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Discovery of a Photoaffinity Probe that Captures the Active Conformation of the Cannabinoid CB_2 Receptor

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The cannabinoid receptor type 2 (CB_2R) is a G protein-coupled receptor with therapeutic potential for the treatment of inflammatory disorders. Fluorescent probes are desirable to study its receptor localization, expression and occupancy. Previously, we have reported a photoaffinity probe LEI-121 that stabilized the inactive conformation of the CB_2R. Here, we report the structure-based design of a novel bifunctional probe that captures the active conformation of the CB_2R upon irradiation with light. An alkyne handle was incorporated to visualize the receptor using click-chemistry with fluorophore-azides. These probes may hold promise to study different receptor conformations in relation to their cellular localization and function.

Introduction

G protein-coupled receptors (GPCRs) constitute a valuable protein family for drug discovery. The cannabinoid receptor type 2 (CB_2R) is a GPCR expressed in cells of the immune system and is considered as a potential drug target due to its anti-inflammatory effects upon activation.[1] However, its low endogenous levels and inducible nature complicate the understanding of its cellular mechanism-of-action, which makes the drug discovery process more challenging. Chemical and biological tools can aid in the cellular characterization of CB_2R.[2] Antibodies are widely used to study GPCR localization and expression, but CB_2R antibodies suffer from poor quality, low selectivity and large batch to batch variability.[3] Therefore, the development of fluorescent chemical probes targeting CB_2R has become an attractive strategy to study its cellular localization, dynamics and occupancy by drugs.[4–6] For example, previously a probe with a silicon-rhodamine fluorophore was reported that was cell-permeable and able to selectively label the CB_2 receptor in CB_2R overexpressing cells as well as in primary cultures of human macrophages.[1]

We have contributed to this field by developing LEI-121 (**1**, Figure 1), a photoaffinity probe that is able to monitor endogenous CB_2R expression levels and their occupancy.[7] LEI-121 is a bifunctional CB_2R-selective probe based on the 5-fluoropyridin-2-yl-benzyl-imidazolidine-2,4-dione scaffold.[8] It includes a diazirine group that forms a covalent bond with CB_2R upon photoactivation. The alkyne click handle allows for ligand binding to the receptor prior to incorporation of a fluorescent tag. This two-step photoaffinity-based protein profiling (ABPP) strategy prevents a decrease of the binding affinity and reduces non-specific binding due to the bulky fluorophore. LEI-121 was able to label CB_2R in gel-based ABPP using recombinant CB_2R, and detected endogenous CB_2R in primary human immune cells using flow cytometry.[7]

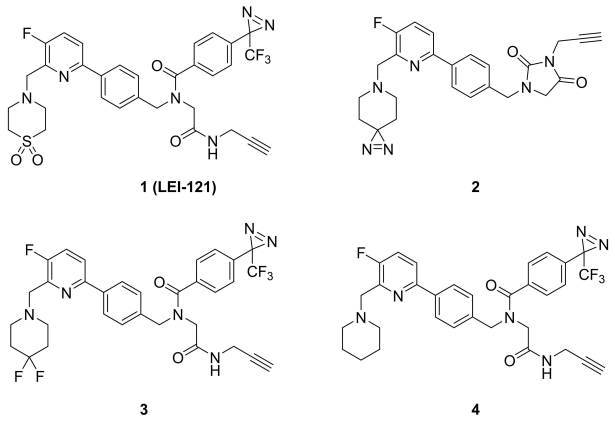

Figure 1. The chemical structures of LEI-121 (**1**) and the newly designed two-step photo affinity probes **2**–**4**.

LEI-121 is an inverse agonist and reduces the constitutive activity of CB_2Rs.[7] To complete the toolbox and allow targeting of active CB_2Rs, we set out to develop a new probe that behaves as an agonist. Here we describe the structure-based design and synthesis of photo-affinity probes (**2**-**4**), which were based on the recently published structure of LEI-102, a close analog of LEI-121, in complex with the CB_2R elucidated by cryogenic electron microscopy (cryo-EM) (Figure 1 and 2).[9] LEI-102 is a high-affinity CB_2R ligand (pK_i of 8.0 ± 0.1) with over

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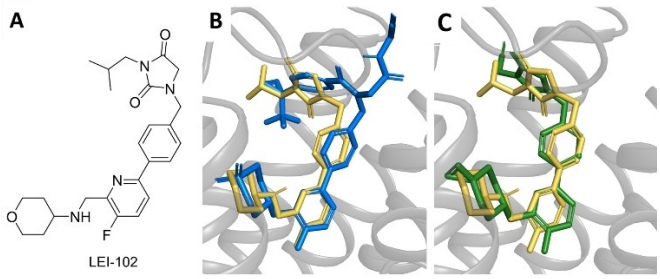


Figure 2. The chemical structure of LEI-102 (A), and the docked poses of LEI-121 (blue, B) and **2** (green, C) overlayed with the cryo-EM structure of LEI-102 (yellow). Docking was performed in the cryo-EM structure of CB2R containing LEI-102 (PDB: 8GUT).

1000-fold selectivity over CB_1R. LEI-102 activates the CB_2 receptor as a partial agonist (pEC_{50} 6.9 ± 0.2, E_{max} 76 ± 1 %).[9]

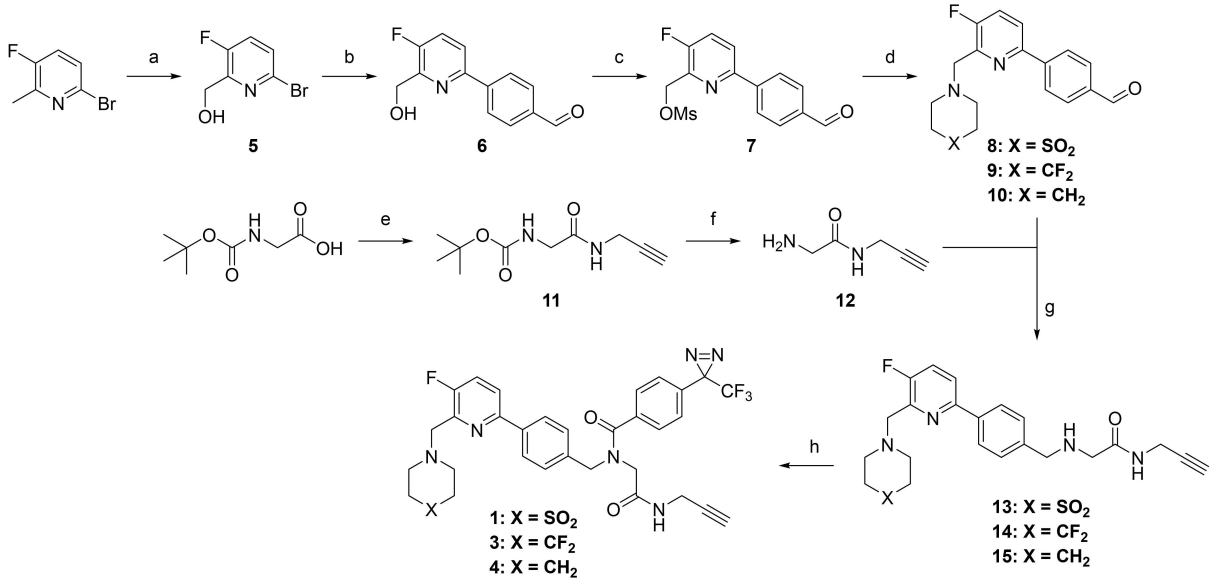
Results and Discussion

Design

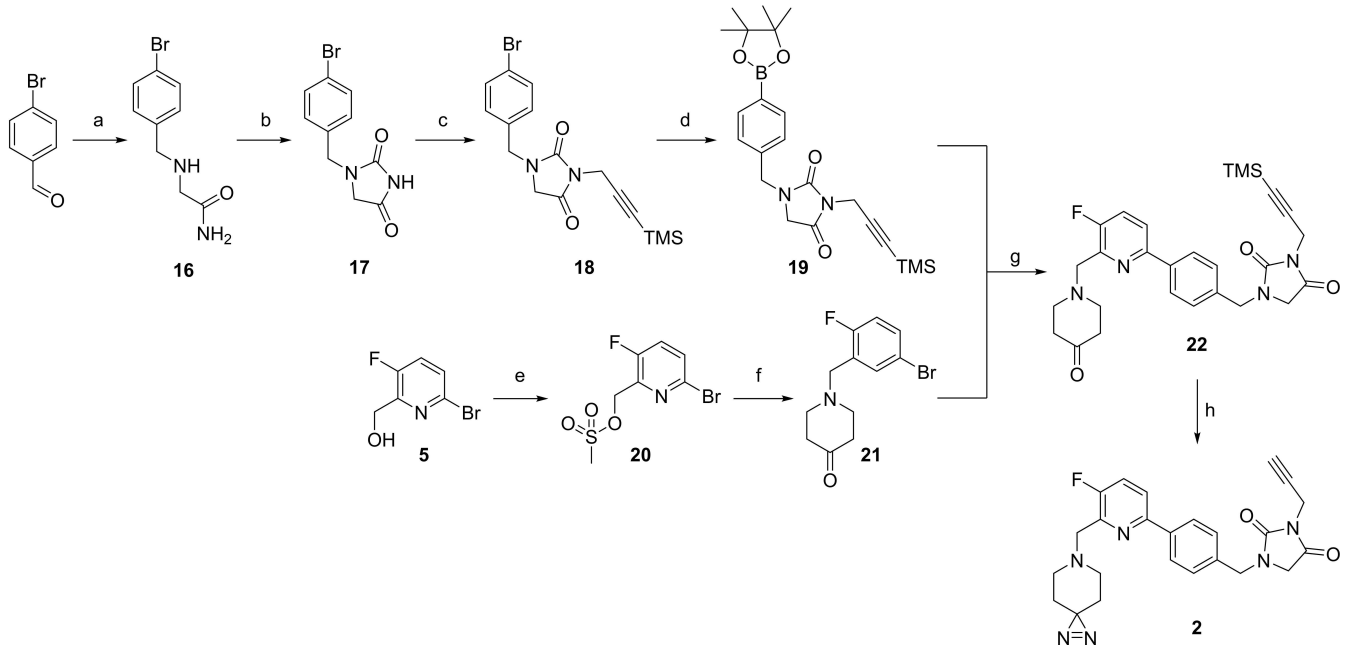
To design novel photoreactive probes, we docked LEI-121 into the recently published CB_2R cryo-EM structure (Figure 2B).[9] The CB_2R-G_i structure was reported in complex with CB_2R selective agonist LEI-102 with a resolution 2.9 Å.[9] Considering previous structure activity relationship (SAR) reports[8], we substituted the thiomorpholine 1,1-dioxide (**1**) of LEI-121 for a diaziridine-piperidine (**2**), a difluoropiperidine (**3**) or a piperidine (**4**) to investigate their effect on the potency and functionality of the probe.[8] Furthermore, compound **2** was designed to more closely resemble the structure of LEI-102, in an attempt to create an agonist probe. We hypothesized that the positioning of the propargylamide in LEI-121 is responsible for its inverse agonistic activity by occupying a subpocket, which may stabilize the inactive receptor conformation. This resembles the change in functionality, we have previously reported with a lipophilic spacer on this scaffold.[10] In compound **2** the propargyl moiety was introduced on the imidazolidine moiety to act as a ligation handle and the diazirine was moved to the piperidine. Of note, compound **2** had a similar binding pose as LEI-102 in the CB_2R structure (Figure 2C).

Synthesis

The synthesis of probes **1**,**3** and **4** started with oxidation of 6-bromo-3-fluoro-2-methylpyridine with *m*-CPBA followed by a Boekelheide rearrangement to yield **5**. Conjugation of **5** and (4-formylphenyl) boronic acid through a Suzuki coupling led to pyridyl benzaldehyde **6** (Scheme 1). Subsequent mesylation (**6**→**7**) and nucleophilic substitution with the desired piperidines gave the thiomorpholine 1,1-dioxide **8**, 4,4-difluoropiperidine **9** and piperidine **10** intermediates. The alkyne click handle was introduced via reductive amination of 2-amino-*N*-propargyl acetamide (**12**), obtained through HBTU mediated condensation of Boc-Gly-OH with propargylamine followed by Boc deprotection, and intermediates **8–10** to gain **13–15**. Finally, conjugation of 4-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)benzoic acid to **13–15** afforded the probes **1**,**3** and **4**. The construction of probe **2** was similar to the synthesis of LEI-102 (Scheme 2).[9] To start, reductive amination of 4-bromobenzaldehyde and 2-amino-acetamide led to compound **16**. After cyclization the formed



Scheme 1. The Synthesis of compounds **1**, **3** and **4**. Reagents and conditions: a) Step 1: *m*-CPBA (1.8 eq), 0 °C-RT, DCM (0.2 M), 4 days; Step 2: TFAA (2.2 eq), 0 °C-55 °C, 3 h; Step 3: K_2CO_3 (2.3 eq), THF:MeOH (20 : 1), 17 h, 35 % (three steps); b) (4-formylphenyl) boronic acid (1.5 eq), $Pd(PPh_3)_4$ (0.1 eq), K_2CO_3 (6 eq), toluene : EtOH (0.4 M, 4 : 1 v/v), 80 °C, 96 h, 80 %; c) DiPEA (2 eq), MsCl (1 eq), DCM (0.2 M), 0 °C, 1 h, 75 %; d) **8**: Thiomorpholine 1,1-dioxide / **9**: 4,4 difluoropiperidine/ **10**: piperidine (1.2 eq), K_2CO_3 (3.6 eq), ACN (0.2 M), 60 °C, 3 h, 70–80 %; e) propargylamine (1 eq), *N*-methylmorpholine (1.1 eq), HOBt (1.1 eq), EDC.HCl (1.1 eq), DCM (0.2 M), RT, 6 h, 74 %; f) HCl (4 M, 1.6 eq) in dioxane, RT, 2.5 h, 46 %; g) acetic acid (1 eq), $NaBH(OAc)_3$ (3.6 eq), THF (0.1 M), RT, 16 h, 43–54 %; h) 4-(3-(trifluoromethyl)-3H-diazirin-3-yl)benzoic acid (1 eq), HBTU (1.5 eq), DiPEA (3 eq), DCM (0.1 M), RT, 1 h, 28–31 %.



Scheme 2. The synthesis of the hydantoin probe **2**. Reagents and conditions: a) Step 1: 2-aminoacetamide hydrochloride (1.0 eq), NaOH (1.1 eq), MeOH:H_2O (0.3 M, 5:1 (v/v)), RT, 18 h; Step 2: $NaBH_4$ (2.1 eq), 18 h, 91% (two steps); b) CDI (2.1 eq), DMAP (2.1 eq), ACN (0.1 M), 60 °C, 70 h, 37%; c) 3-bromo-1-(trimethylsilyl)-1-propyne (1.2 eq), K_2CO_3 (1 eq), DMF (0.3 M), 50 °C, 16 h, 61%; d) bis(pinacol)diborane (1.5 eq), KOAc (4 eq), Pd(dppf)Cl_2 (0.5 eq), 1,4 dioxane (0.1 M), 80 °C, 16 h, used as crude; e) Et_3N (2.3 eq), MsCl (1.7 eq), THF (0.2 M), 0 °C-RT, 1 h, 75%; f) 4-piperidone hydrochloride (1.2 eq), K_2CO_3 (3 eq), ACN (0.2 M), 70 °C, 48 h, 78%; g) K_2CO_3 (1.8 eq), $Pd(PPh_3)_4$ (0.1 eq), toluene:EtOH (0.1 M, 4:1 (v/v)), 50 °C, 16 h, 29%; h) Step 1: NH_3 (g), MeOH (0.2 M), 0 °C, 5 h; Step 2: NH_2SO_3H (1.5 eq), MeOH (0.2 M), 16 h, RT, Step 3: I_2, MeOH, 0 °C 15 min., 27% (three steps).

imidazolidinedione **17** was alkylated with 3-bromo-1-(trimethylsilyl)-1-propyne (**18**) and subsequent borylation gave building block **19**. Simultaneously, previously synthesized (6-bromo-3-fluoropyridin-2-yl)methanol (**5**) was mesylated (**20**) followed by substituted of the mesyl with piperidin-4-one to gain ketone **21**. Suzuki coupling of **19** and **21** gave key intermediate **22**. The reaction conditions used for the conversion of the ketone in **22** to a diazirine led to simultaneous demasking of the alkyne function to give the photoaffinity probe **2**.

Molecular Pharmacology

Next we tested probes **1–4** in a [^{3}H]CP-55,940 radioligand displacement assay to determine their affinity (pK_i or displacement %) for the CB_2R and CB_1R. Compounds with less than 50% displacement on CB_1R at 1 μM were considered inactive. Additionally, their potency (EC_{50}) and maximal efficacy (E_{max}) were measured in a [^{35}S]-GTPγS functional assay. The results are summarized in Table 1.

All probes showed improved affinity for CB_2R compared to LEI-121 (Figure 3, Table 1), while retaining selectivity over CB_1R. This was accompanied by increased activity on hCB_2R by probes **2–4** compared to LEI-121 (Figure 4, Table 1). Probe **2** had the

Table 1. The Affinity and Potency of the two-step probes **1–4** on CB_1R and CB_2R.

Probe	hCB_2R		hCB_1R			
	$pK_i \pm$ SEM	$pEC_{50} \pm$ SEM	Emax (% ± SEM)	Displacement at 1 μM (%)	$pEC_{50} \pm$ SEM	Emax (% ± SEM)
LEI-102	8.6 ± 0.3	6.9 ± 0.2	76 ± 1	< 50	n.d.	n.d.
1 (LEI-121)	7.24 ± 0.06	7.06 ± 0.19	−35 ± 3	4 ± 1	6.08 ± 0.21	−26 ± 4
2	7.89 ± 0.13	8.56 ± 0.56	58 ± 4	42 ± 6	6.97 ± 0.13	35 ± 3
3	7.59 ± 0.09	7.75 ± 0.19	−26 ± 2	42 ± 7	7.00 ± 0.23	−16 ± 2
4	7.42 ± 0.05	7.10 ± 0.33	−28 ± 2	20 ± 2	5.98 ± 0.16	−19 ± 3

Binding affinities (pKi or displacement %) and potency (pEC_{50}) were determined with a [^{3}H]CP-55,940 displacement assay and [^{35}S]GTPγS functional assay respectively on CBR overexpressing CHO membranes. Potency values (pEC50) were obtained for compounds with displacement ≥ 35% using a [^{35}S]GTPγS assay. Efficacy (E_{max}) was normalized to the effect of 10 μM CP-55,940. Data are presented as the mean ± SEM from three independent experiments performed in triplicate.

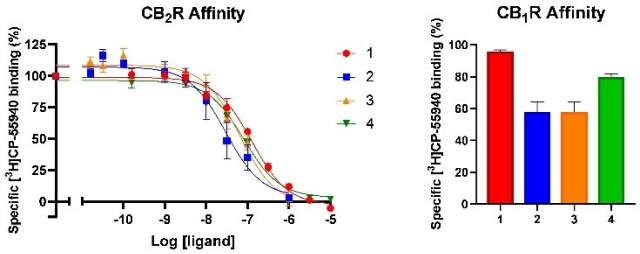


Figure 3. The affinity (pK_i or displacement) of compounds **1–4** on CB_2R (A) and CB_1R (B). Binding was normalized to binding of [^{3}H]CP-55,940 at 10 μM. Percentage displacement was determined with 1 μM compound. Data are presented as the mean ± SEM from three independent experiment performed in triplicate (CB_1R two experiments in duplicate).

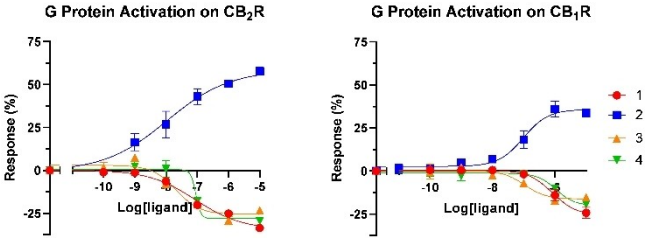


Figure 4. G protein activation of CB_2R and CB_1R by compounds **1–4** were measured with [^{35}S]GTPγS functional assays on CBR overexpressing CHO membranes. The results showed the switch of **2** from inverse agonist to partial agonist. Data points are presented as the mean ± SEM of at least three independent experiments performed in triplicate.

highest affinity (pK_i 7.89 ± 0.13) and activity (pEC_{50} 8.56 ± 0.56), which was 15-fold increased compared to LEI-102.

Compounds **3** and **4** behaved as inverse agonists with an $E_{max} \approx -30\%$, comparable to LEI-121 (**1**).[8] In contrast, probe **2** acted as a partial agonist (E_{max} 58% ± 4) (Figure 4). While probe **2** at 1 μM showed less than 50% radioligand displacement at the CB_1R, the compound did show some activity in the G protein activation assay. Of note, probe **2** was 77-fold selective over CB_1R and elicited 40-fold more potent receptor activation for CB_2R compared to CB_1R.

Since probe **2** was the only compound that behaved as a partial agonist, we studied its ability to visualize CB_2R by two-step pAfBPP. Probe **1** was used as a positive control. To this end, membrane preparations of hCB_2R-overexpressing CHO cells were incubated with probe **1** or **2**. Cross-linking was effected by UV-irradiation (λ = 350 nm, 5 min) using a CaproBox, a device used for controlled irradiation of biological samples with simultaneous cooling at 4 °C, to counteract the heat induced by the irradiation. Next, the membranes were subjected to copper(I)-catalysed click reaction conditions, utilizing Cy5-N_3 as the fluorescent azide to analyse the probe-protein complex by SDS-PAGE and in-gel fluorescence imaging. In this manner, two major bands with an apparent molecular weight of ~47 and ~41 kDa (Figure 5) were visualized for both probes, and these were absent in membranes from wild-type CHO cells. Heat-induced denaturation prior to probe incubation also resulted in a loss of fluorescent bands, indicating that the recognition is dependent on an intact three-dimensional protein conformation. The bands were also absent in non-UV treated samples, demonstrating that the probe does not covalently interact with CB_2Rs in the absence of irradiation. Furthermore, omission of the click-mixture showed that labelling was dependent on copper(I)-catalysed azide alkyne click ligation (Figure 5). Preincubation with CP-55,940 was able to prevent the CB_2R labelling by probes **1** and **2**. Of note, probe **2** did show two off-targets with a molecular weight around 30 kDa not present for the original LEI-121 and derivatives.[7] The identity of the off-targets is unknown, but one of the off-targets is also competed out by CP-55,940.

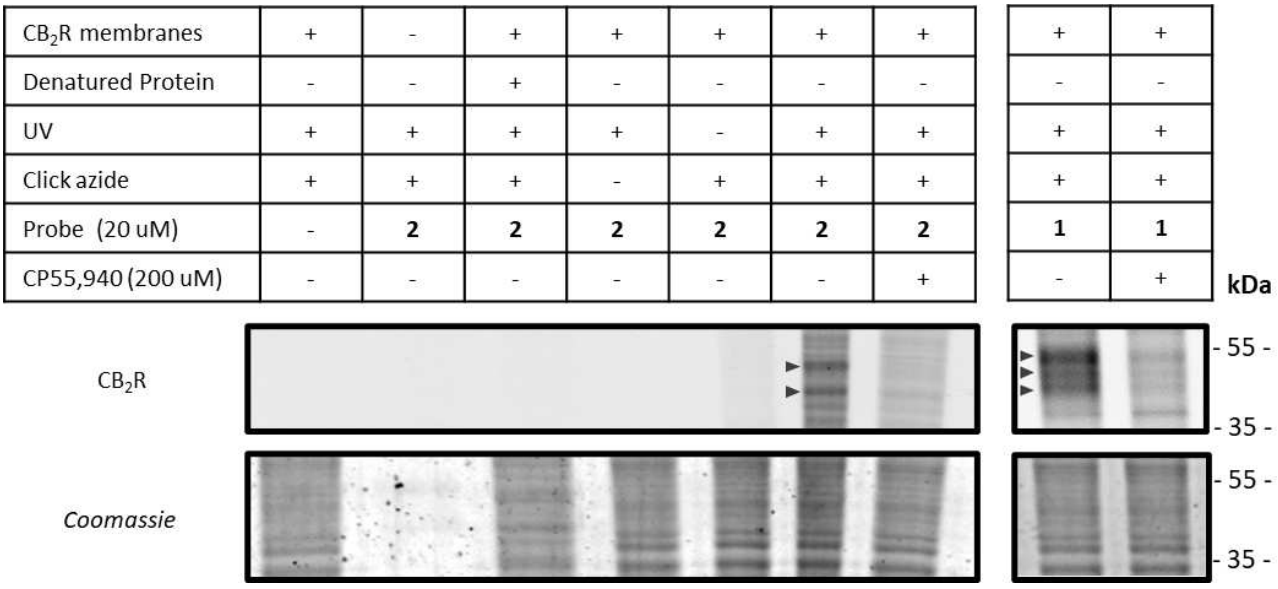


Figure 5. Probes **1** and **2** were able to label CB_2R (45-55 kDa) in CB_2R-overexpressing CHO membranes. Denaturation of the protein prior to probe incubation, or omitting the UV irradiation step prevented labelling of CB_2R by the probes. Additionally, no signal was detected in the absence of either click azide or probe. Labelling was outcompeted by CP-55,940. The gels are representative of two independent experiments.

The labelling pattern of probes **1** and **2** is slightly different. Probe **2** labels two distinct bands, while labelling with probe **1** yields multiple intense bands. GPCRs including CB_2R can have a wide variety of post translational modifications such as N-glycosylation and phosphorylation.[11,12] Phosphorylation patterns change depending on receptor activity, thus a partial agonist probe may recognize a different receptor population than an inverse agonistic probe.[13]

Conclusions

A structure-based approach exploiting the recently published cryo-EM structure of the CB_2R in complex with LEI-102 was used to develop a new bifunctional photoaffinity probe that stabilized the active conformation of CB_2R. Probe **2** had high binding affinity (pK_i 7.89 ± 0.13), selective of CB_1R and behaved as a partial agonist (EC_{50} 8.56 ± 0.56 with, E_{max} 58 % ± 4). Probe **2** labelled the CB_2R upon photoactivation in a slightly different manner compared to LEI-121 (probe **1**), which is an inverse agonist. This may suggest that the stabilization of a different receptor conformations result in a different labelling pattern, which could be dependent on different post-translational modifications.[11,12] We envision that probe **2** may be used to isolate the CB_2R in the active state using a biotin-reporter and affinity enrichment from primary cells and tissues, which may facilitate the identification of post-translational modifications and potential protein interaction partners of the active receptor. To conclude, we have developed a novel photoaffinity probe for the CB_2R that may hold promise to study different receptor conformations in relation to its cellular function.

Experimental Section

General Information: Analytical thin-layer chromatography (TLC) was routinely performed to monitor the progression of a reaction and was conducted on silica gel 60 F254 plates. Reaction compounds on the TLC plates were visualized by UV irradiation (λ_{254}) and/or spraying with potassium permanganate solution (K_2CO_3 (40 g), $KMnO_4$ (6 g), and H_2O (600 mL)), ninhydrin solution (ninhydrin (1.5 g), n-butanol (100 mL) and acetic acid (3.0 mL)) or molybdenum solution ($(NH_4)_6MO_7O_{24}\cdot 4H_2O$ (25 g/L) and $(NH_4)_4Ce(SO_4)_4\cdot 2H_2O$ (10 g/L) in sulfuric acid (10 %)) followed by heating as appropriate. Purification by flash column chromatography was performed using silica gel 60 (40–63 μm, pore diameter of 60 Å). Solutions were concentrated using a rotary evaporator. Analytical purity was determined with liquid chromatography-mass spectrometry (LC–MS) using a Finnigan LCQ Advantage MAX apparatus with electrospray ionization (ESI), equipped with a Phenomenex Gemini 3 μm NX–C18 110 Å column (50x4.6 mm). For purification by mass guided preparative high-performance liquid chromatography (Prep-HPLC) the Waters AutoPurification HPLC/MS apparatus was used with a Gemini prep column 5 μm 18 C 110 Å (150×21.2 mm). All final compounds had a purity > 95 %. ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AV 300 (300/75 MHz), AV 400 (400/100 MHz) or AV 500 (500/125 MHz) spectrometer at ambient temperature using $CDCl_3$ or MeOD as solvent.

Supporting Information

Experimental procedures and analytical data of the molecules described in this article are provided in the Supporting Information.[9,14–16]

Author Contributions

Conducting Experiments: A.Y. (Yu An), L.V.d.P. (Laura V. de Paus), A.P.A.J. (Antonius P.A. Janssen); Writing-Original Draft: L.V.d.P.; Writing Review & Editing: M.v.d.S., L.H.H. (Laura H. Heitman), R.J.B.H.N.v.d.B (Richard J. B. H. N. van den Berg); Supervision: L.H.H. and M.v.d.S.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: cannabinoid CB_2 receptor · Photoaffinity-based probe · affinity-based protein profiling · GPCR

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