The endogenous cannabinoid system (ECS) regulates a broad number of physiological processes, and perturbations in its normal functioning are linked to many disorders.[1] In this respect, the development of high-sensitivity and high-throughput analytical tools that afforded a broader view of the ECS would be highly valuable. As such, small-molecule fluorescent probes could provide dynamic information concerning the direct spatial and temporal expression levels of cannabinoid receptors as has been recently reported for some specific classes of enzymes.[2] Accordingly, the development of small-molecule probes that are able to recognize cannabinoid receptors is an area of current interest, because they could complement and even overcome some of the drawbacks of the available antibodies.[3] Recent attempts toward this goal have been mainly focused on the CB₁ receptor (CB₁= cannabinoid receptor type 2). However, these probes show moderate affinities \( K_i = (260–387) \text{ nM} \), and their application in native systems is limited.[4] Similarly, endocannabinoid-based probes display modest affinities for CB₁ and CB₂ receptors \( [(84.7–450) \text{ nM}] \).[5] A fact that could limit their use in complex systems. Therefore, we focused our efforts on the synthetic high-affinity cannabinoid ligands HU210 \( K_i(\text{CB}_1, R) = 0.061 \text{ nM} \), \( K_i(\text{CB}_2, R) = 0.52 \text{ nM} \).[6] and HU308 \( K_i(\text{CB}_1, R) > 10000 \text{ nM} \), \( K_i(\text{CB}_2, R) = 22.7 \text{ nM} \).[7] (Scheme 1).

Among the different tags, biotin was selected owing to its versatility for detection by a variety of readily available (strept)avidin conjugates. A closer look to the structure of these ligands revealed that the most straightforward possibility was to attach the tag at the free hydroxy groups (Scheme 1).

![Scheme 1. Structures of the synthetic cannabinoid ligands HU210 and HU308 and of synthesized probes 1–3.](image)

Ligand HU210 was prepared as previously described.[8] For the introduction of the tag, we made some attempts to selectively acylate the allylic hydroxy group using either Mitsuobu conditions[9] or the HfCl₄·2THF catalyst.[10] However, none of them gave good yields in our hands and we carried out the reaction between ligand HU210 and \( \text{N}^-\text{-biotinyl-6-aminohexanoic} \) acid in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and 4-dimethylaminopyridine. Under these conditions, formation of biacylated product was not observed, starting material was partly recovered, and compounds 1 and 2 were obtained after separation by column chromatography with 25 % and 14 % yields, respectively. Ligand HU308 was obtained as previously described[11] from commercially available \( \alpha\)-pinene. Esterification of ligand HU308 with \( \text{N}^-\text{-biotinyl-6-aminohexanoic} \) acid gave derivative 3 in 55 % yield. Compounds 1–3 were
tested for their affinities to CB₁ and CB₂ receptors, showing $K_i$ values in the nanomolar range (Table 1). It is especially relevant that probes 1 and 2, derived from ligand HU210, keep a high affinity for both cannabinoid receptors and that probe 3 keeps the selectivity feature of its parent compound, HU308, with high affinity for the CB₂ receptor whilst being inactive at the CB₁ receptor.

Table 1: Binding affinities of synthesized probes 1–3 to the CB₁ and CB₂ receptors.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_i$ (CB₁-R) [nM]</th>
<th>$K_i$ (CB₂-R) [nM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.4 ± 0.4</td>
<td>1.6 ± 0.4</td>
</tr>
<tr>
<td>2</td>
<td>11 ± 2</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>3</td>
<td>&gt; 5000</td>
<td>44 ± 4</td>
</tr>
</tbody>
</table>

[a] $K_i$ values are given as the mean ± standard error from 2–3 independent experiments performed in triplicate.

To propose structural hypotheses for the binding of these compounds, we constructed three-dimensional models of the CB₁ and CB₂ receptors using the crystal structure of the homologous sphingosine 1-phosphate receptor 1 (S1P₁)[15] as a template (the Supporting Information and Figure S1). Docking of ligands HU210 and HU308 into the receptor models (the Supporting Information and Figure S2) places the hydroxy groups pointing toward the extracellular environment. However, the entrance of the ligand into the binding pocket has been proposed very recently to be from the lipid bilayer, in particular through an opening between transmembrane helices (TMs) 1 and 7 (Figure 1A)[11]. Thus, in ligand HU210, the hydroxy group attached to the phenyl ring is more distant to this opening than its allylic hydroxy group. As a consequence, probes 1 (Figure 1A, B) and 3 (Figure 1C) expand their biotin chains through this channel more favorably than probe 2 (not shown). Importantly, attachment of the biotin chain to ligands HU210 or HU308 decreases the binding affinity in a more pronounced manner in CB₁-R than CB₂-R (Table 1). According to the Ballesteros–Weinstein numbering scheme the side chains at positions 1.32 (Q in CB₁-R and K in CB₂-R), 1.35 (I in CB₁-R and V in CB₂-R), 7.34 (V in CB₁-R and A in CB₂-R), and 7.36 (A in CB₁-R and A in CB₂-R) define this channel between TMs 1 and 7. Thus, we hypothesize that this effect is due to the presence of amino acid residue K1.32 in the CB₁ receptor, which stabilizes the carbonyl group of the biotin chain in a more significant manner than residue Q1.32 of the CB₂ receptor.

Compounds 1–3 were used for in vitro labeling of cannabinoid receptors in transiently transfected hippocampal neuronal (HT22) cells (data not shown). In these experiments, probe 1 performed better than 2, either because of the higher exposure of the biotin moiety as suggested by the computational model or because of the slightly higher affinity for CB₁-R (Table 1). Therefore, we selected probes 1 and 3 for their use in native systems, and neurons and microglia were chosen as relevant systems for CB₁ and CB₂ receptors, respectively. Probe 1 is able to label neurons in primary culture (Figure 2A, B). Next, we checked the specificity of the labeling by parallel experiments in the presence of an excess of ligand HU210, which abolished labeling (Figure 2C). As expected, probe 3, with no affinity for CB₁-R, did not show any significant fluorescence in neurons (Figure 2D). Notably, this labeling is fully compatible with the simultaneous use of primary antibodies in colabeling studies (Figure 3). Probe 1 shows the predominant location of CB₁ receptor in the neuronal soma (Figure 3A), whereas the anti-MAP2 antibody labels the entire neuron (Figure 3B), including axon and dendrites, as expected from this marker. Labeling of CB₁ receptor by probe 1 (Figure 3D) is further confirmed by double staining with a commercial antibody against this receptor (Figure 3E, F).

To further explore the potential of the probes in a different native cell system, we assessed their performance in micro-
glial cells, also known as the macrophages of the brain. These cells express CB₂R, which is believed to play a fundamental role in their immune-related functions.

In agreement with the binding profile of the probes, both probes 1 and 3 allowed visualization of CB₂R in microglia (Figure 4A, B) but not in the presence of an excess of ligand HU210 (100 μM). D) Neurons incubated with the CB₂R-selective probe 3. All samples were imaged under the same conditions by using a Zeiss fluorescence inverted microscope. Nuclei are shown in blue. Bars: 25 μm.

Importantly, in microglial cells, labeling with probe 3 clearly reflects the changes in CB₂R expression between a resting state (Figure 5, panels A–C) and an activated (pro-inflammatory) phenotype induced by lipopolysaccharide (LPS) exposure (Figure 5, panels D–F).

Considering the relevance of the ECS in the regulation of the immune system we studied the potential of these probes in flow cytometry. The application of this methodology would facilitate the profiling of cannabinoid receptors at the single-cell level.
cell level and could contribute to shed light on the function of the ECS in the immune system, a field of utmost current interest.\textsuperscript{13} Probes were tested in the monocytic cell line THP-1, which has been described to have a functional endocannabinoid system.\textsuperscript{14} Cells were incubated with probe 1 (1 μM) and then stained with streptavidin-Alexa Fluor 488 and analyzed by flow cytometry (Figure 6). Although the expression of CB\textsubscript{1} and CB\textsubscript{2} receptors is moderate in unstimulated THP-1 cells as assessed by western blot (data not shown), our experiments clearly demonstrate the suitability of probe 1 to visualize cannabinoid receptors in this cell line by flow cytometry.

In conclusion, the probes described herein are, to our knowledge, the first small-molecule tools suitable for visualization of CB\textsubscript{1} and CB\textsubscript{2} receptors in native cell systems of physiological relevance with at least comparable potency to antibodies. Taking into account the practical limitations that these antibodies have, these probes increase the arsenal of chemical tools for the study of cannabinoid receptors and may help to dissect the complex roles of the ECS. Further extension of the versatility of this approach is currently ongoing.

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