>
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<td>[18F]J2-126</td>
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<td>Kᵦ = 1.2 ± 0.8 nM (h)⁸³</td>
<td>NR</td>
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<tr>
<td>19</td>
<td>[11C]KP23</td>
<td>Kᵦ &gt;10000 nM (h)⁸³</td>
<td>Kᵦ = 6.8 ± 5.8 nM (h)⁸⁰</td>
<td>NR</td>
</tr>
</tbody>
</table>

NR = not reported; Kᵦ apparent Kᵦ; m = mouse; r = rat; h = human
### Affinity or activity (species)

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<tr>
<th>Nr.</th>
<th>Name</th>
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<th>CB,R</th>
<th>Functionality</th>
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<td>20</td>
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<td>K_i = 3.3 ± 0.5 nM^{51}</td>
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<td>21</td>
<td>[11C]JAT-778</td>
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<td>K_i = 4.3 ± 0.7 nM^{52}</td>
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<td>22</td>
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<td>K_i = 300 nM (h)^{95}</td>
<td>K_i = 9.0 nM (h)^{95}</td>
<td>NR</td>
</tr>
<tr>
<td></td>
<td>Trans: K_i = 200 nM (h)</td>
<td>Trans: K_i = 0.7 nM (h)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>[18F]1a</td>
<td>K_i &gt; 1000 nM (h)^{66}</td>
<td>K_i = 2.32 ± 2.12 nM (h)^{66}</td>
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<td>[18F]GE-GW405833</td>
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<td>K_i = 27 nM (h)^{100}</td>
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<td>[18C]GW405833^{101}</td>
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<td>NR</td>
<td>NR</td>
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<td>26</td>
<td>GW424166X</td>
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<td>E_C50 = 63 nM (h)^{104}</td>
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<td>27</td>
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<td>E_inh = 95%</td>
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<td>28</td>
<td>Gp-1a</td>
<td>pK_i = 6.97 ± 0.35 (h)^{112}</td>
<td>pK_i = 7.66 ± 0.11 (h)^{12}</td>
<td>Inverse agonist</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pK_i = 6.37 ± 0.08 (m)</td>
<td>pK_i = 7.68 ± 0.23 (m)</td>
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<td><strong>Fluorescent tools</strong></td>
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<td>JWH015</td>
<td>pK_i = 6.47 ± 0.09 (h)^{112}</td>
<td>pK_i = 7.92 ± 0.26 (h)^{12}</td>
<td>Agonist</td>
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<tr>
<td></td>
<td></td>
<td>pK_i = 5.94 ± 0.15 (m)</td>
<td>pK_i = 6.63 ± 0.21 (m)</td>
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<td>NBD-JWH015</td>
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<td>NMCP6</td>
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<td>pK_i = 7.88 ± 0.06 (h)^{12}</td>
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</tr>
<tr>
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<td></td>
<td>pK_i = 6.92 ± 0.20 (m)</td>
<td>pK_i = 10.16 ± 0.16 (m)</td>
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<td><strong>Near-infrared probes</strong></td>
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<tr>
<td>33</td>
<td>mbc94^{127}</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
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<td>NiR-mbc94</td>
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<td>35</td>
<td>NiR760-mbc94</td>
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<td>IR700DX-mbc94</td>
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<td>37</td>
<td>ZW760-mbc94</td>
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<td>NiR760-XLP6</td>
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<td>K_i = 169.1 ± 66.09 nM (m)^{134}</td>
<td>NR</td>
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<td><strong>Biotinylated or 'clickable' probes</strong></td>
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<td>40</td>
<td>2-AGE-biotin</td>
<td>K_i = 221 ± 8 nM (h)^{190}</td>
<td>K_i = 450 ± 11 nM (h)</td>
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<td>41</td>
<td>2-AGE-alkyne</td>
<td>K_i = 84.7 ± 0.8 nM (h)^{190}</td>
<td>K_i = 84.9 ± 0.6 nM (h)^{190}</td>
<td>NR</td>
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<td>42</td>
<td>AEA-biotin</td>
<td>iC_50 = 5000 ± 700 nM (h)^{140}</td>
<td>iC_50 &gt; 10000 nM (h)^{140}</td>
<td>NR</td>
</tr>
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<td>HU210</td>
<td>pK_i = 9.55 ± 0.06 (h)^{12}</td>
<td>pK_i = 9.78 ± 0.04 (h)^{12}</td>
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<tr>
<td></td>
<td></td>
<td>pK_i = 8.96 ± 0.27 (m)</td>
<td>pK_i = 9.27 ± 0.38 (m)</td>
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<td>HU210-biotin</td>
<td>K_i = 2.4 ± 0.4 nM (h)^{141}</td>
<td>K_i = 1.6 ± 0.4 nM (h)^{141}</td>
<td>NR</td>
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<td>HU308</td>
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<td>pK_i = 7.44 ± 0.12 (h)^{13}</td>
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<td>pK_i = 6.08 ± 0.14 (m)</td>
<td>pK_i = 7.15 ± 0.21 (m)</td>
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<td>46</td>
<td>HU308-biotin</td>
<td>K_i &gt; 5000 nM (h)^{141}</td>
<td>K_i = 44 ± 4 nM (h)^{141}</td>
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<td><strong>Electrophilic probes</strong></td>
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<td>47</td>
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<td>NR</td>
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<td>49</td>
<td>MAPF</td>
<td>IC_50 = 20 nM (r)^{155}</td>
<td></td>
<td>NR</td>
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<td>K_i = 29 nM (r)^{140}</td>
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<td>52</td>
<td>AM3661</td>
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<td>53</td>
<td>AM3677</td>
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<td>K_i = 48.5 ± 13 nM (m)^{161}</td>
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<td>54</td>
<td>AM841</td>
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<tr>
<td>55</td>
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<td>K_i = 3.0 nM (r)^{155}</td>
<td>K_i = 34.6 nM (m)^{155}</td>
<td>CB_i: agonist</td>
</tr>
<tr>
<td></td>
<td></td>
<td>K_i = 10.3 nM (h)</td>
<td></td>
<td>CB_i: inverse agonist</td>
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<tr>
<td>56</td>
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<td>K_i = 0.54 nM (h)^{144}</td>
<td>Inverse agonist</td>
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NR = not reported; K_i*: apparent K_i; m = mouse; r = rat; h = human

---

Chapter 2

### Chemical Tools and Strategies to Study the Cannabinoid Receptor Type 2

---
Chapter 2

### Affinity or activity (species)

<table>
<thead>
<tr>
<th>Nr.</th>
<th>Name</th>
<th>CB₂R Kᵢ (nM)</th>
<th>CB₂R Kᵢ (nM)</th>
<th>Functionality</th>
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<tbody>
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<td>19 ± 6</td>
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</tr>
<tr>
<td>59</td>
<td>[125]AM91</td>
<td>5.6 ± 9.38</td>
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<td>60</td>
<td>AM993</td>
<td>4.4</td>
<td>9.6</td>
<td>CB₂: agonist</td>
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<td>Dimethylethyl-AM91</td>
<td>0.4</td>
<td>NR</td>
<td>NR</td>
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<td>62</td>
<td>AM836</td>
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<td>AEA-diazirine (n=1)</td>
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<td>2.65</td>
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*NR = not reported; Kᵢ = apparent Kᵢ; m = mouse; r = rat; h = human*

#### 2.6 Overall summary and conclusions

To conclude, the aim of this chapter was to summarize the chemical tools for the cannabinoid receptor type 2 (CB₂R) that have been published to date: radioligands, PET tracers, fluorescent and near-infrared molecules, biotinylated tools and covalent probes, such as electrophilic and photoaffinity probes. Whereas the reported radioligands have been used predominantly in vitro to determine the molecular pharmacology of unlabeled ligands, several PET tracers have been reported to study the role or expression level of CB₂R in several inflammatory or neurological disorders in vivo. However, most of the reported CB₂R radioligands and PET tracers displayed low metabolic stability, lack CB₂R selectivity and/or specificity, or were metabolized to toxic radiometabolites. To avoid these limitations and other problems associated with radioactive isotopes, chemical tools with other detection elements, such as fluorophores, or biotinylated molecules have been developed. Fluorescent and biotinylated small molecules have been especially used to study CB₂R pharmacology, expression level and localization, but these chemical tools also showed significant losses in receptor affinity and/or selectivity, increased nonspecific binding and/or decreased metabolic stability. In addition, all radioactive, fluorescent and biotinylated probes for CB₂R rely on non-covalent interactions with the receptor, which can be easily disrupted by various experimental conditions. To avoid this, several electrophilic or photoactivatable probes have been developed for CB₂R. These probes undergo a covalent interaction with the receptor and have been mostly used to map the CB₂R binding site and specific amino acid residues that interact with cannabinoid ligands of interest. However, a drawback from these covalent tools is that these do not possess a detection element, which is necessary to study CB₂R expression and ligand engagement. A technique that combines the covalent addition to the receptor’s active site with a detection element, but avoids the problems associated with large fluorescent or biotin tags or radioactive isotopes, is two-step photoaffinity-based protein profiling (pA₃-BPP).
This technique has been widely used to visualize small molecule-(soluble)protein interactions in living systems and is therefore likely to be the most suitable chemical strategy to study CB$_2$R expression and ligand engagement.

References


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