

Review

Rational Design, Synthesis, and Evaluation of Fluorescent CB₂ Receptor Ligands for Live-Cell Imaging: A Comprehensive Review

Pinaki Bhattacharjee  and Malliga R. Iyer * 

Section on Medicinal Chemistry, National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, 5625 Fishers Lane, Rockville, MD 20852, USA

* Correspondence: malliga.iyer@nih.gov

Abstract: The cannabinoid receptors CB₁ and CB₂ are class A G protein-coupled receptors (GPCRs) that are activated via endogenous lipids called endocannabinoids. The endocannabinoid system (ECS) plays a critical role in the regulation of several physiological states and a wide range of diseases. In recent years, drug discovery approaches targeting the cannabinoid type 2 receptor (CB₂R) have gained prominence. Particular attention has been given to selective agonists targeting the CB₂ receptors to circumvent the neuropsychotropic side effects associated with CB₁ receptors. The pharmacological modulation of CB₂R holds therapeutic promise for various diseases, such as inflammatory disorders and immunological conditions, as well as pain management and cancer treatment. Recently, the utilization of fluorescent probes has emerged as a valuable technique for investigating the interactions between ligands and proteins at an exceptional level of spatial and temporal precision. In this review, we aim to examine the progress made in the development of fluorescent probes targeting CB₂ receptors and highlight their significance in facilitating the successful clinical translation of CB₂R-based therapies.

Keywords: endocannabinoid system; cannabinoid type 2 receptor; fluorescent probe; disease



Citation: Bhattacharjee, P.; Iyer, M.R. Rational Design, Synthesis, and Evaluation of Fluorescent CB₂ Receptor Ligands for Live-Cell Imaging: A Comprehensive Review. *Pharmaceuticals* **2023**, *16*, 1235. <https://doi.org/10.3390/ph16091235>

Academic Editors: Tiziano Tuccinardi, Carlotta Granchi, Francesca Gado and Andrea Mastinu

Received: 31 July 2023

Revised: 21 August 2023

Accepted: 25 August 2023

Published: 31 August 2023



Copyright: © 2023 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

The G protein-coupled receptors (GPCRs) are a class of over 700 proteins that include seven transmembrane domains and are produced on the cellular membrane [1]. These cellular components are responsible for transmitting extracellular signals originating from many stimuli, including light, peptide hormones, and neurotransmitters [2,3]. This transmission enables intracellular second messenger systems to activate, facilitating cellular responses to the surrounding environment. G protein-coupled receptors (GPCRs) play a crucial role in the control of several physiological processes, including vision, behavior, mood, energy balance, immunology, and inflammation [4–6].

In recent years, research into the endocannabinoid system (ECS) has expanded significantly among the scientific community. The endocannabinoid system has been investigated for its potential role in a variety of pathophysiological disorders due to its wide range of regulatory effects [7,8]. The cannabinoid receptors, their endogenous lipid ligands (endocannabinoids), and the machinery for their manufacture and metabolism are all parts of the endocannabinoid system. The cannabinoid receptors CB₁ and CB₂ have been extensively studied and recognized as prominent members of the cannabinoid receptor family. These receptors are classified as G protein-coupled receptors (GPCRs), and their primary function involves coupling with inhibitory G proteins. The primary interaction of CB₁ and CB₂ receptors is coupling to inhibitory G proteins (G_i/G_o) and activation of the signaling pathways associated with G_i/G_o [9]. These receptors are activated via both exogenous and endogenous ligands. Apart from being the molecular targets of the exogenous psychoactive component of *Cannabis sativa*, Δ⁹-tetrahydrocannabinol (THC), the endocannabinoid

system is also influenced by naturally occurring endocannabinoids, such as anandamide (AEA) and 2-arachidonyl glycerol (AEA). The cannabinoid receptors CB₁ and CB₂ play an important role in the physiological control of numerous central and peripheral activities [10]. The CB₁ receptor comprises a total of 472 amino acids, is highly expressed in the central nervous system, and is particularly abundant in various brain regions, including the hippocampus, cerebellum, cortex, and basal ganglia [11]. Additionally, CB₁ receptors are found in peripheral tissues such as the liver, lung, pancreas, and ileum [12].

The CB₂ protein encodes the CNR2 gene (GeneID1269) located in 1p36.11.; it consists of a total of 360 amino acids [13]. CB₂R has been increasingly reported in brain pathologies, but its highest concentration is found in immune system cells [14,15]. These receptors play a crucial role in the immunomodulatory effects induced via cannabinoids. The two cannabinoid receptors exhibited a 44% level of homology and a 68% degree of sequence similarity inside their transmembrane regions [16].

There are significant variations across species, as demonstrated by the approximately 80% similarity in amino acid sequences between humans and rats. Two isoforms of CB₂ have been discovered in humans, namely CB₂A and CB₂B. CB₂A is primarily found in the testis and is also expressed, albeit to a lesser extent, in certain regions of the brain, such as the amygdala, caudate, putamen, nucleus accumbens, cortex, hippocampus, and cerebellum. On the other hand, CB₂B is the main isoform found in the spleen and leukocytes, but it is also present in other peripheral tissues [13,17].

Although CB₁R and CB₂R have enormous therapeutic promise, very few medicines that target them are currently available on the market. The similarities between the active-state orthosteric ligand-binding pockets of CB₁R and CB₂R make it challenging to develop selective ligands based on their activation [18–20]. Despite their importance, very little is known about the signaling processes involved or the patterns of expression in different tissues. Therefore, a comprehensive knowledge of CB₁R and CB₂R tissue expressions and their signaling pathways is critical to avoid off-target or undesired psychotropic effects. Hence, subtype-selective probes are the most effective means of addressing these problems. CB₂R agonists have shown vast potential, offering directions for future selective medication development [17,21,22]. Understanding ligand–protein interactions, target engagement, and the mechanism of action is crucial to the development of novel medications. GPCR expression in endogenous tissue under physiological conditions is limited, making it challenging to determine target interactions. The adaptability of proteins when activated emphasizes this even further. Biomarkers are a valuable tool for determining target engagement *in vivo*. Although it has been hypothesized that alterations in the ECS might be used as a biomarker for psychiatry, there are presently no biomarkers for CB₂R, which explains the necessity of specialized imaging techniques with minimal off-target interactions [23]. The imaging of CB₂R in live cells remains underexplored due to its limited expression in healthy tissue and the lack of robust pharmacological techniques [24].

Fluorescent compounds have allowed for significant advances in the visualization of proteins, receptors, and ion channels within and outside of cells. Using these molecules, researchers have gained insight into the subcellular localization and, in certain cases, dynamic mobility of a number of membrane-bound proteins. Typically, antibodies or the overexpression of a fluorescent protein linked to a target protein are employed to mark membrane-bound proteins [25].

Molecular probes that have been fluorescently tagged have found widespread use in both *in vitro* and *in vivo* settings. Finding an area where chemical modifications may be made without affecting probe affinity is the primary difficulty in designing fluorescent probes. Traditional antibodies are unable to see intracellular protein targets, but small-molecule-derived probes are capable due to the incorporation of cell-permeable fluorescent dyes. By using them, we may learn about the molecular processes behind pharmacological responses alongside where and how frequently targets are expressed. To better understand how ligands interact with their targets, methods such as fluorescent confocal microscopy,

flow cytometry, and time-resolved Förster resonance energy transfer (TR-FRET) have been employed [24].

Recent efforts to investigate CB₂R expression at the tissue level have relied on positron emission tomography (PET) tracers; however, this approach lacks the necessary cellular resolution. PET probes need a reduced nanomolar affinity in order to be suitable for imaging applications, sometimes leading to false-positive outcomes [26,27]. Fluorescent imaging probes, on the other hand, have recently emerged as powerful instruments for studying the subcellular localization, structure, dynamics, and function of proteins and GPCRs in live cells. Such probes also have the potential to provide high-throughput data on binding kinetics and equilibrium without the use of radioactive materials. Fluorescence is often used in the investigation of small molecule–target interactions, facilitated via techniques such as fluorescence resonance energy transfer (FRET) and bioluminescence resonance energy transfer (BRET), which are widely utilized in this field. Both the bioluminescence resonance energy transfer (BRET) and fluorescence resonance energy transfer (FRET) techniques have been used in the viewing of various G protein-coupled receptors (GPCRs), therefore illustrating the suitability of both approaches for ligand–protein imaging. As a result, the precise evaluation of CB₂R pharmacology in live cells is still hampered by the limited robust antibodies, the reversible high-affinity probe with desirable fluorescence features, and the requisite specificity for CB₂R. Phytocannabinoid-derived CB₂R ligands, which are typically used as starting points for probe development, present significant challenges due to their highly lipophilic nature. When combined with the usage of highly lipophilic dyes, this may have a synergistic effect that reduces the dyes' overall characteristics and makes them less useful for a wide range of applications. Furthermore, the fluorescent probe should not have any interspecies variations between rodent and human CB₂R to provide excellent translatability of preclinical pharmacological animal data to the clinic and perhaps deploy a CB₂R fluorescence probe for dosage selection in humans [27–31].

2. CB₂ Receptor: Molecular Characterization and Spatial Distribution

Cannabinoids have an influence on almost all physiological systems in the human body. Unsurprisingly, extensive research has shown the presence of CB receptors throughout the human body. The first categorization assigned reactions inside the central nervous system (CNS) to the CB₁ receptor, whereas those occurring in the peripheral regions were attributed to the CB₂ receptor. As previously mentioned, the CB₂ receptor was first cloned from a human leukocyte cell line, and it has been shown that the CB₂ receptor is expressed in peripheral blood leukocytes to variable degrees [10,32].

The most prominent manifestation is seen on B-cells, with natural killer (NK) cells exhibiting a similar level of expression in close proximity. Myeloid cells have moderate levels of receptor expression, whereas resting T cell subsets show low levels of receptor expression. It is not unexpected that certain components of the immune system, including the tonsils, spleen, thymus, and lymph nodes, have significantly elevated levels of CB₂ receptor mRNA expression. A comprehensive investigation has provided more evidence that the CB₂ receptor is present in distinct areas of the brain, spinal cord, and dorsal root ganglia, as well as the gastrointestinal (GI) tract, liver, bone, and reproductive systems (Figure 1) [33,34].

The comprehensive understanding of the CB₂ receptor's existence and function inside the central nervous system remains inadequate. Initially, there was a prevailing belief that the expression of CB₂ receptors was absent in nonimmune cells of the central nervous system. This belief stemmed from the findings of Munro et al., who, during their cloning of the receptor, failed to locate CB₂ receptor mRNA in any region of the brain [10]. However, many studies have recently shown that CB₂R plays a functionally relevant role in the central nervous system and various pathologies associated with the brain [34–37].

CB₂ Receptor expression in human

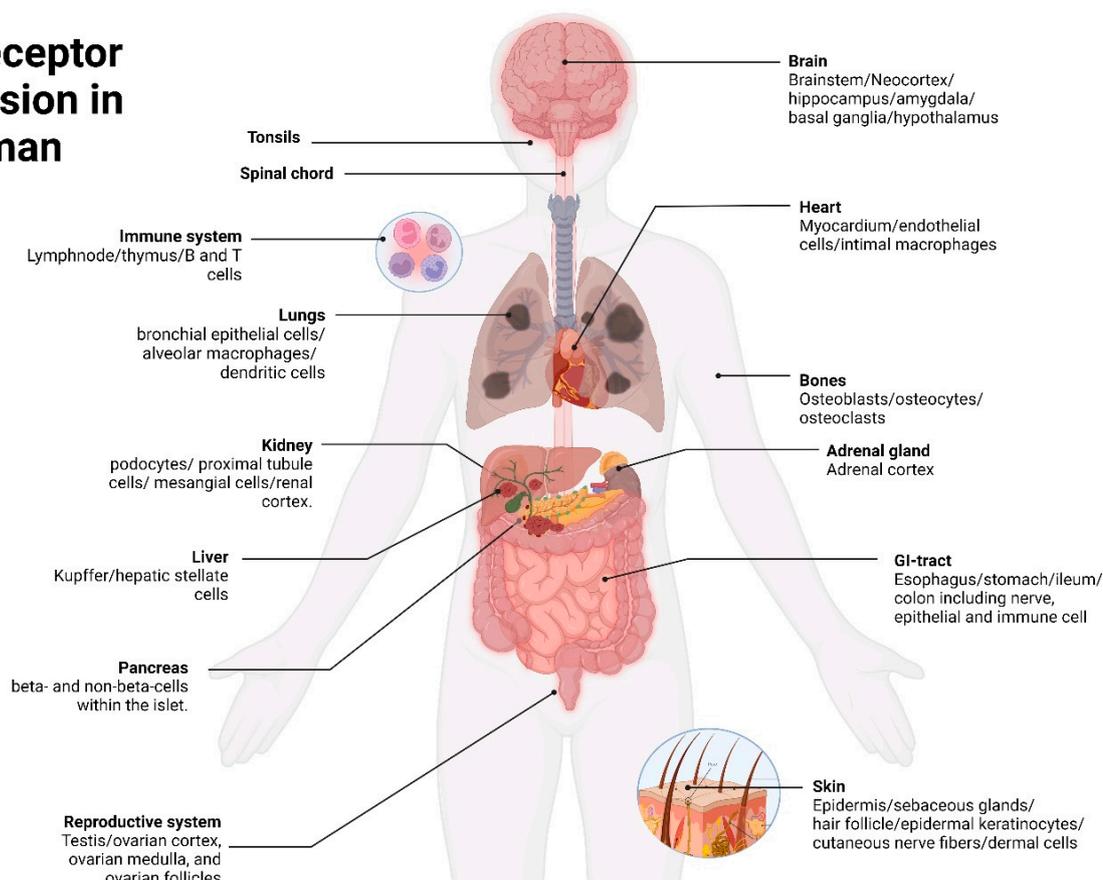


Figure 1. CB₂ receptor expression in human physiological system.

The CB₂ receptors located in peripheral tissues and cells, including those of the immune system, hematopoietic cells, bone, liver, peripheral nerve terminals, and keratinocytes, play a crucial role in the suppression of cytokine/chemokine secretion, as well as the migration of neutrophils and macrophages. Additionally, they contribute to the deceleration of persistent inflammatory mechanisms and the regulation of chronic pain [38]. The limited dependability of the antibodies used in immunochemistry investigations to study CB₂R receptor expression underscores the need for further investigation to enhance our understanding of the participation of the CB₂ receptor in the central nervous system and neuroinflammation. Numerous CB₂ ligands have been synthesized, and their receptor function has been studied in detail (Figure 2); however, drugs solely targeting CB₂ have not yet made it to the clinic [16,17,22].

The therapeutic potential of CB₂ and its role in various pathologies have been extensively reviewed [17,21,39–42]. Our focus here is to review the imaging probes that were developed to study CB₂ expression and function, as well as to highlight challenges and opportunities to guide future improvements.

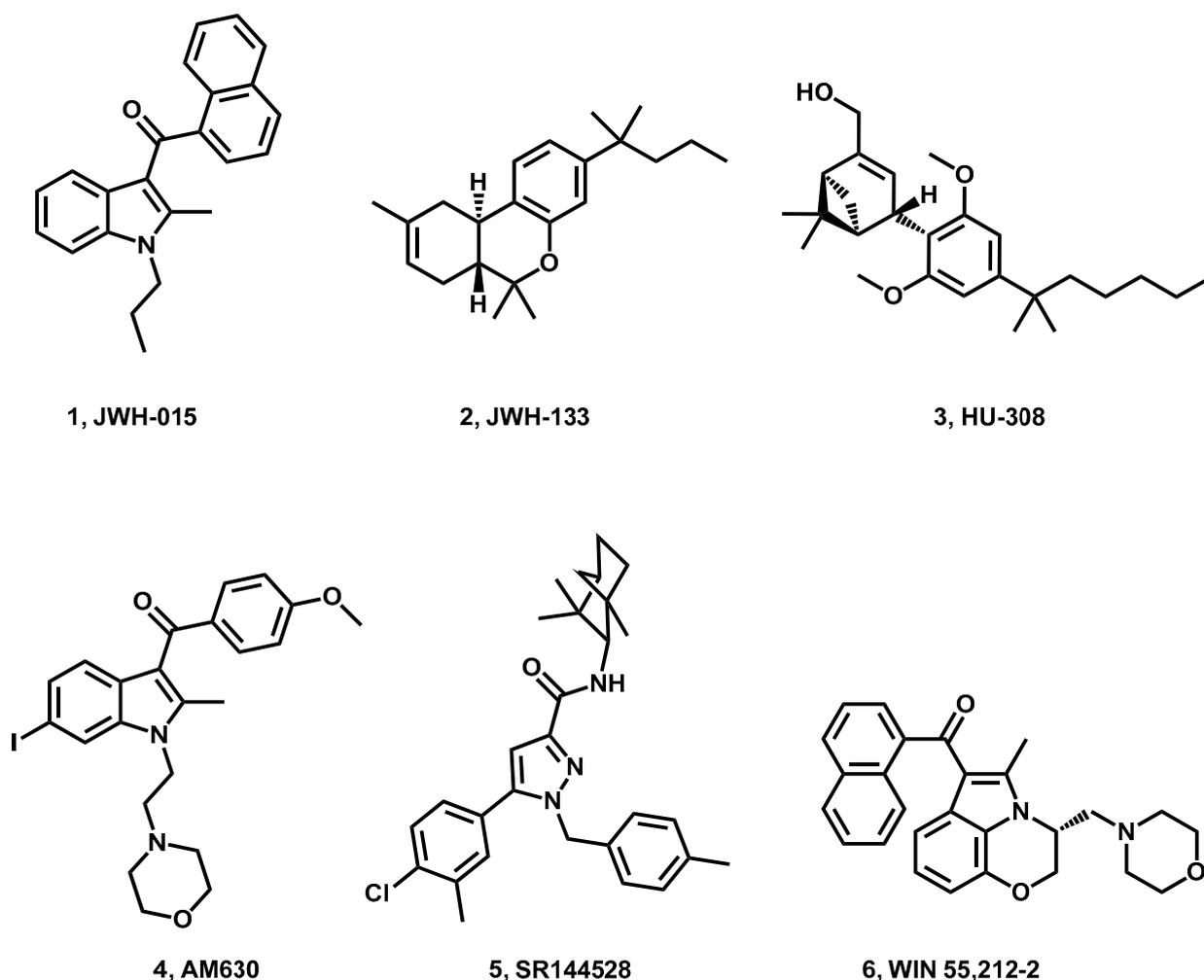


Figure 2. Examples of CB₂R probe (agonist/antagonist).

3. Fluorescent Probe Design Principles

Numerous CB₂ agonists have been documented in the scientific literature, and an in-depth investigation of patents revealed the existence of several CB₂ receptor modulators now undergoing clinical development at varying stages [22,43]. To study the brain CB₂ receptors effectively, the ligand must demonstrate the capability to effectively penetrate the blood–brain barrier, enabling it to exercise its intended effects on activated microglia [28]. Furthermore, for its effective clinical translation, it must undergo rigorous testing with the right patient cohort [42].

The CB₂ receptor has garnered significant interest as a promising candidate for the noninvasive detection of neuroinflammation due to its reported upregulation in resident microglial cells after cerebral ischemia, injury, and other neuroinflammatory disorders. When creating small-molecule fluorescent ligands, it is essential to consider many parameters. The successful interaction between the ligand and the fluorophore is crucial for both specific binding to the desired target and the generation of a strong fluorescence signal, enabling effective imaging of the receptor. The pharmacophore must possess high potency and selectivity while also accommodating chemical functionalization by coupling to a linker and fluorophore. Functionalization may be included in the system via chemical methods in certain cases. The determination of spacer length should ideally be derived from an optimization procedure. However, limited research is available that has provided a systematic review of the length of the spacer used for the fluorescent probe being investigated [44,45].

Based on an extensive review of the existing literature, it is evident that spacers are composed of a minimum of six atoms [46]. Extended linkers have been used to accom-

moderate larger fluorophores, such as near-infrared fluorophores (NIRF) or quantum dots (QDs) [47]. The physicochemical parameters of the fluorescent probe may be influenced by the chemical composition of the linker. Multiple investigations have posited that the presence of elevated lipophilicity could play a role in the nonspecific binding of some fluorescent probes [48,49].

The best selection of a fluorophore should prioritize characteristics such as high fluorescence intensity, photostability, and compatibility with the available microscope and the specific system under investigation. Commonly used fluorophores for fluorescence imaging include fluorescein, coumarin, dansyl, cyanine, rhodamine derivatives, and NBD, as previously indicated. When selecting an appropriate fluorophore, it is crucial to ensure that the excitation and emission wavelengths do not fall within the spectral range where cells demonstrate significant amounts of autofluorescence and light scattering. It is essential to exercise caution in order to prevent interference from pre-existing fluorescent signals inside the target cells, including those associated with a GFP-tagged protein [50]. The fluorescent ligand's modular design approach, as previously described by Gazzi et al., encompassed three primary components: (i) a recognition element (pharmacophore) capable of accommodating subsequent chemical functionalization while retaining its affinity and selectivity towards the target; (ii) a suitable fluorescent dye; and (iii) a linker that serves to separate these two functionalities. This enables the positioning of the dye in the extracellular area, external to the receptor. The incorrect escape vector provided via the linker attachment, when combined with a big and charged fluorescent dye that is typically larger than the recognition element, results in a significant disruption of the interaction between the receptor and the ligand and may even cause a total loss of binding affinity. Hence, the careful identification of optimal linker attachment sites, together with the subsequent optimization of linker length and composition, is of utmost importance in order to prevent any adverse interactions with the receptor [25,51,52].

4. CB₂R Targeting Fluorescent Probes

Even though CB₂R expression is significantly increased in pathological conditions, no particular drugs targeting receptors have been introduced to the market despite its obvious relevance. This knowledge gap is largely due to a lack of understanding of receptor biology, particularly in the context of tissue and disease. The problem is made worse by very low expression levels in native cells, a dearth of authorized chemical probes, and a paucity of tailored antibodies [53–56].

Given the aforementioned problems, researchers have been diligently endeavoring to produce chemical tools that may address the unresolved questions in CB₂R pharmacology. This effort aims to optimize and facilitate the creation of effective medications targeting CB₂R. Previous literature exists in which authors have documented advancements in the development of selective CB₂R ligands that have been refined for use as pharmacological and imaging instruments, such as positron emission tomography (PET), fluorescent, photochromic, and covalent chemical probes [27].

These probes are expected to enhance our perception of the receptor's activities and its role in regulating the physiological processes and pathophysiological pathways associated with various diseases. This, in turn, will contribute to the advancement of our imaging techniques and knowledge of the receptor's behavior. The focus of research has been on enhancing the progress of developing specific fluorescent ligands for CB₂R. This is because the current techniques used to study CB₂R, such as radioligands and antibodies, have certain limitations [57].

Fluorescent ligands are powerful tools for visualizing receptor dynamics in living cells, and they are compatible with methods like confocal microscopy, fluorescence resonance energy transfer, and flow cytometry [29].

The use of the identified CB₂R fluorescent ligands as imaging tools has been impeded in the past due to the presence of significant nonspecific binding at membranes. The lipophilicity of cannabinoids and their pharmacophore makes it challenging to de-

velop fluorescent ligands that are devoid of nonspecific binding. Despite these challenges, researchers aim to strike an equilibrium between pharmacological selectivity and physicochemical properties through linker modifications connecting the pharmacophore with fluorescent dyes [58].

4.1. Fluorescent Indole Derivatives

Indoles have been extensively used as fluorescent ligands among the CB₂ receptor pharmacophores. This is mostly due to the presence of the indole moiety in many CB₂R reference ligands, including JWH-015 (1) and WIN 55, 212-2 (6). JWH-015 is a very potent agonist of the cannabinoid receptor characterized by the presence of a naphthoylindole scaffold (Figure 3). It has about 28 times more of an affinity for the CB₂ receptor compared to the CB₁ receptor. The first development of fluorophores for cellular imaging was the invention and synthesis of a molecule that incorporated the fluorescent dye nitrobenzofurazan (NBD), owing to its notable selectivity. The NBD fluorophore was conjugated to the naphthalene moiety via a brief amide linkage in order to inhibit the formation of a nonfluorescent molecule with PET activity. The affinity of CB₂R of 7 was assessed using a displacement test against [³H]-CP 55,940 in CHO cells that were engineered to express CB₂R. The novel compound NBD JWH-015 (7) exhibited a displacement rate of just 25% at a concentration of 10 μM. Unfortunately, the generated probe exhibited a significant decrease in CB₂R affinity, exceeding a 250-fold reduction compared to the untagged ligand. Consequently, its suitability as a selective CB₂R fluorescence instrument was compromised. Upon visualization using a confocal microscope, it was seen that the majority of the fluorescence was concentrated inside the cytoplasm. This localization may be attributed to the compound's lipophilic nature, which facilitated its quick absorption via the cells. The compound's precursors were discovered to retain CB₂R activity; however, this aspect was not further investigated. Instead, subsequent studies have shown that indole compounds mostly exhibit tolerance for fluorophores at position C-7 despite their antagonistic properties [59].

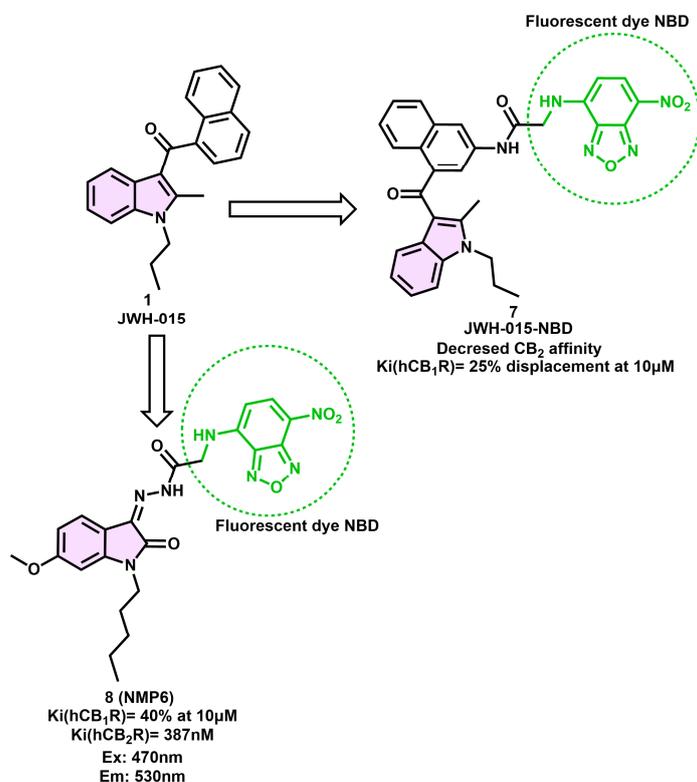


Figure 3. Indole-based CB₂R fluorescent probe.

The reduced affinity prompted the use of a powerful CB₂R antagonist scaffold, namely the 6-methoxy-N-pentyl isatin acylhydrazone, which was then linked with the NBD dye (Figure 3) [60]. The synthesis of NMP6 (8) was carried out through a two-step process, wherein NBD was used to bind to the sizable hydrophobic pocket, hence potentially enhancing CB₂R affinity. In the competitive binding experiment conducted on Chinese hamster ovary (CHO-K1) cells, NMP6 (8) exhibited a K_i value of 387 nM when tested against [³H]-CP 55,940. The excitation and emission wavelengths of NMP6 (8) in acetonitrile were measured to be 470 nm and 530 nm, respectively. NMP6 continued to exhibit nanomolar affinity and good selectivity for CB₂R, therefore prompting further evaluation in CB₂R cellular imaging experiments. NMP6 (8) demonstrated its ability to detect CB₂R via the use of confocal microscopy in nontransfected cells and cytometric analysis in B lymphocytes. The binding of CB₂R was seen to be impeded in competition experiments or when preincubated with GW842166X, a selective CB₂R agonist. These findings provide evidence of the selectivity of NMP6 (8) towards CB₂R. Although the scaffold has shown a consistent affinity for CB₂R, there has been a lack of exploration about the use of other analogs incorporating the isatin scaffold for CB imaging [61].

The functionalization of the other locations on the indole ring was also examined in order to produce fluorescent ligands. The results of N1 functionalization experiments indicate that linkers longer than a hexamethylene chain are not well tolerated. However, further investigation into the C5-7 positions using various linkers has yielded promising compounds. Notably, a consistent pattern has emerged: C5-substituted indoles exhibit agonistic activity, while the C7-substituted derivatives demonstrate inverse agonistic activity as observed in a cAMP assay. The use of positions 5 and 7 of the indole moieties was employed for the purpose of the prospective attachment of linkers and fluorophores. Following a structure–activity relationship (SAR) investigation of the indole scaffold, Cooper et al. discovered ligands for the CB₂R receptor that exhibited a nanomolar affinity [61]. Notably, these ligands had bulky substituents at either position 1 or 3 of the indole scaffold, such as ethyl morpholine, methoxyphenyl, or methyl tetrahydropyran. However, the binding between CB₂R and the linkers was significantly impeded when decorated with BODIPY 630/650-X fluorescent tags in both places. Regrettably, the conjugation of both these indole derivatives with the fluorophore BODIPY 630/650-X in either the C5 or C7 positions via alkylene, PEG, or dipeptide linkers, respectively, yielded fluorescent ligands lacking an affinity for CB₂R (K_i > 10,000 nM). This outcome indicates that only smaller substituents are permissible at these specific locations. In light of the commitments made via the C5 or C7 functionalized indole derivatives, it is possible to explore the same sites using fluorescent tags other than BODIPY.

4.2. Pyrazole-Based Fluorescent Probes

The subclass of fluorescent CB₂R ligands is derived from various fluorophore enhancements included in the biarylpyrazole framework of SR 144528 (5). In 1998, Rinaldi-Carmona et al. developed the first selective CB₂R antagonist with a high affinity (K_i CB₂R = 0.6 nM), which was later shown to act as an inverse agonist [62].

Nevertheless, although it possesses a pharmacological profile that is very desirable, it is deficient in functional groups that would facilitate convenient fluorophore conjugation. In the year 2008, the compound MBC94 (9) was first produced, whereby a benzyl bromide group was substituted for a methyl group to enhance its coupling capabilities. The compound has a terminal amino group that may be readily conjugated to imaging scaffolds, resulting in a modest decrease in affinity by a factor of 15 (K_i = 15 nM) [63,64].

The coupling of the near-infrared (NIR) dye IRDye 800CW NHS ester to MBC94 (9) resulted in the formation of NIRmbc94 (10). This compound represents the first instance of a CB₂R selective probe and is among the earliest fluorescent ligands for CB₂R, exhibiting a high affinity in the nanomolar range (K_i = 260 nM). The probe has favorable photophysical characteristics, demonstrating a peak absorbance at 779 nm and emitting light at 797 nm when placed in a water medium. The NIR microscopy studies exhibited favorable imaging

capabilities, as shown by a signal-to-noise ratio (S/N) of 1.6. Moreover, it demonstrated an intriguing pharmacological profile due to its absence of nonspecific binding and its excellent selectivity in competitive binding studies with SR144528 as the displacing ligand.

CB₂-expressing DBT cells derived from a mouse astrocytoma cell line were labeled with NIR-*mbc94* (**10**), but no signal was seen when the NIR dye was incubated in the cells. The fluorescence signal exhibited a decrease in intensity when cells lacking CB₂R expression were subjected to incubation with an equivalent dose. All the experimental results provided support for the possible use of NIR-*mbc94* (**10**) in conducting the high-throughput screening (HTS) of chemical libraries including compounds that bind to CB₂R. An initial evaluation of chemicals was conducted, confirming the validity of this theory and indicating potential new discoveries. Nevertheless, there are several drawbacks associated with NIR-*mbc94* (**10**), mostly stemming from the use of its fluorophore, IRDye 800CW. This particular dye is known for its high cost and notable stability concerns. Zhang et al. successfully addressed these challenges by selecting NIR760 as a novel probe for conjugation with MBC-94 (**9**) [65].

The recently developed fluorophore, NIR760, has comparable characteristics to IR Dye 800CW, although with a different linker location relative to the pharmacophore. The absorbance of the novel fluorescent probe is measured at a wavelength of 766 nm, while its fluorescence emission occurs at 785 nm, exhibiting a quantum yield of 15.2%. The recently developed compound NIR760-*mbc94* (**11**) exhibited a high level of affinity in the nanomolar range, as seen in its dissociation constant (K_d) of 26.9 nM. Additionally, it demonstrated a considerable degree of specific binding, as indicated by a 40% drop in fluorescence intensity following a 30-min pretreatment with 10 μ M of SR144528, as depicted in Figure 4. Significantly, the CB₂R fluorescent probe NIR760-*mbc94* (**11**) was used as the first agent for in vivo testing in a CB₂-mid DBT murine tumor model with the aim of evaluating its viability as a tool for cancer imaging. The maximal absorption of the tumor was seen 24 h after intravenous injection, but NIR760-*mbc94* (**11**) exhibited the highest ratio of the tumor area to the normal area (T/N) after 72 h, progressively rising over time. This finding provides evidence of a prolonged half-life. The fluorescent CB₂R probe NIR760-*mbc94* (**11**) was subjected to testing for inflammatory imaging both in vitro and in vivo. The novel fluorescent ligand was used in the in vivo tests to investigate an inflammatory mouse model generated via a complete Freund's adjuvant (CFA). An evaluation of specific binding at the cellular level was conducted on ordinary RAW-264.7 (mouse macrophages) and LPS-activated cell lines. The antagonist SR144528 was used, resulting in decreases in the fluorescence signal of 36% in ordinary cells and 23% in activated cells. The use of in vivo imaging in a mouse inflammation model revealed a progressive enhancement in image contrast over time, specifically in the fluorescence identified in an inflamed paw compared to an uninfamed paw. This finding serves to validate the previously observed metabolic stability and indicates a peak blockage of 30% occurring at the 36-h mark. The use of immunofluorescence labeling on frozen slices of paw tissue confirmed prior findings while also reiterating the modest level of specific binding shown by NIR*mbc94* (**10**) [66].

The authors propose that the net negative charge of the NIR760 probe may be responsible for certain limitations. To address this issue, they have made advancements by creating a zwitterionic CB₂R NIR probe named ZW760-*mbc94* (**12**). This new probe retains a similar structure to NIR760 but substitutes alkyl sulfonic acid groups with trimethyl amine cations. The compound ZW760-*mbc94* has a strong binding affinity to the CB₂R receptor with a measured dissociation constant (K_d) of 53.9 nanomolar (nM). As anticipated, the introduction of ZW760-*mbc94* (**12**) to CB₂-mid DBT cells was hindered by about 50% when 4-quinolone-3-carboxamide (4Q3C), a specific CB₂R blocking agent, was used. This inhibition was greater than the earlier outcome seen with NIR760-*mbc94*. In an experimental study using an in vivo mouse model, CB₂-mid DBT tumor cells were injected subcutaneously. The fluorescence intensity of ZW760-*mbc94* (**12**) was shown to be greater in comparison to animals that were pre-injected with 4Q3C to block certain receptors. Notably, ZW760-*mbc94* (**12**) exhibited significant absorption in the liver and kidneys, which are organs involved in metabolism. In the ex vivo biodistribution research, a discernible

inhibitory impact of around 47% was only found in the cancer cells. This finding suggests that ZW760-mbc94 (**12**), in comparison to its precursor chemical NIR760-mbc94 (**11**), has enhanced and targeted binding capabilities at both the cellular and animal levels [67].

The previously mentioned research team successfully developed a novel phototherapeutic alternative, known as IR700DX-mbc94 (**13**), using the near-infrared (NIR) probe IR700DX. This innovative approach shows promise in targeting tumor cells that exhibit high levels of CB₂R expression (Figure 5) [68].

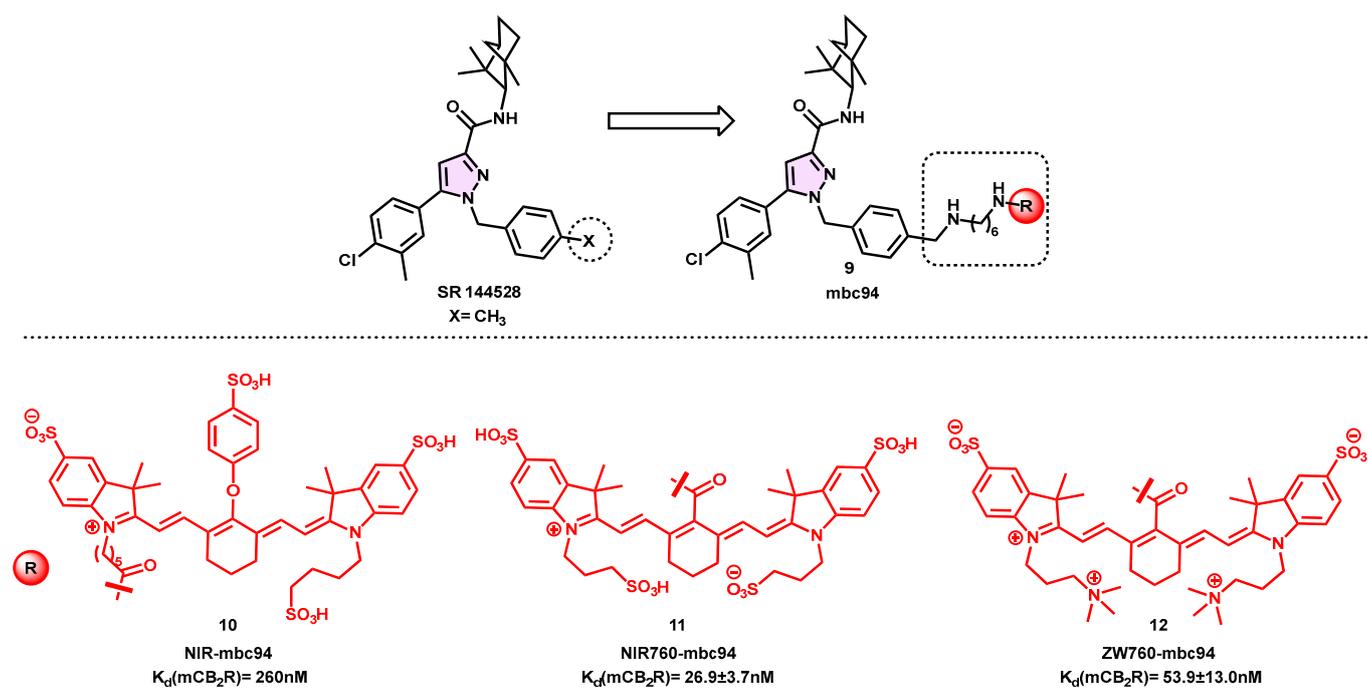


Figure 4. Biarylpyrazole-based CB₂ fluorescent probe.

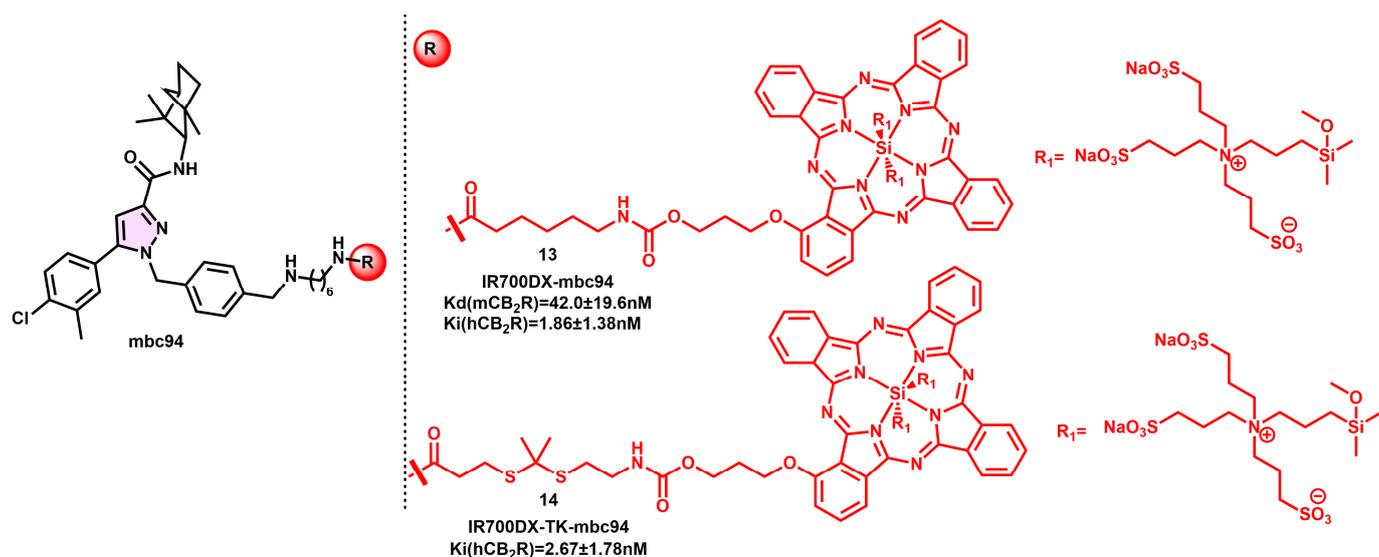


Figure 5. MBC94 CB₂R-selective fluorescent derivatives with various NIR fluorophores.

The compound exhibited absorbance and emission wavelengths of 682 and 690 nm, respectively, when dissolved in methanol. The nontoxicity of IR700DX-mbc94 (**13**) was established by the absence of irradiation in both CHO-K1/CB₂ and CB₂-mid DBT cells. The chemical had a dissociation constant (K_d) of 42.0 ± 19.6 nM and indicated cytotoxic

and inhibitory effects on CB₂ positive cells upon irradiation, contingent upon its binding to CB₂R. Research was conducted to investigate targeted phototherapy *in vivo* utilizing IR700DX-*mbc94* (**13**). The results of this investigation demonstrated the production of singlet oxygen (¹O₂) and free radicals under irradiation, leading to a kind of cell death resembling necrosis [69].

The IR700DX-*mbc94* (**13**) compound has promising characteristics as a possible agent for selective phototherapy in cancer cells. It demonstrates little cytotoxicity prior to irradiation and exhibits a preference for CB₂ + cells. The IR700DX-*mbc94* (**13**) compound was subsequently altered to include a dimethyl thioketal moiety inside the linker region. The recently discovered probe IR700DX-TK-*mbc94* (**14**) exhibited a high affinity in the nanomolar range for displacing [³H]-CP55,940 from hCB₂R, similar to the affinity seen with IR700DX-*mbc94* (**13**) (Figure 5). In the CB₂R+ animal model of a delayed brain tumor, CB₂-mid DBT cells treated with IR700DX-TK-*mbc94* (**14**) showed a significant 59% reduction in cell viability after irradiation. In contrast, IR700DX-*mbc94* (**13**) treatment resulted in little cell death. The observed increase in cell mortality when exposed to IR700DX-TK-*mbc94* (**14**) was constant across all incubation durations and concentrations.

The analysis of structure–activity relationships (SARs) revealed that the chromenopyrazole scaffold exhibits tolerance towards N-pyrazole aromatic and alkyl substituents. However, this tolerance leads to the development of high-affinity ligands that are selective for CB₁R, selective for CB₂R, or nonselective [31,69]. The study documented the enhancement of CB₂R selectivity via the phenolic alkylation of chromenopyrazole. Nevertheless, the presence of both the N-phenyl and O-alkyl groups in the same ligand resulted in a reduction in CBR affinity.

Several CB₂ fluorescent probes were produced, and wide-field fluorescence microscopy imaging tests were conducted to assess the capability of probe **18** in identifying CB₂R at the individual cell level. The fluorescent ligand with the greatest affinity was Cy5-containing (**18**) (hCB₂R pK_i = 7.38 ± 0.05, K_i = 41.8 ± 4.5 nM at hCB₂R; 5857 ± 1265 nM at hCB₁R), exhibiting a selectivity of 131-fold over CB₁R (Figure 6). In the context of a cAMP BRET experiment, compound **18** exhibited strong potency as an inverse agonist for the CB₂R receptor [31]. The HEK-293 cells expressing CB₂R were subjected to incubation with a concentration of 1 μM of the fluorescent ligand **18**. This resulted in evident labeling on the cell surface, but no noticeable accumulation was seen inside the cells. The specific binding of compound **18** to the CB₂R receptor was shown by the little fluorescence detected when the cells were co-incubated with a high affinity, nonfluorescent CB₂R inverse agonist, SR144528 (30 μM), and compound **18**. The specific binding of CB₂R was further confirmed by the little fluorescence produced when incubating **18** with HEK-293 cells that were transfected with an empty vector, indicating the absence of CB₂R. Given that **18** functions as an inverse agonist, the probability of receptor internalization is deemed exceedingly improbable. Moreover, considering the physical attributes of **18**, such as its substantial molecular weight and extensive polar surface area, the ease of cellular permeability seems implausible. In the imaging experiments, surface CB₂R-restricted labeling was observed, which was confirmed by the presence of clear colocalization with cell surface CB₂R. This colocalization was detected using a non-cell-permeable primary antibody that specifically targeted an extracellular epitope tag on the receptor. Furthermore, it became apparent that there was an absence of intracellular labeling despite the established knowledge that CB₂R is expressed intracellularly in the absence of ligand activation. Fluorescent ligand **18** has a high affinity for CB₂R, demonstrating selectivity and powerful inverse agonist action. Additionally, it possesses excellent imaging capabilities. Consequently, it is anticipated that this ligand will prove to be a significant tool for *in vitro* and *ex vivo* investigations, particularly in the context of examining CB₂R expression in whole-cell binding applications.

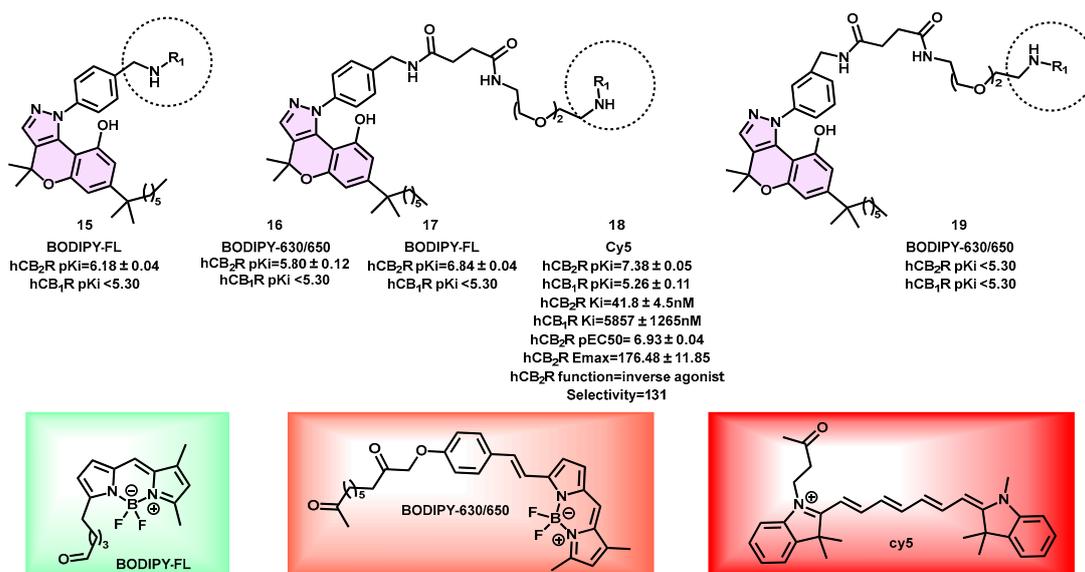


Figure 6. Pyrazoline-based CB₂ fluorescent probe.

4.3. Fluorescent Oxoquinoline Derivatives

The use of oxoquinolines has been extensively employed in the acquisition of highly specific and powerful CB₂R ligands [70].

Consequently, a substantial number of comprehensive SARs have been documented for this particular subclass, enabling the development of fluorescence instruments based on oxoquinoline with notable affinity and selectivity. In the first instances of success, the fluorophore, namely NIR760, was inserted directly into the aromatic moiety using an appropriate linker. The pyrazolo [1,5-*a*]pyrimidin-7-one moiety has been shown to have a strong affinity for CB₂R binding and selectivity while also possessing the advantageous characteristic of being highly tolerant to chemical modifications at the C2 position [71].

NIR760-XLP6 (**20**) was synthesized by attaching NIR760 to the pyrazolo [1,5-*a*]pyrimidin-7-one core using a 4 aminohexylphenylamide linker (Figure 7) [72]. The study demonstrated the significant affinity and selectivity of CB₂R (with a dissociation constant, K_d, of 169.1 nM) in the relevant DBT-CBR cells. This was supported by the presence of nonspecific binding, which was validated using *in vitro* fluorescence imaging. Both the binding test and biodistribution investigation yielded further data supporting the favorable and specific binding of NIR760-XLP6 (**20**) to CB₂R. Significantly, the measured tumor-to-normal (T/N) ratio reached a maximum value of 7.9 in the DBT-CB₂ tumor at 72 h after intravenous administration. The preference of NIR760-XLP6 (**20**) for CB₂R over CB₁R in a tumor mouse model was supported by *in vivo* and *ex vivo* optical imaging, indicating a moderate CB₂R specific binding. The fluorescent probe NIR760-XLP6 (**20**) was assessed for its potential as an imaging tool for pancreatic duct adenocarcinoma (PDAC). The evaluation showed that the probe achieved a good level of imaging contrast in both xenograft tumor and PDAC lymph node metastasis models. Additionally, the study confirmed the overexpression of CB₂R in this kind of tumor. During the investigation, a substantial quantity of nonspecific binding of NIR760-XLP6 (**20**) was found. This is a typical difficulty in the creation of CB₂R fluorescence probes [73].

The NIR760 fluorophore was used in conjunction with a selective CB₂R quinolone structure at position 6 via a triazole-PEGylated linker to produce NIR760-Q (**22**) [74].

In this instance, the binding affinity and targeting specificity of CB₂R were assessed in Jurkat cells, which inherently express CB₂R. The NIR760-Q (**22**) compound has a high affinity in the nanomolar range, with K_d = 75.51 nM (Figure 7). Additionally, it demonstrates moderate specific binding, which aligns with the observed pattern seen in other charged NIR fluorescent dyes [74]. In comparison to the CB₂R probe that was previously published, namely NIR760-*mbc*94 (**11**), NIR760-Q (**22**) exhibits improved absorption and emission

properties while maintaining similar binding affinity and specificity. This study made the first use of optical imaging targeting CB₂R in human tumor cells that endogenously express CB₂R. The aggregated data suggest that NIR760-Q (**22**) exhibits promise as an imaging probe for the targeted imaging of CB₂R, with potential applications in translational investigations.

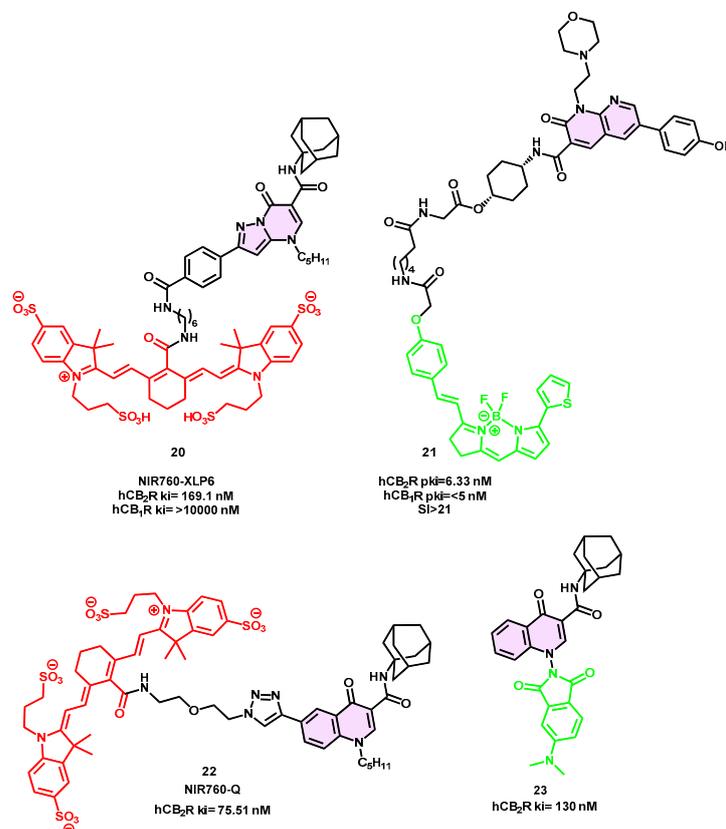


Figure 7. Oxaquinoline-based fluorescent probe for CB₂R.

In recent times, there has been an exploration of the potential attachment points for fluorescent dyes, specifically focusing on positions N1 and C3 of the oxaquinoline core [61]. Cooper et al. synthesized a collection of derivatives of 1,8-naphthyridin-2-(1H)-one-3-carboxamide, including the fluorophore BODIPY630/650-X at certain locations [75].

In comparison to previously known CB₂R scaffolds, the naphthyridine moiety exhibits more hydrophilicity, which is expected to be advantageous in preventing undesired non-specific binding. Regrettably, it has been observed that all fluorescent ligands connected to the N1 position have shown little affinity for CB₂R. However, the derivative **21**, which is coupled to the C3 position and utilizes a glycine bridge, has demonstrated a significant affinity and selectivity for CB₂R (pK_i = 6.33). This derivative functions as an inverse agonist [75].

Compound **21** has a noteworthy characteristic as it belongs to the category of infrequent CB₂R ligands that demonstrate enhanced affinity following the incorporation of a fluorophore. Notwithstanding these favorable premises, it was shown that ligand **21** exhibited suboptimal imaging characteristics for CB₂R in cellular contexts, which might be attributed to its pronounced membrane association and nonspecific intracellular accumulation [75].

In a recent structure–activity relationship (SAR) study focused on 4-oxo-1,4-dihydroquinoline analogs, a fluorescent CB₂R ligand was successfully synthesized with attachment at the N1 position [70,76].

Spinelli et al., in a recent study, reported the synthesis of a comprehensive collection of CB₂R fluorescent ligands. This was achieved by attaching 4-DMAP, NBD, and fluorescein cores to the N-adamantyl-4-oxo-1,4-dihydroquinoline-3-carboxamide pharmacophore. The lead drug, which contains a 4-DMAP fluorophore and a hexamethylene linker, had a favorable binding affinity towards the CB₂R receptor ($K_i = 130$ nM) and exhibited a low level of radioligand displacement compared to the CB₁R subtype (Figure 7). The researchers have introduced derivative **23** as an innovative candidate for the use of fluorescent-based competition binding tests and the identification of CB₂R in certain cell types, as assessed using fluorescence-activated cell sorting (FACS) analysis. The obtained results from a preliminary competitive binding assay were used to measure the IC₅₀ of two reference compounds, namely WIN55,212-2 and GW405833. Additionally, a saturation binding assay was conducted on CB₂R-HEK293 cells, using GW405833 as the reference compound and [³H]-CP55,940 as the radioligand. Compound **7** was used as the fluo-ligand at a concentration of 100 nM with an incubation time of 90 min. The results obtained were found to be comparable to the K_i values reported in the existing literature [76].

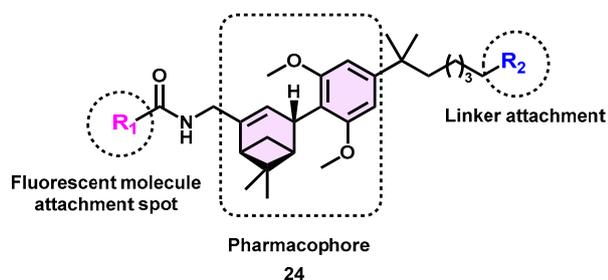
Furthermore, experiments including saturation and binding tests were conducted in tumor cells that naturally express CB₂R, providing further support for findings that have been previously documented for cells that have been genetically modified. Additional immunofluorescence tests were conducted using compound **23** at a concentration of 15 μ M as a fluorescent probe for seeing CB₂R in cells. These results demonstrated the significant specificity and reliability of compound **23** as a viable alternative to conventional radioligand assays.

4.4. Miscellaneous Fluorescent Probes

Since the presence of the original tetrahydrocannabinol (THC) core or analogs is often associated with a lack of CBR subtype selectivity and interference with other targets of the ECS, finding selective ligands harboring this moiety among all the CB₂R functionalized probes is not frequent. Because of its unusual biological features and the fact that CBR subtype selectivity may be obtained via strategic substitutions, the tricyclic cannabinoid scaffold has recently received a great deal of interest. A series of fluorescent ligands was produced and physiologically tested, beginning with the bioisostere chromenopyrazole core [53].

Carreira and colleagues recently developed a series of fluorescent CB₂R probes (**24a–f**) using NBD, DY-480XL, Alexa647, Alexa488, and AttoThio12 dyes (Figure 8, Table 1). The selection of fluorescent molecules was based on many criteria, including a significant Stokes shift, absorption and emission maxima that are red-shifted, a high molar extinction coefficient, appropriateness for Förster resonance energy transfer (FRET), and the possibility of use in ultra-high-resolution microscopy. Among all the synthetic molecules synthesized, a specific agonist, **24f**, has shown exceptional imaging characteristics and a remarkable binding affinity with the CB₂ receptor with $K_i = 4.7$ nM and a notable selectivity (K_i CB₂/CB₁ ratio = 228). CB₂R-selective fluorescent probes with unique photophysical characteristics were designed by attaching fluorescent dyes to amine building blocks through short linkers. The researchers used a previously reported high-recognition component that combines the characteristics of two CBR ligands, namely HU-308 and AM841. The docking simulations conducted on the DY-480-XL-carrying fluorescent azide derivative **24c** provided insights into the localization of the fluorescent tag in the extracellular region. Additionally, it was observed that the azide moiety forms a hydrogen bond with Leu191, which is likely a key factor contributing to the strong binding affinity exhibited by the two most effective azide derivatives as compared to their alkane counterparts. It is interesting to note that only changing the fluorescent dye moiety resulted in different efficacy (partial agonism for the molecule with AttoThio12 and agonism for the molecule attached with DY-480XL), all while maintaining extremely high functional selectivity over hCB₁R. The efficacy of their probe showed it to be a valuable instrument in several experimental settings, including competition studies, flow cytometry, and the time-resolved confocal imaging of both human

macrophages and mouse cells. In addition, the researchers assessed the kinetic binding characteristics using a newly developed TR-FRET-based assay, which is well suited to conducting high-throughput screening. Perhaps the most important point is that synthetic probes make it feasible to identify and monitor CB₂R in cells that naturally express the receptor, which is not achievable with existing antibody technology [53].



Properties of the synthesized Fluorescent probes:

1. Affinity and specificity for CB₂R
2. Selectivity for CB₂R than CB₁R
3. Enhanced photophysical properties
4. Applicability across species, techniques and cell type

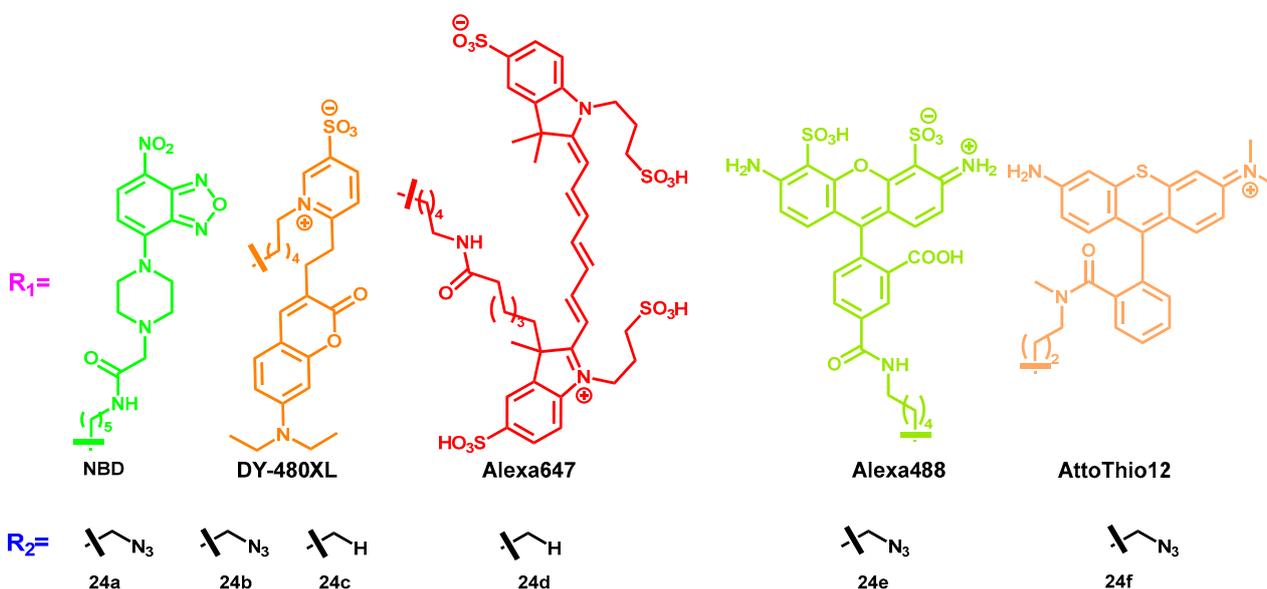


Figure 8. THC-based CB₂ selective fluorescent probes and their properties.

Table 1. Binding affinity (K_i) and selectivity values of the THC-based CB₂ selective fluorescent probe.

Compound	Dye	K _i (nM)			EC ₅₀ (nM)		
		hCB ₂ R	hCB ₁ R	mCB ₂ R	hCB ₂ R	hCB ₁ R	mCB ₂ R
24a	NBD	4.2	>10,000	n.d.	n.d.	>10,000	n.d.
24b	DY-480XL	99	4031	1986	>10,000	>10,000	>10,000
24c	DY-480XL	21	2378	1459	171	2152	118
24d	Alexa647	2565	>10,000	>10,000	25	2565	370
24e	Alexa488	268	>10,000	1204	n.d.	n.d.	n.d.
24f	Atto Thio12	4.7	1075	1.1	5.6	>10,000	17

Fluorescent imaging probes have been recognized as very sensitive technologies that provide a significant level of spatiotemporal resolution. In order to provide a reliable and verified translational pathway from preclinical pharmacological animal data to clinical applications, the Carreira research group endeavored to design a novel series of pyridine-based fluorescent probes for CB₂R [54].

In a previous study, a set of fluorescent probes using a phytocannabinoid as the foundation was developed [53]. However, these probes exhibit suboptimal pharmacokinetic properties, limiting their potential for practical applications in translational research. Con-

sequently, more progress was made using a reverse-design methodology, starting with a pharmacologically proven in vivo active compound class. This strategy aimed to meet many essential criteria, including affinity, potency, selectivity, chemical stability, water solubility, and membrane permeability. A ligand, denoted as **25**, has a preferential binding affinity in the picomolar range for both human and mouse CB₂R receptors, and it demonstrates complete agonistic efficacy in the picomolar range (Figure 9) [77,78].

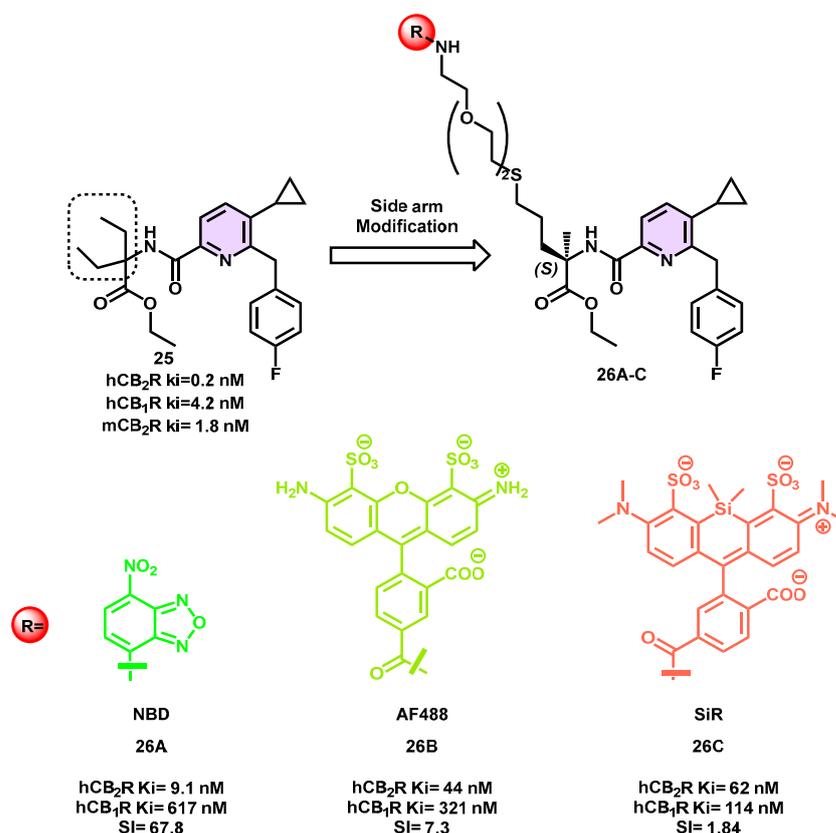


Figure 9. Pyridine-based CB₂R fluorescent probe.

To facilitate the attachment of a fluorophore, a conjugation handle was necessary, based on the aforementioned lead. Initially, the use of docking research was employed to identify a potential linker and attachment site for the pharmacophore inside selective CB₂R ligands based on pyrazine and pyridine [54].

A variety of fluorescent ligands were synthesized, whereby dyes were attached to the geminal diethyl group of the appropriately substituted pyridine moiety through a PEG linker. The process of optimizing the probes and selecting appropriate dyes was facilitated by conducting a thorough assessment of the absorption and emission spectra in a buffer solution. From the conducted investigations, it was discovered that all probes exhibited water solubility and did not demonstrate any inclination to form aggregates. A wide variety of lipophilicity values, ranging from 5.3 to 10.6, were detected for compound **26A–C** (Figure 9). This observation suggests that compound **26C**, which has an Alog *p* value of 10.6 and lacks negative charges according to its design, exhibits high permeability. These ligands were then subjected to in vitro biological evaluation. Among the derivatives examined, derivative **26A** emerged as the most effective ligand in terms of its potency, selectivity, and agonist functional features. However, due to the superior imaging characteristics of their respective dyes, only compounds **26B** and **26C** were subjected to further testing via fluorescence imaging. Overexpressing hCB₂R-CHO cells and endogenously expressing native cells from human macrophages and mouse splenocytes were used with fluoroprobe **26C** for live-cell imaging of intracellular CB₂R distribution using super-resolution confocal

microscopy. During the FACS investigation, it was shown that both derivatives **26B** and **26C** exhibited a high degree of target specificity with little unspecific binding. Furthermore, probe **26C** was effectively used for the purpose of visualizing hCB₂R in viable cells via the application of confocal imaging techniques. When injected intravenously, **26C** was shown to have a low toxicity profile in zebrafish larvae. It floated about the circulatory system for a while before it finally found its way to CB₂R-expressing macrophages. This highlights the probe's in vivo use and related issues, including biosafety, biodistribution, and cellular uptake. By providing a high-specificity, low-nanomolar-affinity probe for CB₂R with full agonist efficacy, probe **26C** provides researchers with a novel and useful platform to study this receptor in a variety of relevant biological and pharmacological contexts [54].

Additional research and investigation into the development of a fluorescent probe that specifically targets CB₂ receptors are needed. The Carrier group successfully pioneered the first ligand-directed covalent (LDC) labeling of CB₂R using an innovative synthetic approach and the implementation of platform reagents. The affinity labeling technique, sometimes referred to as ligand-directed covalent (LDC) chemistry, enables the precise and selective fluorescent labeling of proteins that are naturally expressed within intricate and multifaceted systems. The LDC alteration facilitates the imaging and examination of CB₂R while also preserving its capacity to bind various ligands at the orthosteric location. The use of in silico docking and molecular dynamics simulations is employed to provide guidance in the design of probes and evaluate the viability of tagging the CB₂R with LDC. The study showcased the use of fluorogenic O-nitrobenzoxadiazole (O-NBD)-functionalized probes (**27a–e**) in a TR-FRET experiment to label the peripheral lysine residue of CB₂R selectively and covalently (Figure 10). The confirmation of proof-of-concept was conducted rapidly using O-NBD probes, which subsequently led to the inclusion of advanced electrophiles that are appropriate for experimentation in living cells. In pursuit of this objective, innovative methodologies were devised to synthesize N-sulfonyl pyridone (NSP) and N-acyl-N-alkyl sulfonamide (NASA) LDC probes. These procedures facilitated the introduction of fluorophores via covalent bonding, making them ideal for cellular investigations. The LDC probes underwent characterization via the radioligand binding and TR-FRET assays.

The tendency of **27a–e** to bind more strongly to hCB₂R than the mouse ortholog may be explained by the pattern of molecular interactions shown in MD simulations. This may be because of the amino acid substitutions Val261^{6.51} Ala, Ser90^{2.60} Asn, and Asn93^{2.60} Ile that occurred during the evolution from humans to mice. The mouse ortholog with an alanine residue lacks the Val261^{6.51} side chain's hydrophobic interactions with the dimethyl group of the azidoheptyl substituent on the arene, which stabilize the molecule. Both the **27a** and **27c** probes produced weaker signals than **27b**. Similar to probe **27b**, both probes **27d** and **27e** were effective in covalently labeling their targets. Therefore, probes **27b**, **27d**, and **27e** were the most suited candidates for further research because of their high affinity and selectivity for CB₂R in the radioligand assay and their robust signal in the TR-FRET test. The shorter, less lipophilic linker of **27b** was regarded as preferable to the linkers of **27d** and **27e** in this group due to its anticipated benefits in target selectivity and overall physicochemical qualities, most notably decreased lipophilicity.

Furthermore, the probes were used for the explicit purpose of visualizing CB₂R in conventional and imaging flow cytometry, as well as in confocal fluorescence microscopy, using both overexpressing and endogenously expressing microglial live cells.

A significant improvement in labeling yields was seen when the incubation period of other sets of synthesized probes (**28a–e**) was prolonged to 20 h (Figure 11). Significantly, after a 20-h incubation period, **28e** exhibited a comprehensive covalent transfer of its fluorophore, as shown by the FRET experiment. Upon doing the experiment under conditions that mimicked physiological temperatures (namely, 37 °C), it was observed that the rate of cargo transfer was significantly increased. Namely, after a duration of 120 min, the cargo transfer reached a level of 53.5% ± 9.6%, indicating a substantial enhancement in the process. In the TR-FRET test, 119-fold selectivity for CB₂R over CB₁R was established via the measurement of saturation binding. The plasma membrane of the cells exhibited

a strong DY-480XL signal, together with intracellular puncta that may indicate the presence of internalized receptors. The signal seen demonstrated colocalization between the SNAP-dye signal in the far-red range and the signal of interest. This colocalization provided confirmation that the tagging of SNAP tagged CB₂R was effective using compound 29 [79].

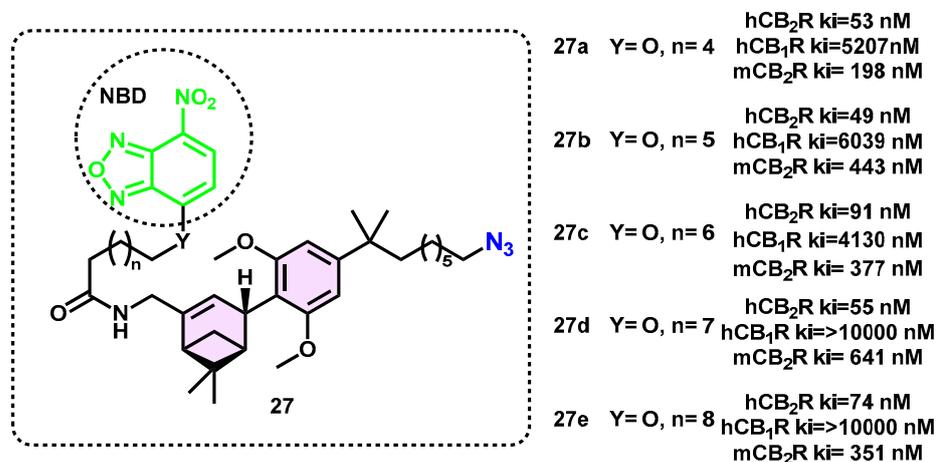


Figure 10. O-NBD and N-NBD probes for CB₂R cell imaging.

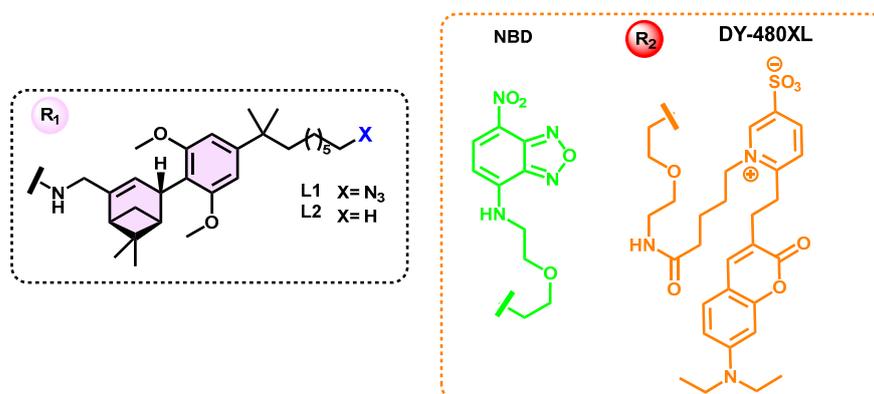
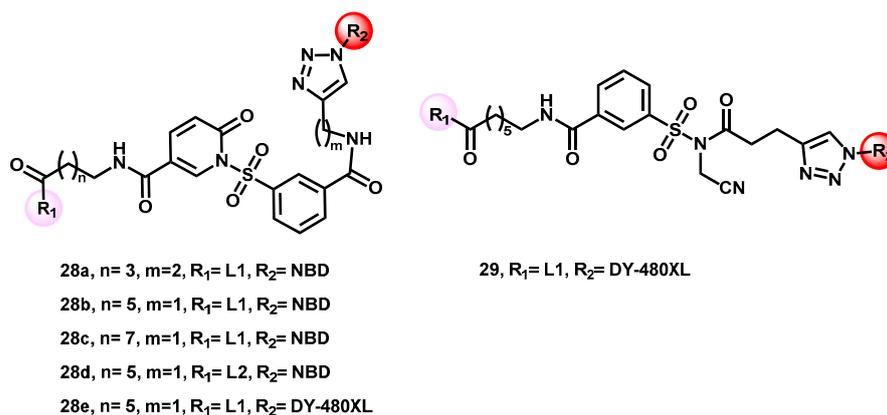


Figure 11. N-SP and NASA probes for CB₂R live-cell imaging.

5. Future Perspective and Critical Discussion

Small-molecule fluorescent ligands for use as tags for GPCRs have been in development throughout the last two decades. Innovation in chemical biology tools has been crucial to fostering this development. For instance, the last decades have seen significant progress in organic dye production, which is very helpful for the development of fluorescent ligands

for GPCRs. Among the factors that drove the discipline out of its “comfort-zone” of radioligand substituents and into biochemical applications are the aforementioned advancements. This review delves into the use of fluorescence imaging techniques to study CB₂ receptor expression, contributing to the advancement of our existing knowledge of CB₂R and its therapeutic applications. Additionally, it sheds light on novel discoveries that have emerged from these investigations. The activation of CB₂ has been shown extensively to suppress proliferation and promote autophagy and cell death, making it a promising target for therapy for cancer and other diseases [17,80]. According to the Drugs database of Global Data’s Pharma Intelligence Center, cannabinoid receptors have gained significant attention as a promising therapeutic target for the development of cannabis-based medications aimed at managing pain, neurological diseases, and inflammation. In the coming decade, one can expect a significant increase in the study of cannabinoid receptors to maximize their therapeutic potential. Cannabinoids based on CB₂R have significant potential for the treatment of inflammation and pain management since they demonstrate quantifiable qualities of antipruritic, antinociceptive, and anti-inflammatory effects. It is anticipated that the systematic availability of more clinical data will facilitate the exploration and use of this intriguing collection of phyto- and synthetic cannabinoids. Many of the CB₂ ligands suffer from poor pharmacokinetic properties, including high lipophilicity, low solubility, tight plasma protein binding, high in vivo clearance, and low oral bioavailability. In addition, residual CB₁ agonism in subselective ligands further limits their application. Clearly, a balance must be established between excellent pharmacokinetic properties and high activity and selectivity for CB₂R. An enhanced understanding of the kinetics involved in the binding of ligands to their respective targets, as well as the mechanisms behind biased signaling and allostereism in the CB₂R receptor, together with the availability of more structural information pertaining to both antagonist- and agonist-bound CB₂R, is expected to facilitate the development of more targeted approaches to drug design. The findings of this study have the potential to generate renewed optimism about the therapeutic capabilities of CB₂R and contribute to a more comprehensive understanding of the endocannabinoid system (ECS). The application of AI and machine learning techniques may further enable the design and expeditious synthesis of smart ligands for bespoke applications in the immediate future.

Author Contributions: Conceptualization, design of the study, and writing—original draft preparation and editing, P.B. and M.R.I. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by intramural funds provided by the National Institute on Alcohol Abuse and Alcoholism (NIAAA), the National Institutes of Health, to M.R.I. (ZIAAA000360).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data sharing is not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

GPCRs = G protein-coupled receptors; ECS = endocannabinoid system; CB₁R = Cannabinoid type 1 receptor; CB₂R = Cannabinoid type 2 receptor; hCB₁R = Human Cannabinoid type 1 receptor; hCB₂R = Human Cannabinoid type 2 receptor; THC = tetrahydrocannabinol; AEA = Arachidonoyl Ethanolamide/Anandamide; CNS = central nervous system; FRET = Förster resonance energy transfer; TR-FRET = time-resolved Förster resonance energy transfer; PET = positron emission tomography; BRET = bioluminescence resonance energy transfer; NK cells = natural killer cells; NBD = nitrobenzoxadiazole; CHO-K1 cells = Chinese hamster ovary cells; cAMP = Adenosine-3', 5'-cyclic monophosphate,

or cyclic AMP; SAR = structure-activity relationship; PDAC = pancreatic duct adenocarcinoma; DMAP = 4-Dimethylaminopyridine; FACS = fluorescence-activated cell sorting; PEG = Polyethylene glycol; LDC = ligand-directed covalent; NIRF = near-infrared fluorophores; QDs = quantum dots; DBT = delayed brain tumor; SI = Selectivity index; NASA = N-acyl-N-alkyl sulfonamide; NSP = N-sulfonyl pyridone.

References

1. Manglik, A.; Kobilka, B.K.; Steyaert, J. Nanobodies to Study G Protein-Coupled Receptor Structure and Function. *Annu. Rev. Pharmacol. Toxicol.* **2017**, *57*, 19–37. [[CrossRef](#)]
2. Rosenbaum, D.M.; Rasmussen, S.G.F.; Kobilka, B.K. The Structure and Function of G-Protein-Coupled Receptors. *Nature* **2009**, *459*, 356–363. [[CrossRef](#)]
3. Weis, W.I.; Kobilka, B.K. The Molecular Basis of G Protein-Coupled Receptor Activation. *Annu. Rev. Biochem.* **2018**, *87*, 897–919. [[CrossRef](#)]
4. Marinissen, M.J.; Gutkind, J.S. G-Protein-Coupled Receptors and Signaling Networks: Emerging Paradigms. *Trends Pharmacol. Sci.* **2001**, *22*, 368–376. [[CrossRef](#)]
5. Lagerström, M.C.; Schiöth, H.B. Structural Diversity of G Protein-Coupled Receptors and Significance for Drug Discovery. *Nat. Rev. Drug Discov.* **2008**, *7*, 339–357. [[CrossRef](#)]
6. Katritch, V.; Cherezov, V.; Stevens, R.C. Structure-Function of the G Protein-Coupled Receptor Superfamily. *Annu. Rev. Pharmacol. Toxicol.* **2013**, *53*, 531–556. [[CrossRef](#)] [[PubMed](#)]
7. Pacher, P.; Bátkai, S.; Kunos, G. The Endocannabinoid System as an Emerging Target of Pharmacotherapy. *Pharmacol. Rev.* **2006**, *58*, 389–462. [[CrossRef](#)]
8. Pacher, P.; Kunos, G. Modulating the Endocannabinoid System in Human Health and Disease—Successes and Failures. *FEBS J.* **2013**, *280*, 1918–1943. [[CrossRef](#)]
9. Lu, H.-C.; Mackie, K. Review of the Endocannabinoid System. *Biol. Psychiatry Cogn. Neurosci. Neuroimaging* **2021**, *6*, 607–615. [[CrossRef](#)]
10. Munro, S.; Thomas, K.L.; Abu-Shaar, M. Molecular Characterization of a Peripheral Receptor for Cannabinoids. *Nature* **1993**, *365*, 61–65. [[CrossRef](#)]
11. Bellocchio, L.; Soria-Gómez, E.; Quarta, C.; Metna-Laurent, M.; Cardinal, P.; Binder, E.; Cannich, A.; Delamarre, A.; Häring, M.; Martín-Fontecha, M.; et al. Activation of the Sympathetic Nervous System Mediates Hypophagic and Anxiety-like Effects of CB₁ Receptor Blockade. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 4786–4791. [[CrossRef](#)]
12. Rodríguez de Fonseca, F.; Del Arco, I.; Bermudez-Silva, F.J.; Bilbao, A.; Cippitelli, A.; Navarro, M. The Endocannabinoid System: Physiology and Pharmacology. *Alcohol Alcohol.* **2005**, *40*, 2–14. [[CrossRef](#)]
13. Liu, Q.R.; Pan, C.H.; Hishimoto, A.; Li, C.Y.; Xi, Z.X.; Llorente-Berzal, A.; Viveros, M.P.; Ishiguro, H.; Arinami, T.; Onaivi, E.S.; et al. Species Differences in Cannabinoid Receptor 2 (CNR2 Gene): Identification of Novel Human and Rodent CB₂ Isoforms, Differential Tissue Expression and Regulation by Cannabinoid Receptor Ligands. *Genes Brain Behav.* **2009**, *8*, 519–530. [[CrossRef](#)] [[PubMed](#)]
14. Onaivi, E.S.; Ishiguro, H.; Gong, J.-P.; Patel, S.; Perchuk, A.; Meozzi, P.A.; Myers, L.; Mora, Z.; Tagliaferro, P.; Gardner, E.; et al. Discovery of the Presence and Functional Expression of Cannabinoid CB₂ Receptors in Brain. *Ann. N. Y. Acad. Sci.* **2006**, *1074*, 514–536. [[CrossRef](#)]
15. Roche, M.; Finn, D.P. Brain CB₂ Receptors: Implications for Neuropsychiatric Disorders. *Pharmaceuticals* **2010**, *3*, 2517–2553. [[CrossRef](#)]
16. Wu, Y.-R.; Tang, J.-Q.; Zhang, W.-N.; Zhuang, C.-L.; Shi, Y. Rational Drug Design of CB₂ Receptor Ligands: From 2012 to 2021. *RSC Adv.* **2022**, *12*, 35242–35259. [[CrossRef](#)]
17. Gasperi, V.; Guzzo, T.; Topai, A.; Gambacorta, N.; Ciriaco, F.; Nicolotti, O.; Maccarrone, M. Recent Advances on Type-2 Cannabinoid (CB₂) Receptor Agonists and Their Therapeutic Potential. *Curr. Med. Chem.* **2023**, *30*, 1420–1457. [[CrossRef](#)]
18. Li, X.; Hua, T.; Vemuri, K.; Ho, J.-H.; Wu, Y.; Wu, L.; Popov, P.; Benchama, O.; Zvonok, N.; Locke, K.; et al. Crystal Structure of the Human Cannabinoid Receptor CB₂. *Cell* **2019**, *176*, 459–467.e13. [[CrossRef](#)]
19. Hua, T.; Vemuri, K.; Nikas, S.P.; Laprairie, R.B.; Wu, Y.; Qu, L.; Pu, M.; Korde, A.; Jiang, S.; Ho, J.-H.; et al. Crystal Structures of Agonist-Bound Human Cannabinoid Receptor CB₁. *Nature* **2017**, *547*, 468–471. [[CrossRef](#)]
20. Li, X.; Chang, H.; Bouma, J.; de Paus, L.V.; Mukhopadhyay, P.; Paloczi, J.; Mustafa, M.; van der Horst, C.; Kumar, S.S.; Wu, L.; et al. Structural Basis of Selective Cannabinoid CB₂ Receptor Activation. *Nat. Commun.* **2023**, *14*, 1447. [[CrossRef](#)]
21. Dhopeshwarkar, A.; Mackie, K. CB₂ Cannabinoid Receptors as a Therapeutic Target—What Does the Future Hold? *Mol. Pharmacol.* **2014**, *86*, 430–437. [[CrossRef](#)] [[PubMed](#)]
22. Han, S.; Thatte, J.; Buzard, D.J.; Jones, R.M. Therapeutic Utility of Cannabinoid Receptor Type 2 (CB₂) Selective Agonists. *J. Med. Chem.* **2013**, *56*, 8224–8256. [[CrossRef](#)] [[PubMed](#)]
23. Hamilton, A.J.; Payne, A.D.; Mocerino, M.; Gunosewoyo, H. Imaging Cannabinoid Receptors: A Brief Collection of Covalent and Fluorescent Probes for CB. *Aust. J. Chem.* **2021**, *74*, 416–432. [[CrossRef](#)]
24. Guberman, M.; Kosar, M.; Omran, A.; Carreira, E.M.; Nazaré, M.; Grether, U. Reverse-Design toward Optimized Labeled Chemical Probes—Examples from the Endocannabinoid System. *Chimia* **2022**, *76*, 425. [[CrossRef](#)]

25. Giepmans, B.N.G.; Adams, S.R.; Ellisman, M.H.; Tsien, R.Y. The Fluorescent Toolbox for Assessing Protein Location and Function. *Science* **2006**, *312*, 217–224. [[CrossRef](#)]
26. Evens, N.; Bormans, G.M. Non-Invasive Imaging of the Type 2 Cannabinoid Receptor, Focus on Positron Emission Tomography. *Curr. Top. Med. Chem.* **2010**, *10*, 1527–1543. [[CrossRef](#)]
27. Basagni, F.; Rosini, M.; Decker, M. Functionalized Cannabinoid Subtype 2 Receptor Ligands: Fluorescent, PET, Photochromic and Covalent Molecular Probes. *ChemMedChem* **2020**, *15*, 1374–1389. [[CrossRef](#)]
28. Soethoudt, M.; Stolze, S.C.; Westphal, M.V.; van Stralen, L.; Martella, A.; van Rooden, E.J.; Guba, W.; Varga, Z.V.; Deng, H.; van Kasteren, S.I.; et al. Selective Photoaffinity Probe That Enables Assessment of Cannabinoid CB2 Receptor Expression and Ligand Engagement in Human Cells. *J. Am. Chem. Soc.* **2018**, *140*, 6067–6075. [[CrossRef](#)] [[PubMed](#)]
29. Stoddart, L.A.; Kilpatrick, L.E.; Briddon, S.J.; Hill, S.J. Probing the Pharmacology of G Protein-Coupled Receptors with Fluorescent Ligands. *Neuropharmacology* **2015**, *98*, 48–57. [[CrossRef](#)]
30. Kherlopian, A.R.; Song, T.; Duan, Q.; Neimark, M.A.; Po, M.J.; Gohagan, J.K.; Laine, A.F. A Review of Imaging Techniques for Systems Biology. *BMC Syst. Biol.* **2008**, *2*, 74. [[CrossRef](#)]
31. Singh, S.; Oyagawa, C.R.M.; Macdonald, C.; Grimsey, N.L.; Glass, M.; Vernall, A.J. Chromenopyrazole-Based High Affinity, Selective Fluorescent Ligands for Cannabinoid Type 2 Receptor. *ACS Med. Chem. Lett.* **2019**, *10*, 209–214. [[CrossRef](#)]
32. Galiègue, S.; Mary, S.; Marchand, J.; Dussossoy, D.; Carrière, D.; Carayon, P.; Bouaboula, M.; Shire, D.; Le Fur, G.; Casellas, P. Expression of Central and Peripheral Cannabinoid Receptors in Human Immune Tissues and Leukocyte Subpopulations. *Eur. J. Biochem.* **1995**, *232*, 54–61. [[CrossRef](#)]
33. Patel, K.D.; Davison, J.S.; Pittman, Q.J.; Sharkey, K.A. Cannabinoid CB(2) Receptors in Health and Disease. *Curr. Med. Chem.* **2010**, *17*, 1393–1410. [[CrossRef](#)]
34. Van Sickle, M.D.; Duncan, M.; Kingsley, P.J.; Mouihate, A.; Urbani, P.; Mackie, K.; Stella, N.; Makriyannis, A.; Piomelli, D.; Davison, J.S.; et al. Identification and Functional Characterization of Brainstem Cannabinoid CB2 Receptors. *Science* **2005**, *310*, 329–332. [[CrossRef](#)]
35. Cabral, G.A.; Raborn, E.S.; Griffin, L.; Dennis, J.; Marciano-Cabral, F. CB2 Receptors in the Brain: Role in Central Immune Function. *Br. J. Pharmacol.* **2008**, *153*, 240–251. [[CrossRef](#)]
36. López, A.; Aparicio, N.; Pazos, M.R.; Grande, M.T.; Barreda-Manso, M.A.; Benito-Cuesta, I.; Vázquez, C.; Amores, M.; Ruiz-Pérez, G.; García-García, E.; et al. Cannabinoid CB2 Receptors in the Mouse Brain: Relevance for Alzheimer's Disease. *J. Neuroinflammation* **2018**, *15*, 158. [[CrossRef](#)]
37. Ferranti, A.S.; Foster, D.J. Cannabinoid Type-2 Receptors: An Emerging Target for Regulating Schizophrenia-Relevant Brain Circuits. *Front. Neurosci.* **2022**, *16*, 925792. [[CrossRef](#)]
38. Schmöle, A.-C.; Lundt, R.; Gennequin, B.; Schrage, H.; Beins, E.; Krämer, A.; Zimmer, T.; Limmer, A.; Zimmer, A.; Otte, D.-M. Expression Analysis of CB2-GFP BAC Transgenic Mice. *PLoS ONE* **2015**, *10*, e0138986. [[CrossRef](#)]
39. Kibret, B.G.; Ishiguro, H.; Horiuchi, Y.; Onaivi, E.S. New Insights and Potential Therapeutic Targeting of CB2 Cannabinoid Receptors in CNS Disorders. *Int. J. Mol. Sci.* **2022**, *23*, 975. [[CrossRef](#)]
40. Yang, P.; Wang, L.; Xie, X.-Q. Latest Advances in Novel Cannabinoid CB(2) Ligands for Drug Abuse and Their Therapeutic Potential. *Future Med. Chem.* **2012**, *4*, 187–204. [[CrossRef](#)]
41. Smoum, R.; Grether, U.; Karsak, M.; Vernall, A.J.; Park, F.; Hillard, C.J.; Pacher, P. Editorial: Therapeutic Potential of the Cannabinoid CB2 Receptor. *Front. Pharmacol.* **2022**, *13*, 1039564. [[CrossRef](#)]
42. Bie, B.; Wu, J.; Foss, J.F.; Naguib, M. An Overview of the Cannabinoid Type 2 Receptor System and Its Therapeutic Potential. *Curr. Opin. Anaesthesiol.* **2018**, *31*, 407–414. [[CrossRef](#)]
43. Morales, P.; Hernandez-Folgado, L.; Goya, P.; Jagerovic, N. Cannabinoid Receptor 2 (CB2) Agonists and Antagonists: A Patent Update. *Expert Opin. Ther. Pat.* **2016**, *26*, 843–856. [[CrossRef](#)]
44. Berque-Bestel, I.; Soulier, J.-L.; Giner, M.; Rivail, L.; Langlois, M.; Sicsic, S. Synthesis and Characterization of the First Fluorescent Antagonists for Human 5-HT4 Receptors. *J. Med. Chem.* **2003**, *46*, 2606–2620. [[CrossRef](#)]
45. Middleton, R.J.; Briddon, S.J.; Cordeaux, Y.; Yates, A.S.; Dale, C.L.; George, M.W.; Baker, J.G.; Hill, S.J.; Kellam, B. New Fluorescent Adenosine A1-Receptor Agonists That Allow Quantification of Ligand-Receptor Interactions in Microdomains of Single Living Cells. *J. Med. Chem.* **2007**, *50*, 782–793. [[CrossRef](#)]
46. Middleton, R.J.; Kellam, B. Fluorophore-Tagged GPCR Ligands. *Curr. Opin. Chem. Biol.* **2005**, *9*, 517–525.
47. Höltke, C.; von Wallbrunn, A.; Kopka, K.; Schober, O.; Heindel, W.; Schäfers, M.; Bremer, C. A Fluorescent Photoprobe for the Imaging of Endothelin Receptors. *Bioconjug. Chem.* **2007**, *18*, 685–694.
48. Iliopoulos-Tsoutsouvas, C.; Kulkarni, R.N.; Makriyannis, A.; Nikas, S.P. Fluorescent Probes for G-Protein-Coupled Receptor Drug Discovery. *Expert Opin. Drug Discov.* **2018**, *13*, 933–947.
49. Leopoldo, M.; Lacivita, E.; Berardi, F.; Perrone, R. Developments in Fluorescent Probes for Receptor Research. *Drug Discov. Today* **2009**, *14*, 706–712.
50. McCarron, S.T.; Chambers, J.J. Modular Chemical Probes for Visualizing and Tracking Endogenous Ion Channels. *Neuropharmacology* **2015**, *98*, 41–47. [[CrossRef](#)]
51. Ma, Z.; Du, L.; Li, M. Toward Fluorescent Probes for G-Protein-Coupled Receptors (GPCRs). *J. Med. Chem.* **2014**, *57*, 8187–8203. [[CrossRef](#)] [[PubMed](#)]

52. Briddon, S.J.; Kellam, B.; Hill, S.J. Design and Use of Fluorescent Ligands to Study Ligand-Receptor Interactions in Single Living Cells. *Methods Mol. Biol.* **2011**, *746*, 211–236. [[PubMed](#)]
53. Sarott, R.C.; Westphal, M.V.; Pfaff, P.; Korn, C.; Sykes, D.A.; Gazzi, T.; Brennecke, B.; Atz, K.; Weise, M.; Mostinski, Y.; et al. Development of High-Specificity Fluorescent Probes to Enable Cannabinoid Type 2 Receptor Studies in Living Cells. *J. Am. Chem. Soc.* **2020**, *142*, 16953–16964. [[CrossRef](#)]
54. Gazzi, T.; Brennecke, B.; Atz, K.; Korn, C.; Sykes, D.; Forn-Cuni, G.; Pfaff, P.; Sarott, R.C.; Westphal, M.V.; Mostinski, Y.; et al. Detection of Cannabinoid Receptor Type 2 in Native Cells and Zebrafish with a Highly Potent, Cell-Permeable Fluorescent Probe. *Chem. Sci.* **2022**, *13*, 5539–5545. [[PubMed](#)]
55. Zhang, H.-Y.; Shen, H.; Jordan, C.J.; Liu, Q.-R.; Gardner, E.L.; Bonci, A.; Xi, Z.-X. CB2 Receptor Antibody Signal Specificity: Correlations with the Use of Partial CB2-Knockout Mice and Anti-Rat CB2 Receptor Antibodies. *Acta Pharmacol. Sin.* **2019**, *40*, 398–409. [[CrossRef](#)]
56. Cécyre, B.; Thomas, S.; Ptito, M.; Casanova, C.; Bouchard, J.-F. Evaluation of the Specificity of Antibodies Raised against Cannabinoid Receptor Type 2 in the Mouse Retina. *Naunyn Schmiedeberg's Arch. Pharmacol.* **2014**, *387*, 175–184.
57. Cooper, A.; Singh, S.; Hook, S.; Tyndall, J.D.A.; Vernall, A.J. Chemical Tools for Studying Lipid-Binding Class A G Protein-Coupled Receptors. *Pharmacol. Rev.* **2017**, *69*, 316–353.
58. Contino, M.; Abate, C.; Colabufo, N.A.; Leonetti, F.; Stefanachi, A. Cannabinoid Receptor Subtype 2 (CB2R): Features and Targets for Medical Applications. In *Medicinal Usage of Cannabis and Cannabinoids*; Elsevier: Amsterdam, The Netherlands, 2023; pp. 319–333.
59. Yates, A.S.; Doughty, S.W.; Kendall, D.A.; Kellam, B. Chemical Modification of the Naphthoyl 3-Position of JWH-015: In Search of a Fluorescent Probe to the Cannabinoid CB2 Receptor. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 3758–3762. [[CrossRef](#)] [[PubMed](#)]
60. Petrov, R.R.; Ferrini, M.E.; Jaffar, Z.; Thompson, C.M.; Roberts, K.; Diaz, P. Design and Evaluation of a Novel Fluorescent CB2 Ligand as Probe for Receptor Visualization in Immune Cells. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 5859–5862. [[CrossRef](#)]
61. Cooper, A.G.; MacDonald, C.; Glass, M.; Hook, S.; Tyndall, J.D.A.; Vernall, A.J. Alkyl Indole-Based Cannabinoid Type 2 Receptor Tools: Exploration of Linker and Fluorophore Attachment. *Eur. J. Med. Chem.* **2018**, *145*, 770–789.
62. Rinaldi-Carmona, M.; Barth, F.; Millan, J.; Derocq, J.M.; Casellas, P.; Congy, C.; Oustric, D.; Sarran, M.; Bouaboula, M.; Calandra, B.; et al. SR 144528, the First Potent and Selective Antagonist of the CB2 Cannabinoid Receptor. *J. Pharmacol. Exp. Ther.* **1998**, *284*, 644–650.
63. Sexton, M.; Woodruff, G.; Horne, E.A.; Lin, Y.H.; Muccioli, G.G.; Bai, M.; Stern, E.; Bornhop, D.J.; Stella, N. NIR-Mbc94, a Fluorescent Ligand That Binds to Endogenous CB(2) Receptors and Is Amenable to High-Throughput Screening. *Chem. Biol.* **2011**, *18*, 563–568.
64. Bai, M.; Sexton, M.; Stella, N.; Bornhop, D.J. MBC94, a Conjugable Ligand for Cannabinoid CB 2 Receptor Imaging. *Bioconjug. Chem.* **2008**, *19*, 988–992. [[CrossRef](#)]
65. Zhang, S.; Shao, P.; Bai, M. In Vivo Type 2 Cannabinoid Receptor-Targeted Tumor Optical Imaging Using a near Infrared Fluorescent Probe. *Bioconjug. Chem.* **2013**, *24*, 1907–1916. [[CrossRef](#)] [[PubMed](#)]
66. Zhang, S.; Shao, P.; Ling, X.; Yang, L.; Hou, W.; Thorne, S.H.; Beaino, W.; Anderson, C.J.; Ding, Y.; Bai, M. In Vivo Inflammation Imaging Using a CB2R-Targeted near Infrared Fluorescent Probe. *Am. J. Nucl. Med. Mol. Imaging* **2015**, *5*, 246–258.
67. Wu, Z.; Shao, P.; Zhang, S.; Bai, M. Targeted Zwitterionic near Infrared Fluorescent Probe for Improved Imaging of Type 2 Cannabinoid Receptors. *J. Biomed. Opt.* **2014**, *19*, 36006. [[CrossRef](#)]
68. Zhang, S.; Jia, N.; Shao, P.; Tong, Q.; Xie, X.-Q.; Bai, M. Target-Selective Phototherapy Using a Ligand-Based Photosensitizer for Type 2 Cannabinoid Receptor. *Chem. Biol.* **2014**, *21*, 338–344. [[PubMed](#)]
69. Jia, N.; Zhang, S.; Shao, P.; Bagia, C.; Janjic, J.M.; Ding, Y.; Bai, M. Cannabinoid CB2 Receptor as a New Phototherapy Target for the Inhibition of Tumor Growth. *Mol. Pharm.* **2014**, *11*, 1919–1929. [[CrossRef](#)] [[PubMed](#)]
70. Spinelli, F.; Capparelli, E.; Abate, C.; Colabufo, N.A.; Contino, M. Perspectives of Cannabinoid Type 2 Receptor (CB2R) Ligands in Neurodegenerative Disorders: Structure-Affinity Relationship (SAfIR) and Structure-Activity Relationship (SAR) Studies. *J. Med. Chem.* **2017**, *60*, 9913–9931.
71. Aghazadeh Tabrizi, M.; Baraldi, P.G.; Saponaro, G.; Moorman, A.R.; Romagnoli, R.; Preti, D.; Baraldi, S.; Ruggiero, E.; Tintori, C.; Tuccinardi, T.; et al. Discovery of 7-Oxopyrazolo[1,5-a]Pyrimidine-6-Carboxamides as Potent and Selective CB(2) Cannabinoid Receptor Inverse Agonists. *J. Med. Chem.* **2013**, *56*, 4482–4496. [[CrossRef](#)] [[PubMed](#)]
72. Ling, X.; Zhang, S.; Shao, P.; Li, W.; Yang, L.; Ding, Y.; Xu, C.; Stella, N.; Bai, M. A Novel Near-Infrared Fluorescence Imaging Probe That Preferentially Binds to Cannabinoid Receptors CB2R over CB1R. *Biomaterials* **2015**, *57*, 169–178. [[CrossRef](#)] [[PubMed](#)]
73. Guo, X.; Ling, X.; Du, F.; Wang, Q.; Huang, W.; Wang, Z.; Ding, X.; Bai, M.; Wu, Z. Molecular Imaging of Pancreatic Duct Adenocarcinoma Using a Type 2 Cannabinoid Receptor-Targeted Near-Infrared Fluorescent Probe. *Transl. Oncol.* **2018**, *11*, 1065–1073. [[PubMed](#)]
74. Wu, Z.; Shao, P.; Zhang, S.; Ling, X.; Bai, M. Molecular Imaging of Human Tumor Cells That Naturally Overexpress Type 2 Cannabinoid Receptors Using a Quinolone-Based near-Infrared Fluorescent Probe. *J. Biomed. Opt.* **2014**, *19*, 76016. [[CrossRef](#)] [[PubMed](#)]
75. Cooper, A.G.; Oyagawa, C.R.M.; Manning, J.J.; Singh, S.; Hook, S.; Grimsey, N.L.; Glass, M.; Tyndall, J.D.A.; Vernall, A.J. Development of Selective, Fluorescent Cannabinoid Type 2 Receptor Ligands Based on a 1,8-Naphthyridin-2-(1H)-One-3-Carboxamide Scaffold. *Medchemcomm* **2018**, *9*, 2055–2067.

76. Spinelli, F.; Giampietro, R.; Stefanachi, A.; Riganti, C.; Kopecka, J.; Abatematteo, F.S.; Leonetti, F.; Colabufo, N.A.; Mangiatordi, G.F.; Nicolotti, O.; et al. Design and Synthesis of Fluorescent Ligands for the Detection of Cannabinoid Type 2 Receptor (CB2R). *Eur. J. Med. Chem.* **2020**, *188*, 112037.
77. Haider, A.; Kretz, J.; Gobbi, L.; Ahmed, H.; Atz, K.; Bürkler, M.; Bartelmus, C.; Fingerle, J.; Guba, W.; Ullmer, C.; et al. Structure-Activity Relationship Studies of Pyridine-Based Ligands and Identification of a Fluorinated Derivative for Positron Emission Tomography Imaging of Cannabinoid Type 2 Receptors. *J. Med. Chem.* **2019**, *62*, 11165–11181. [[CrossRef](#)]
78. Haider, A.; Gobbi, L.; Kretz, J.; Ullmer, C.; Brink, A.; Honer, M.; Woltering, T.J.; Muri, D.; Iding, H.; Bürkler, M.; et al. Identification and Preclinical Development of a 2,5,6-Trisubstituted Fluorinated Pyridine Derivative as a Radioligand for the Positron Emission Tomography Imaging of Cannabinoid Type 2 Receptors. *J. Med. Chem.* **2020**, *63*, 10287–10306. [[CrossRef](#)]
79. Kosar, M.; Sykes, D.A.; Viray, A.E.G.; Vitale, R.M.; Sarott, R.C.; Ganzoni, R.L.; Onion, D.; Tobias, J.M.; Leippe, P.; Ullmer, C.; et al. Platform Reagents Enable Synthesis of Ligand-Directed Covalent Probes: Study of Cannabinoid Receptor 2 in Live Cells. *J. Am. Chem. Soc.* **2023**, *145*, 15094–15108. [[CrossRef](#)]
80. Khan, M.I.; Sobocińska, A.A.; Czarnecka, A.M.; Król, M.; Botta, B.; Szczylik, C. The Therapeutic Aspects of the Endocannabinoid System (ECS) for Cancer and Their Development: From Nature to Laboratory. *Curr. Pharm. Des.* **2016**, *22*, 1756–1766. [[CrossRef](#)]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.