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MBC94, a Conjugable Ligand for Cannabinoid CB_2 Receptor Imaging

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Abstract

Cannabinoid CB_2 receptor is a particularly attractive target for noninvasive imaging of neuroinflammation and monitoring of therapeutic efficacy. Its expression is low to undetectable in healthy brain and induced in resident microglial cells (the macrophage of the brain) after cerebral ischemia, injury, and in neuroinflammatory disease. Additionally, immune cells migrating across the blood–brain barrier typically express CB_2 receptors, which adds to the expression pool of this target and provides a reliable indicator of inflammation in the brain. Here, we synthesized a novel conjugable CB_2 receptor ligand, mbc94, which has a terminal amino group that allows for facile conjugation to imaging moieties. A near-infrared (NIR) dye labeled mbc94, NIRmbc94, was developed for CB_2 targeted imaging. Preliminary evidence, including *in vitro* fluorescence imaging and a competition study, showed that NIRmbc94 specifically labeled CB_2-expressing cells.

Two cannabinoid receptors, CB_1 and CB_2, have been identified at the molecular level (1, 2). They are G protein-coupled receptors that share an overall 44% amino acid homology and a 68% amino acid homology within their transmembrane domains (1). CB_1 receptors are abundantly expressed by neurons, whereas CB_2 receptors are abundantly expressed by immune cells (3, 4).

The high level of CB_2 receptor expression in immune cells and much lower expression in other cell types, particularly in the CNS, makes this receptor an attractive target for imaging and monitoring of therapy (3, 4) for neurological diseases. Specifically, CB_2 receptor expression is high in spleen, tonsils, and thymus and low—or even undetectable—in brain, thyroid, retina, placenta, skeletal muscle, kidney, liver, adrenal gland, heart, prostate, and ovary (4). This expression profile provides great opportunities for imaging with low background. Furthermore, CB_2 receptor expression is highly plastic and may be induced under specific disease conditions, for example, in tumor cells (5) and CNS-resident

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Supporting Information Available: Details of experimental section. This material is available free of charge via the Internet at http://pubs.acs.org.

microglia. Accordingly, CB_2 receptor has become a predominant target for drug development aimed at treating pain (6, 7), chronic inflammation (8), osteoporosis (9), malignant gliomas (10), tumors of immune origin (11), and immunological disorders (12,13), and thus developing a tool that allows for its precise mapping in tissue is essential.

Together with the characterization of the CB_2 receptor, a considerable effort has been made to develop CB_2 receptor–ligands. The term cannabinoid was first used to describe terpenophenolic compounds in *Cannabis sativa* L., among which (−)-trans-Δ^9-tetrahydrocanabinol (Δ^9-THC) is the main bioactive constituent (14). Many anti-inflammatory effects of Δ^9-THC have been described, including inhibition of tumor necrosis factor-α, interleukin-2, nitric oxide, and arachidonic acid production from macrophages and T cells (8, 15).

CB_2 receptor ligands can be divided into three main groups: plant-derived, endogenous, and synthetic. The best-known plant-derived cannabinoid is Δ^9-THC, but cannabinol and cannabidiol also induce profound biological effects (16). Two endocannabinoids, arachidonoylethanolamide (anandamide) and 2-arachidonoyl glycerol (2-AG), have been identified (Figure 1) (17). Both molecules have greater affinity at CB_1 than CB_2 receptors (18). 2-AG acts as a full agonist at CB_1 and CB_2 receptor, and anandamide acts as a partial agonist (showing mixed agonist–antagonist properties) toward these receptors (19). Many synthetic cannabinoid receptor ligands have been developed, including HU-210, CP55940, WIN55212–2, SR141617A, AM630, and SR144528 (Figure 2). HU-210, CP55940, and WIN55212–2 are cannabinoid receptor agonists with no or marginal CB_1/CB_2 selectivity (18). AM630 and SR144528 are both selective CB_2 receptor ligands and behave as inverse agonists rather than “silent” or “neutral” antagonists. The CB_2/CB_1 affinity ratio is less for AM630 (CB_2/CB_1 affinity = 165) than for SR144528 (CB_2/CB_1 affinity > 700) (18). Accordingly, SR144528 has been widely used as a pharmacological tool to determine CB_2 receptor-mediated effects (18). However, the use of SR144528 for CB_2 receptor-targeted imaging has never been tested directly, since SR144528 is not conjugable. In other words, signaling moieties, such as fluorescent dyes, lanthanide chelates, and nanoparticles, cannot be easily coupled to SR144528. Thus, to further study CB_2 receptor and diseases associated with an increase in the expression of this receptor, development of a conjugable SR144528 analogue constitutes an essential step. Here, we synthesized such a conjugable SR144528 analogue, mbc94. To our knowledge, this is the only fully conjugable CB_2 receptor ligand in existence. It has a terminal amino group allowing easy conjugation to other molecules, including imaging moieties that can provide opportunities for CB_2 receptor-targeted imaging. A near-infrared (NIR) dye, IRDye 800CW NHS ester, was selected to label mbc94 for optical imaging. The resulting imaging agent, NIRmbc94, was used to label CB_2-expressing cells. Preliminary fluorescence imaging of live cells and competition study showed that indeed NIRmbc94 specifically labeled CB_2-expressing cells.

The synthetic pathway of the conjugable SR 144528 analogue, mbc94, is shown in Scheme 1. Compound **3** was prepared from 4′-chloro-3′methylacetophenone and diethyl oxalate as previously described (20). Formation of pyrazoles **4** and **5** was achieved by following literature procedures (21). Fenchylamine **8** was prepared by converting fenchone to a formamide **7**, followed by hydrolysis of the amide bond as previously described (22).

Compound **9** was prepared using thionyl chloride before, but the yield was relatively low (70%) (21). We synthesized **9** by regular peptide coupling using 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate (HBTU), and the yield was improved to 84%. The following N-alkylation with α,α-dibromo-*p*-xylene produced **10**, and another N-alkylation with 1,6-hexane diamine yielded the conjugable CB_2 receptor ligand, mbc94.

The use of NIR (650–900 nm) light has gained increasingly wide acceptance in molecular imaging during recent years (23) in part because tissues are relatively transparent in this region of the electromagnetic spectrum, with hemoglobin, water, and lipids exhibiting low absorption coefficients (24). Therefore, we labeled mbc94 with a NIR dye, IRDye 800CW NHS ester, for NIR optical imaging. The reaction was monitored by analytical HPLC at 780 nm and the product, IRDye 800CW-mbc94 (NIRmbc94), was purified using a semipreparative HPLC and characterized by NMR and mass spectrometry. The absorption and emission spectra were then taken (Figure 3). NIRmbc94 has maximum absorption at 779 nm and emission at 797 nm (water), allowing deep tissue emission for enhanced *in vivo* imaging.

To test whether NIRmbc94 reliably binds to the CB_2 receptor, we already had in place a system wherein the receptor was both absent and present. Specifically, the highly malignant mouse astrocytoma cell line, DBT, lacks the targeted receptor (wild-type), and we generated a clone that stably expresses our target, 2D4DBT. First, we demonstrated that NIRmbc94 gave a significant signal ($S/N = 1.6$), whereas the “free” dye (IRDye 800CW acid) does not produce significant fluorescence, as illustrated by microscopy in the NIR (thus indicating a lack of nonspecific binding due to the dye: Figure 4). Next, as a preliminary indicator of specific targeting of our receptor, both wild-type cells and 2D4 clones were incubated 5 μM concentration of NIRmbc94. Figure 5B,E shows that the fluorescence signal is relatively low in wild-type DBT cells compared to the clone (A,D).

A competition study gave further evidence for specific binding of NIRmbc94 to CB_2 receptors. Specifically, in a preliminary competitive binding experiment, the fluorescence signal was significantly reduced when 100 nM SR144528 was added to compete with 5 μM NIRmbc94 at the receptor site. The lack of fluorescence is due to the higher-affinity unlabeled ligand, SR144528 (25), occupying the receptor site and thus inhibiting binding by NIRmbc94. This preliminary pharmacological characterization is typical, and the data indicate that we have indeed labeled our target of interest. More detailed pharmacological and biological characterization, including binding affinity (K_d) and reliable measurement of receptor expression (B_{max}) are in order and forthcoming.

In conclusion, we developed a conjugable CB_2 receptor ligand, mbc94, which has a terminal amino group, making it universally conjugable. An NIR dye-labeled mbc94, NIRmbc94, specifically labeled CB_2-expressing DBT cells, whereas the same cells incubated with same concentration of free NIR dye did not show any significant signal. In addition, the reduced fluorescence signal was observed from non-CB_2 expressing wildtype DBT cells incubated with NIRmbc94 compared to CB_2-expressing DBT cells incubated with the same concentration of NIRmbc94. Finally, the specific binding of NIRmbc94 to CB_2 receptors was confirmed by *in vitro* competition study. A preliminary competition study in which cells

were coincubated with NIRmbc94 and SR144528 showed signal reduction compared to cells incubated with NIRmbc94 only. Overall, mbc94 constitutes a promising conjugable CB_2 receptor ligand. NIRmbc94 specifically binds to CB_2 receptors and can potentially be used to image CB_2-expressing cells *in vivo*, including immune and cancer cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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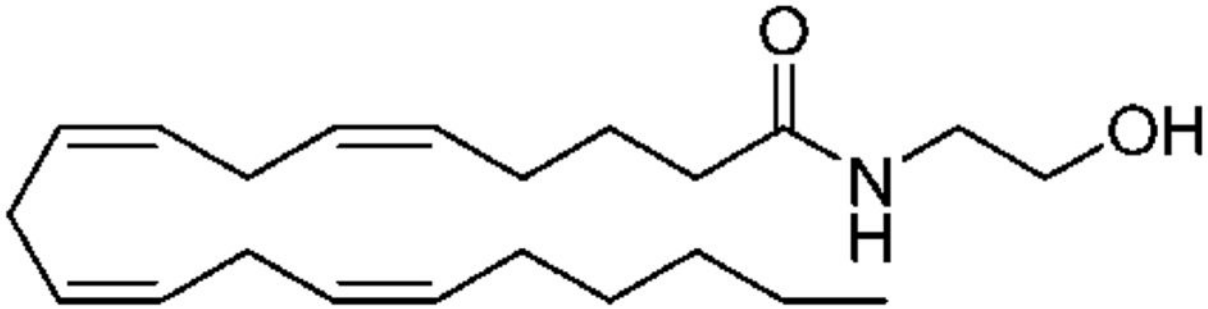
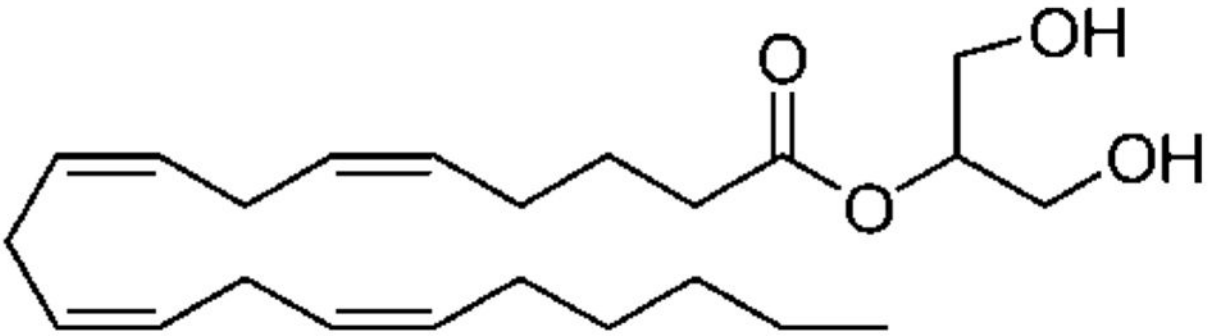


Anandamide



2-Arachidonyl-glycerol (2-AG)

Figure 1.
Structures of endogenous CB_2 receptor ligands.

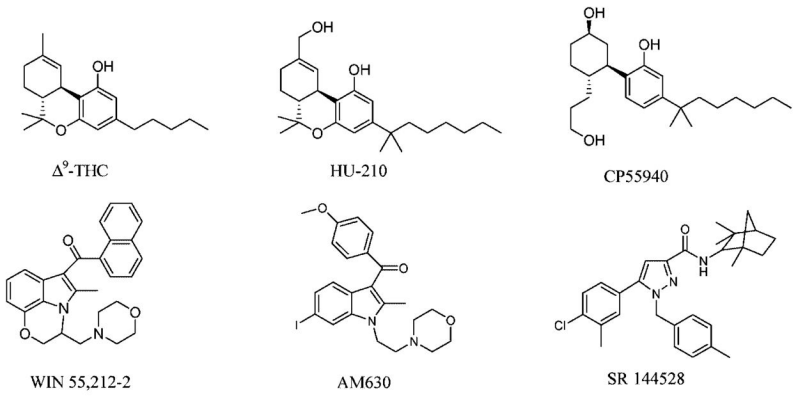


Figure 2.
Structures of plant-derived and synthetic CB_2 receptor ligands.

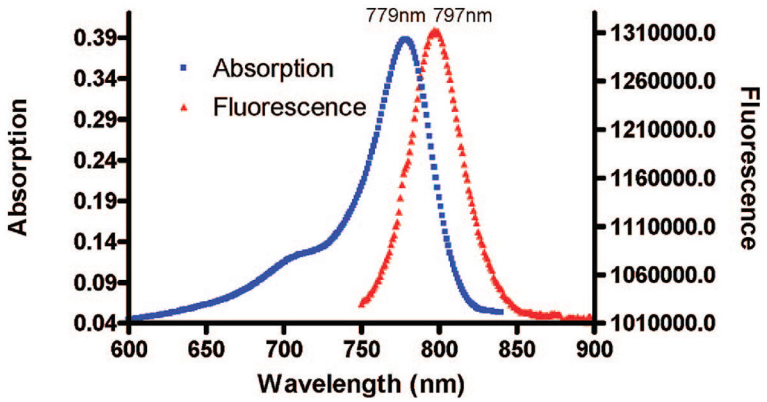


Figure 3.
NIRmbc94 absorption and fluorescence in water.

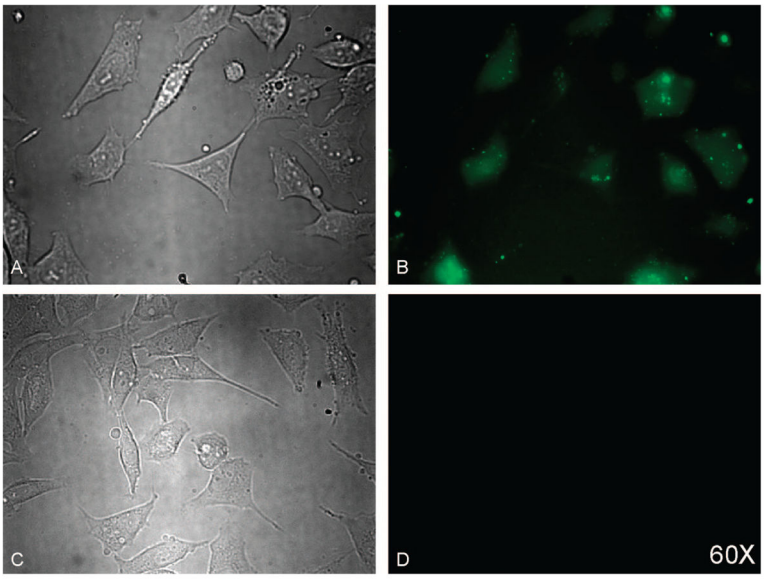


Figure 4.

Fluorescence imaging of DBT cells incubated with NIRmbc94 or free NIR dye: (A) phase contrast microscopy of cells dosed with NIRmbc94; (B) fluorescence imaging of cells dosed with 5 μM NIRmbc94; (C) phase contrast microscopy of cells dosed with free NIR dye; (D) fluorescence imaging of cells dosed with 5 μM free NIR dye (control).

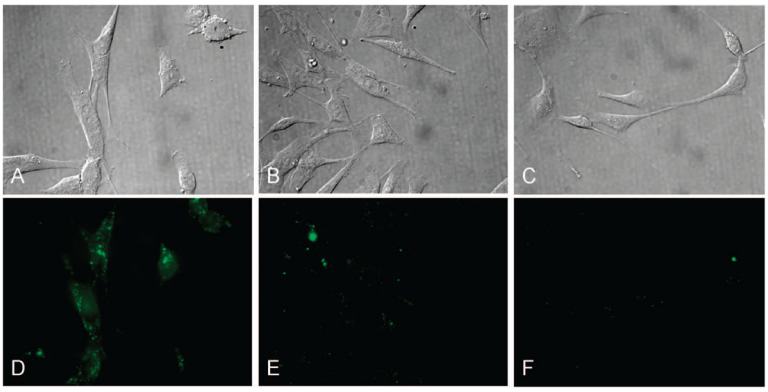
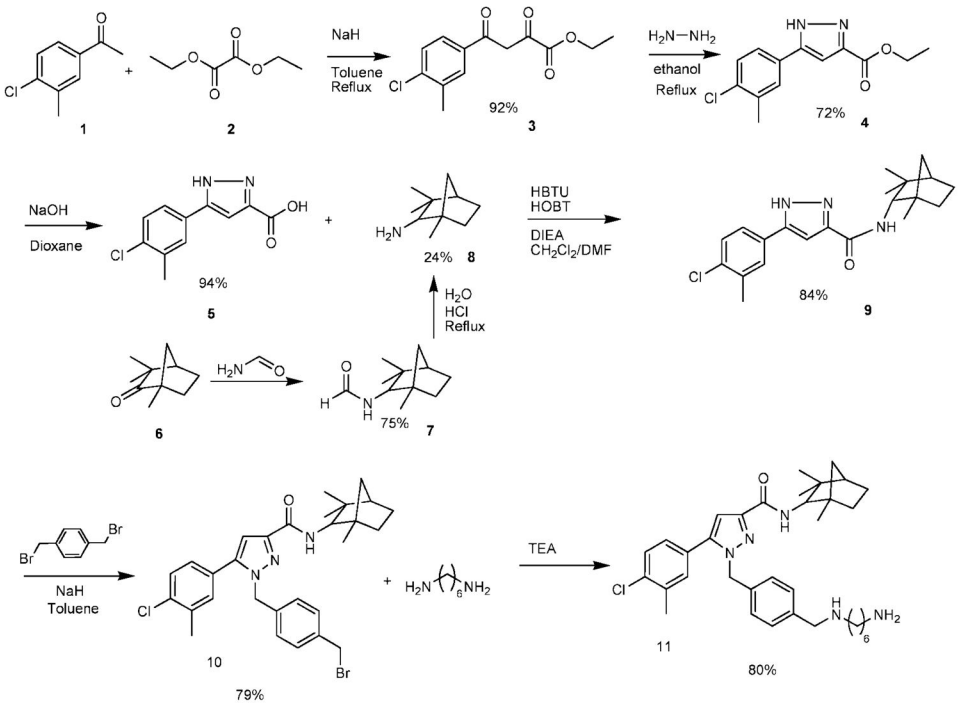
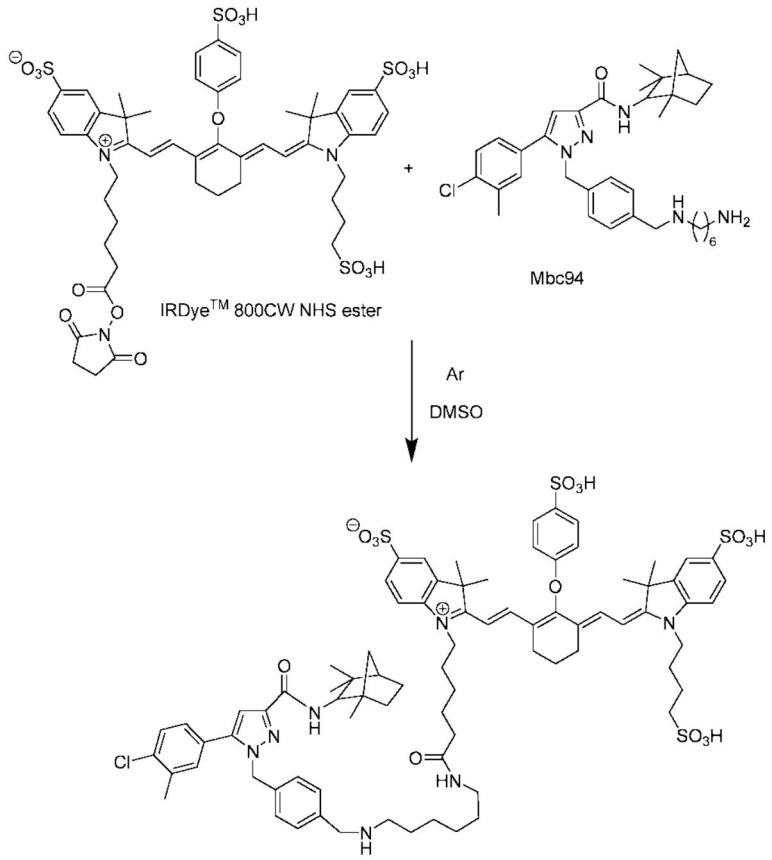


Figure 5.

Fluorescence imaging of 2D4 (CB_2 expressing) and wild-type (WT, non-CB_2 expressing) DBT cells: (A) phase contrast microscopy of 2D4 DBT cells dosed with 5 μM NIRmbc94; (B) phase contrast microscopy of WT DBT cells dosed with 5 μM NIRmbc94; (C) phase contrast microscopy of 2D4 DBT cells dosed with 5 μM NIRmbc94 and 100 nM SR144528; (D) fluorescence imaging of 2D4 DBT cells dosed with 5 μM NIRmbc94; (E) fluorescence imaging of WT DBT cells dosed with 5 μM NIRmbc94; (F) fluorescence imaging of 2D4 DBT cells dosed with 5 μM NIRmbc94 and 100 nM SR144528.



Scheme 1.
Synthesis of mbc94



Scheme 2.
Synthesis of NIRmbc94