Cholesterol as a modulator of cannabinoid receptor CB₂ signaling

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Signaling through integral membrane G protein-coupled receptors (GPCRs) is influenced by lipid composition of cell membranes. By using novel high affinity ligands of human cannabinoid receptor CB₂, we demonstrate that cholesterol increases basal activation levels of the receptor and alters the pharmacological categorization of these ligands. Our results revealed that (2-(6-chloro-2-((2,2,3,3-tetramethylcyclopropane-1-carbonyl)imino)benzo[d]thiazol-3(2H)-yl)ethyl acetate ligand (MRI-2646) acts as a partial agonist of CB₂ in membranes devoid of cholesterol and as a neutral antagonist or a partial inverse agonist in cholesterol-containing membranes. The differential effects of a specific ligand on activation of CB₂ in different types of membranes may have implications for screening of drug candidates in a search of modulators of GPCR activity. MD simulation suggests that cholesterol exerts an allosteric effect on the intracellular regions of the receptor that interact with the G-protein complex thereby altering the recruitment of G protein.

Abbreviations

| Abbreviation | Definition |
|--------------|------------|
| GPCR | G protein-coupled receptor |
| ICL | Intracellular loop (in GPCR) |
| TM | Transmembrane domain (in GPCR) |
| HEK293 | Human embryonic kidney |
| CHO | Chinese hamster ovary |
| E. coli | Escherichia coli |
| Sf9 cells | A clonal isolate of Spodoptera frugiperda baculovirus infected insect cell |
| MD | Molecular dynamics |
| hCB₂ | Human cannabinoid receptor CB₂ |
| hCB₁ | Human cannabinoid receptor CB₁ |
| MBP | Maltose binding protein of E. coli |
| CP-55,940 | Synthetic cannabinoid agonist 2-[(1R,2R,5R)-5-Hydroxy-2-(3-hydroxypropyl)cyclohexyl]-5-(2-methyloctan-2-yl)phenol |
| [³¹H]-CP55,940 | Radiolabeled synthetic ligand CP-55,940 |
| SR-144,528 | Synthetic cannabinoid inverse agonist 5-(4-Chloro-3-methylphenyl)-1-[(4-methylphenyl)methyl]-N-[(1S,2S,4R)-1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl]-1H-pyrazole-3-carboxamide |
| [³⁵S]-GTP₇S | Radiolabeled synthetic analog of guanosine triphosphate |
| MB-CD | Methyl-β-cyclodextrin |
| Gₐ₁₁ and Gₐ₁₂ | Subunits of heterotrimeric G protein |
| GEF assay | Determines rates of nucleotide exchange on the Gₐ subunit of G protein |
| POPC | 1-Palmitoyl-2-oleoyl-glycerol-3-phosphocholine |
| POPG | 1-Palmitoyl-2-oleoyl-sn-glycerol-3-phospho-(1’-rac-glycerol) |
| CHS | Cholesterol hemisuccinate |
| RMSD | Root-mean-square deviation |
| RMSF | Root mean square fluctuation |

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Lipid composition of membranes plays an important role in modulation of G protein-coupled receptors (GPCR) \(^1\). In particular, cholesterol content varies significantly among different types of cell membranes, and was shown to influence the ligand-induced signal transduction through GPCRs \(^2\). Early stage pharmacological characterization of GPCR ligands typically involves screening of prospective drug candidates in cell- and membrane-based assays \(^3\). Hence, it is important to understand how a physiological response of the target receptor to small molecule ligands is modulated by the lipid composition of membranes.

Membranes of mammalian cells typically used for expression of recombinant GPCR for cell- and membrane-based assays may contain significant amount of cholesterol \(^4\). Plasma membranes of human embryonic kidney (HEK293) and Chinese hamster ovary (CHO) cells were reported to contain as much as 25–40 mol% of cholesterol relative to phospholipid \(^5\). The lipid and cholesterol content of cultured mammalian cells fluctuates depending on nutrient composition of growth media, temperature of cultivation, age of cell culture and other growth parameters \(^6\). Other commonly used expression hosts such as *Escherichia coli* \(^7\), *Spodoptera frugiperda* (Sf9), and *Trichoplusia ni* (Tn) synthesize only trace amounts of cholesterol or do not produce it at all \(^8\).

A large proportion of pharmacological drugs, currently on the market or under development, target membrane proteins. Since pharmacological profiles of drug candidates could be influenced by the properties of cell membranes harboring these receptors, variations in composition of the lipid bilayer may result in inconsistencies of the receptor response \(^9\). This is especially true for hydrophobic ligands targeting GPCR since interaction between the drug and the lipid may influence the pharmacological characteristics of the compound such as its binding affinity and selectivity \(^10\). While it was demonstrated that lipids and cholesterol can shift the response of the receptor to ligand binding, it is not well known if the pharmacological categorization of ligands, i.e. agonism, antagonism or inverse agonism, depends on composition of the lipid matrix. Here, we sought to elucidate whether the functional response of the cannabinoid receptor CB2 to a series of novel specific ligands is affected by the cholesterol content of membranes.

Cholesterol is critical for the formation of lateral domains (clusters) \(^11\), and it may induce negative curvature elastic stress in lipid bilayers \(^12\). The cholesterol-dependent increase in membrane stiffness increases the decay length of protein-induced perturbations in the lipid matrix \(^13\). Furthermore, cholesterol may directly interact with GPCR at sites identified in several receptors \(^14\). Cholesterol was reported to negatively modulate the activity of type 1 cannabinoid receptor (CB1) in nerve cells \(^15\). Furthermore, it was proposed that the CB1 receptor possesses a specific cholesterol binding site \(^16\). However, the activity of the structurally close cannabinoid receptor CB2 was reported to be not influenced by changes in membrane cholesterol content \(^17\). The CB2 receptor is known for its role as a regulator of inflammation and is commonly found in tissues containing variable levels of cholesterol \(^18\). Therefore, it is important to understand if CB2 is sensitive to variation in cholesterol content.

Here, we examined the effects of cholesterol on CB2 by analyzing G protein activation by this receptor in response to novel, rationally designed CB2 ligands (MRI-2646, MRI-2654, MRI-2687, MRI-2653, MRI-2659). We will demonstrate that cholesterol increases the basal activation levels of CB2 receptor, thereby altering pharmacological classification of these ligands from inverse agonists to partial agonists. We also use MD simulation to probe structural effects of cholesterol and ligands.

**Results**

Based on the rational modification of the A-836339 thiazole scaffold, some of us previously reported that the synthetic compounds MRI-2687 and MRI-2594 show contrasting activity on the CB2 receptor \(^19\). The two compounds only differ in arm 1 featuring the extended central 6-methylbenzothiazole ring in MRI-2687 and 4,5-dimethylthiazole ring in MRI-2594, respectively. Unexpectedly, MRI-2687 behaved as an inverse agonist, whereas MRI-2594 acted as an agonist on CB2 (similar to A-836339) \(^20\). Detailed molecular docking analysis showed that these two ligands adopt similar binding poses within CB2, wherein the different central rings reside in the same position as arm 1 of the receptor bound AM10257 CB2 crystal structure \(^21\). The arm 1 of MRI-2687 along with its 6-Me substitution forms π–π interactions with the side chain of Trp2586.48 and confines its conformation to a similar rotamer as in the CB2-AM10257 structure. As opposed to MRI-2687, the lack of a large substituent on arm 1 of MRI-2594 prevents it from extending sufficiently deep to constrain the conformation of Trp2586.48 and allows its unrestrained movement and, thereby, leading to receptor activation. The proposed differential interactions with the toggle switch residue Trp2586.48 prompted us to investigate the effect of these novel ligands on CB2 receptor further in various cell- and membrane-based assays.

To assess the function of CB2 in vitro, we measured the activation of the receptor by quantifying the rates of nucleotide exchange on the G subunit of G protein that interacts with CB2. The full agonist CP-55,940 is not well suited for studying modulatory effects of the lipid bilayer on receptor function since its strong activating effect may mask moderate effects exerted by the lipid matrix. This is an important consideration since, unlike full synthetic agonists, many endogenous agonists of GPCR only partially activate these receptors. We hypothesized that our novel series of high-affinity ligands would allow detection of moderate modulating effects of the lipid matrix on CB2 activation. Specifically, we sought to investigate the role of different substitutions at the 6-position of the benzothiazole ring in this series of ligands. Elaborating on our earlier approach \(^22\), we synthesized structurally-related compounds (Fig. 1) and evaluated their effects on CB2 receptor activity.

In this study, we used the recently developed cannabinoid ligands which were synthesized as shown in Fig. 1A. The R group at the 6-position of the benzothiazole were varied to include Me, OMe, OCF3, Cl, and Br, synthesized as shown in Fig. 1B.

The affinity of the MRI ligands for CB2 receptor was determined to be in 0.053–0.1 nM range in hCB2-expressing-CHO cell membranes by a displacement binding experiment (Fig. 2a).

Then, we assessed the functional effect of the compounds on G-protein signaling in [35S]-GTPγS binding assays using hCB2-expressing-CHO cell membranes obtained from PerkinElmer (see “Methods”). MRI-2646
had no effect on basal signaling (Fig. 2b) whereas MRI-2654, MRI-2687, MRI-2653, and MRI-2659 reduced basal signaling to 55% with high potency (Fig. 2a) as a function of inverse agonism. Unlike other structural analogues, MRI-2594 demonstrated full agonism with high affinity (hCB2R, Ki = 0.031 nM) and potency (EC50: 0.09 nM) (Fig. 2a). We were intrigued by the seemingly neutral antagonist activity of MRI-2646 in CHO cells even though its structural features were in line with the other benzothiazole ligands which behaved as inverse agonist. Hence, in the present work, we used this novel, high affinity cannabinoid ligand to explore the role of lipid environment in determining functional activity.

Based on our preliminary studies, we concluded that ligands such as MRI-2646 may act either as a weak partial agonist or a neutral antagonist, depending on the cell type used in the assay. To better understand the reason for these discrepancies, we assessed the activation of CB2 receptor by the in vitro G protein activation assay that measures the rates of nucleotide exchange on the Gα subunit of G protein (GEF assay) as described previously33,34. The assay reports the rates of formation of the complex of [35S]-GTPγS, a homolog of GTP, with the Gα subunit of G protein. Typical assay conditions require small (nanogram) quantities of the receptor protein either in cell membranes or reconstituted into lipid bilayers. The readout of the assay is the amount of the non-hydrolysable complex of Gα with [35S]-GTPγS which, under selected experimental conditions, is proportional to the amount of activated receptor in the assay.

For convenience, the results of the GEF assay are normalized such that activation of CB2 in the presence of CP-55,940 was set to 100%, and residual activity in the presence of saturating concentrations of the full inverse agonist SR-144,528 was set to 0%.

We first compared two different types of membranes: E. coli BL21 (DE3) and a commercially available preparation of CHO cell membranes expressing CB2 (Millipore EMD, Cat. No HTS020M) in the GEF assay (Fig. 3). Depending on the source of membranes, a significant difference in activation behavior of CB2 was observed. In this assay, the tested ligands (with the exception of the previously described full agonist MRI-259431 and strong inverse agonist MRI-2659) behaved as partial agonists of the CB2 receptor in E. coli membranes. However, these same ligands acted as partial inverse agonists on CB2 expressed in CHO cells (Fig. 3). The activities of the ligands relative to each other, which were roughly inversely correlated to the ligand substituent size, were not greatly changed, and correlated roughly to the R-group size.

Intrigued by these observations, we compared several other available sources of membranes expressing CB2. MRI-2646 ligand was selected as a representative of a cohort of related benzothiazole ligands for all subsequent measurements since it activated CB2 in E. coli cell membranes the most while being a partial inverse agonist of CB2 in CHO membranes. Several preparations of membranes expressing CB2 were compared (Fig. 4). While MRI-2646 behaved as a neutral antagonist of CB2 in membranes from CHO cells obtained from PerkinElmer, it acted as a partial inverse agonist in two other commercial preparations of CB2 in CHO membranes procured from EMD Millipore and Applied Cell Sciences (CHO-K1 membranes). Likewise, MRI-2646 was a partial inverse agonist of CB2 expressed in membranes Expi293F and Expi293F GNTI cells. On the other hand, this ligand was a partial agonist of CB2 expressed in baculovirus infected insect S9 cells and in E. coli BL21 (DE3) cells.

Therefore, MRI-2646 exerts differential effects on CB2: in mammalian cell membranes this ligand acts as an inverse agonist or neutral antagonist while in bacterial- and insect-cell membranes it behaves as a partial agonist of CB2 receptor. Likely contributing factors to such discrepancies may include: (i) differences in lipid composition
Figure 2. Affinity and functional effects of novel MRI ligands on CB2 receptor in CHO cell membranes. (a) Binding affinities (nM) and E_max (% and nM) of [35S]-GTPγS binding to CHO membranes (PerkinElmer, Cat. No ES111-M400UA) expressing hCB2. (b) [35S]-GTPγS binding to membranes as a factor of ligand concentration. Binding of [35S]-GTPγS was determined as described in “Methods”. Non-specific binding was defined as 0% activity. The assay of GTPγS non-specific binding contains non-radioactive GTPγS.

| Compound  | CB2R binding  | GTPγS binding |
|-----------|---------------|---------------|
|           | hCB2R-K (nM) | E_max (%)     | EC50 (nM)     |
| CP55,940  | 0.85±0.02    | 275±4         | 0.21±0.05     |
| MRI-2594  | 0.031±0.007  | 276±4         | 0.09±0.02     |
| MRI-2646  | 0.079±0.011  | -2±4          | N.D.          |
| MRI-2654  | 0.079±0.009  | -23±3         | 0.76±0.04     |
| MRI-2687  | 0.100±0.010  | -35±1         | 1.23±0.09     |
| MRI-2693  | 0.094±0.011  | -46±2         | 0.50±0.09     |
| MRI-2659  | 0.053±0.010  | -55±1         | 0.25±0.10     |

Figure 3. GEF of CB2 expressed in: (a) E. coli membranes and (b) CHO membranes (Millipore EMD). 2 μg of total protein per assay. Each point represents an average of four independent measurements (n = 4). Ligands were added at a concentration of 2 μM to ensure saturation of the receptor, and G protein was added as described in “Methods”. The dotted line indicates the rates of activation of G protein in the absence of a ligand. The rates of activation with CP-55,9040 are set to 100%, and rates of activation in the presence of SR-144,528 to 0%.
(in particular, cholesterol content) between mammalian, insect, and bacterial membranes; (ii) different pattern of post-translational modifications (palmitoylation, glycosylation) of receptor molecules expressed in different expression hosts; (iii) differences in expression levels of the receptor and densities of ligand-binding sites in membrane preparations from different sources, and (iv) composition of endogenous G proteins in membrane preparations. The endogenous membrane-associated G protein contained in preparations of CHO, HEK and Sf9 cell membranes expressing CB2 contributes to the GEF signal. E. coli cells do not produce G protein. The subtle role of the sterics and electronics of the 6-Cl substituent in this intricate modulatory mechanism cannot be ruled out either.

Effects of lipid composition. We considered the effects of lipid composition of CB2-containing membranes obtained from different expression hosts. Specifically, the CHO cell membranes are known to contain high concentrations of cholesterol, unlike the E. coli cell membranes that are devoid of cholesterol. We quantified the relative content of lipids and cholesterol in several preparations of membranes of mammalian, insect, and E. coli cells (Supporting Fig. 1). Lipids were extracted from membranes, and their composition determined by 1H-NMR as described in Legend to Supporting Fig. 1. Consistent with the previously published data, cholesterol was not detected in membrane preparations from E. coli and from Sf9 cells while membrane preps obtained from CHO cells and suspension culture of HEK Expi293F expressing CB2 contained 39% and 26% cholesterol relative to phospholipids, respectively. While the bacterial-, insect- and mammalian cell membranes differ significantly not only in content of cholesterol but also in composition of phospholipids, a variability in cholesterol content between membranes from different expression cell lines correlates strongly with the signaling pattern of CB2 activated by the novel ligand. Therefore, we hypothesized that the cholesterol content of membranes affects the activation of the cannabinoid receptor CB2.

Endogenous vs. exogenous G protein. Besides lipids, the content of endogenous G proteins in cell membrane preparations from different sources may also affect the readout of the [35S]-GTPyS binding and the GEF G-protein activation assays used in this study (see “Methods”). The [35S]-GTPyS binding assay measures the binding of the radiolabeled nucleotide analogue to the endogenous G protein that is already present in membrane preparations while the GEF assay relies on the exogenous G protein subunits of Gαi1 and Gβ1γ2 added in large excess relative to receptor. Therefore, the GEF assay typically affords a good signal-to-noise ratio and enables comparison of multiple samples at standardized conditions. The addition of G protein is necessary to analyze the activation of CB2 in E. coli membranes since these membranes do not contain endogenous G protein. On the other hand, the membranes obtained from mammalian and insect cell cultures contain endogenous G proteins and, therefore in these membranes, the GEF assay reports on the rates of activation of a combined pool of endogenous as well as exogenous G protein.
To assess the contribution of endogenous G protein to the total GEF signal, we performed the GEF assay on membranes of Expi293F, CHO (Perkin Elmer) and Sf9, in the absence as well as in the presence of exogenous G protein (Fig. 5).

As expected, in the absence of exogenous G protein the magnitude of the signal was significantly lower (three to tenfold) compared to the standard GEF assay. Yet, the pattern of activation of CB2 by the MRI-2646 was similar in assays performed with- and without addition of exogenous G protein. While in membrane preps from Expi293F cells, MRI-2646 acted as an inverse agonist of CB2 (Fig. 5a); in CHO cell membranes (PerkinElmer) it behaved as a neutral antagonist (Fig. 5b); and in membranes of Sf9 cells expressing CB2, it acted as a partial agonist (Fig. 5c).

Receptor density in membranes. We next examined the density of ligand binding sites (by saturation 3H-CP-55,940 radioligand binding assay, Supporting Fig. 2). The density of the ligand binding sites varied between ~22 and 50 pmol/mg of membrane protein. However, there was no correlation between the density of the ligand binding sites and behavior of the MRI-2646 ligand as an inverse agonist, neutral antagonist or partial agonist of CB2. Thus, the differential pattern of activation of CB2 by the MRI-2646 cannot be explained by differences in expression levels and density of binding sites of receptor in membrane preparations.

The results above suggest that the functional effect of MRI-2646 on CB2 varies following the same pattern as the content of cholesterol in membranes of cell lines expressing CB2. While in membranes devoid of cholesterol, the MRI-2646 is a partial agonist, and, in cholesterol-containing membranes it acts either as an inverse agonist or a neutral antagonist of CB2.

Treatment with methyl-β-cyclodextrin. The content of cholesterol in cell membrane preparations can be altered by pre-treatment with methyl-β-cyclodextrin (MβCD) (Supporting Fig. 3). In membranes of Expi293F cells expressing CB2, treated with 20 mM MβCD, the rates of activation of G protein on CB2 decreased by about twofold although the levels of the CB2 receptor in these membranes were unchanged (Supporting Fig. 3b). In the MβCD-treated membranes, the activity of CB2 in the presence of MRI-2646 was higher than the basal signaling in the absence of ligands, indicating that MRI-2646 ligand acted as a partial agonist of CB2 receptor at these conditions.

MβCD can also be used as a carrier of cholesterol in order to enrich cell membranes with cholesterol. To test the effect of the exogenously added cholesterol on activation of CB2, we pre-treated the E. coli membranes expressing CB2 with a solution of MβCD/cholesterol, and measured the rates of activation of G protein (Fig. 6a). In the MβCD/cholesterol-treated membranes of E. coli, the MRI-2646 reproducibly acted as a neutral antagonist of CB2 while in the untreated membranes it exhibited agonistic effects (Fig. 6b). There was no noticeable change in the levels of CB2 in membranes, and the density of ligand binding sites did not change upon treatment with MβCD/cholesterol (Supporting Fig. 4a,b). These results provide a more direct proof that the activation of CB2 bound to MRI-2646 is modulated by cholesterol content of membranes.
To assess the possible role of post-translational modifications of CB2 on its activation by the MRI ligands, the recombinant CB2 receptor was isolated from two different expression cell lines, E. coli BL21 (DE3) and Expi293F GNTI-42. The protein was purified and reconstituted into lipid bilayers containing 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (POPG) at a molar ratio of 3/1, either supplemented or not supplemented with cholesterol, as described in “Methods”. We reported previously that the presence of phospholipids with a negatively charged headgroup stabilizes CB2 protein in lipid bilayers. Therefore, purified CB2 was reconstituted into POPC/POPG (3/1, mol/mol) liposomes containing 0, 20 and 40 mol% cholesterol (reported as total content of lipids) as described in “Methods”. In one case, the protein purified from E. coli cells was reconstituted into lipids extracted from brain tissue (Avanti Polar Lipids).

The ratio of protein-to-lipid in the resulting samples was in the range of 1:850 to 1:1100 mol/mol. The levels of protein, and the density of ligand binding sites for receptor preparations reconstituted in liposomes with different content of cholesterol varied only slightly (Supporting Fig. 5a,b).

The results of the GEF assay performed on these liposome-reconstituted CB2 samples demonstrate that the MRI-2646 ligand acts as a partial agonist of bacterially expressed CB2 in liposomes devoid of cholesterol (Fig. 7a). However, in the lipid matrix containing 20% or 40% of cholesterol or in liposomes composed of lipids extracted from bovine brain (Fig. 7b–d), the basal (without ligand) activation of CB2 receptor was increased. In

**Liposome-reconstituted CB2 and post-translational modifications.** To assess the possible role of post-translational modifications of CB2 on its activation by the MRI ligands, the recombinant CB2 receptor was isolated from two different expression cell lines, E. coli BL21 (DE3) and Expi293F GNTI-42. The protein was purified and reconstituted into lipid bilayers containing 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1‘-rac-glycerol) (POPG) at a molar ratio of 3/1, either supplemented or not supplemented with cholesterol, as described in “Methods”. We reported previously that the presence of phospholipids with a negatively charged headgroup stabilizes CB2 protein in lipid bilayers. Therefore, purified CB2 was reconstituted into POPC/POPG (3/1, mol/mol) liposomes containing 0, 20 and 40 mol% cholesterol (reported as total content of lipids) as described in “Methods". In one case, the protein purified from E. coli cells was reconstituted into lipids extracted from brain tissue (Avanti Polar Lipids). The ratio of protein-to-lipid in the resulting samples was in the range of 1:850 to 1:1100 (mol/mol). The levels of protein, and the density of ligand binding sites for receptor preparations reconstituted in liposomes with different content of cholesterol varied only slightly (Supporting Fig. 5a,b).

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all cholesterol-containing liposomes, including those containing the brain lipid extract, the MRI-2646 ligand acted as a partial inverse agonist of the receptor. For comparison, the activation behavior of CB2 isolated from the Expi293F GNTI- cells\(^4\) and reconstituted into liposomes was studied (Fig. 7e). In liposomes without cholesterol, the MRI-2646 acted as a partial agonist of CB2, similar to its action on the bacterially expressed protein. At the same time, MRI-2646 acted as a partial inverse agonist on HEK cell-expressed CB2 protein reconstituted into liposomes with 40% of cholesterol (Fig. 7f and Supporting Fig. 5, 6). These results corroborate the above described inverse agonism of MRI-2646 on CB2 in membrane preps containing cholesterol. Therefore, it can be concluded that cholesterol is involved in modulating the activation behavior of CB2. Post-translational modifications of CB2 do not seem to play a significant role in modulation the activation of the receptor by MRI-2646 in the presence of cholesterol.

**Structural effects of cholesterol on CB2 in molecular dynamics simulation.** Using molecular dynamics (MD) simulations, we evaluated the effect of membrane cholesterol on three of the ligands’ interaction with the known toggle switch residue Trp258\(^{48}\) as well as the displacement and fluctuation of the ICL3-TM6 region of CB2, which would interact with the G-protein upon its recruitment. We hypothesized there would be a significant effect of membrane cholesterol on these regions in free CB2. We anticipated three alternatives for the effect of ligand type on the simulation results: (1) no obvious effect of ligand; (2) effects specific to the experimentally observed ligand pharmacological category; or (3) effects correlating mainly to ligand size. MRI-2646, MRI-2659, and MRI-2594 were simulated, chosen for their diversity.

We conducted equilibrium all-atom molecular MD simulations of the cryo-EM structure of CB2 (PDB: 6PT0\(^{44}\)) with and without 40% membrane cholesterol and a POPC/POPG, 3/1, mol/mol ratio. The cholesterol molecules in the extracellular part of the TM5-6 region reported in the cryo-EM structure were retained, particularly since this is a known cholesterol binding site\(^{45,46}\). In both of these conditions, simulations were done with no bound ligand as well as with each of the three chosen ligands. Each ligand was placed respectively in the orthosteric site (Fig. 8) by analogy with the configuration of the structurally similar AM10257 ligand agonist present in the X-ray structure of CB2\(^{31}\). There were eight total simulations.

The R-groups of the ligands were in close proximity to the toggle switch residue Trp258\(^{48}\). In each simulation, the protein underwent an initial relaxation phase from the initial antagonist-bound cryo-EM conformation within 10 ns, as shown by evolution of whole protein RMSD over time (Supporting Fig. 7). Each ligand remained stable in its initial position throughout (Supporting Fig. 7). We discarded the first 50 ns of each production simulation, to include the relaxation phase, for an aggregated grand total of 2 µs of simulation trajectory analyzed across all conditions. Key binding site interactions are shown in Fig. 8a.

In GEF experiments, membrane cholesterol increased the constitutive activity of CB2, and the relative order of MRI-2594, MRI-2646, and MRI-2659 by activity was preserved, even though these ligands would be categorized differently relative to the benchmark of constitutive activity. Since G-protein recruitment is thought to depend...
on the toggle switch Trp2586.48, we hypothesized that the rotameric state of this residue would be differentially changed by ligand type, particularly since the ligand substituent is in close proximity to it.

We found that the distribution of Trp2586.48 side-chain $\chi_1$ rotamer angles (from the N–Cα–Cβ–Cγ dihedral) during the simulations varied by ligand arm 1 substituent size and presence of cholesterol. In ligand-free, cholesterol-free CB2, the distribution of side-chain angles had a peak at $\sim 280^\circ$ (and very small peak at roughly $180^\circ$ which will not be discussed further, since it is unlikely to relate to experimental results). In each case the average magnitude was increased when cholesterol was present (Fig. 9a). The difference in mean $\chi_1$ angles between cholesterol conditions for each bound ligand are shown in Fig. 9a and plotted in Fig. 9b. As the ligand size grew, the effect of cholesterol on the rotamer angle decreased.

Some amount of conformational change from the reference cryo-EM structure is to be expected in simulation, but we hypothesized that residues interfacing with G-protein, and therefore most likely to change the activation rate, would be preferentially affected as a function of the presence of cholesterol. To test this idea, we compared the average root-mean-square deviation (RMSD) of each Ca, with respect to the original cryo-EM structure at each simulation frame, between 0% cholesterol and 40% cholesterol conditions by subtraction. This comparison was made for free and each ligand-bound state. If a residue in the 40% cholesterol structure deviated more than the same residue in the 0% cholesterol structure, the resulting quantity would be positive, and if less, negative. Even though the reference cryo-EM structure is antagonist-bound, if there were no effect of cholesterol or ligand, the RMSD would be expected to be similar across conditions, resulting in zero RMSD difference. We found that residues that would interact with the G-protein complex, primarily in the ICL3 loop and the N-terminal side of TM6 (residues 222–236), were preferentially displaced in the presence of cholesterol across all ligand conditions (Fig. 10). The difference in average per-residue RMSD was relatively variable in this region compared to the rest of the structure, where this quantity was mostly close to zero (Fig. 10). The number of residues in this region with $> 1$ Å mean change in RMSD as a function of cholesterol presence was 9, 6, 2, and 3 in free, MRI-2594, MRI-2646, and MRI-2659-bound simulations respectively. This number decreased with ligand substituent size—though we cannot rule out other factors, particularly including the chemical composition of the substituent.

Given that RMSD change as a function of the presence of cholesterol was related to substituent size, we hypothesized that the root-mean-square fluctuation (RMSF) of each residue with respect to the average structure would also be affected in a systematic way (Supporting Fig. 8). A decrease in RMSF of specific residues would suggest a localized decrease in entropy. The ICL3 and first part of TM6 (residue 221–236) were preferentially displaced in the presence of cholesterol across all ligand conditions (Fig. 10). The difference in average per-residue RMSD was relatively variable in this region compared to the rest of the structure, where this quantity was mostly close to zero (Fig. 10). The number of residues in this region with $> 1$ Å mean change in RMSD as a function of cholesterol presence was 9, 6, 2, and 3 in free, MRI-2594, MRI-2646, and MRI-2659-bound simulations respectively. This number decreased with ligand substituent size—though we cannot rule out other factors, particularly including the chemical composition of the substituent.
presence of cholesterol also decreased as ligand substituent size increased (1.01, 0.58, and 0.40 Å for MRI-2594, MRI-2646, and MRI-2659 respectively, shown in Supporting Fig. 8).

Discussion
Here we demonstrated that cholesterol increases the basal activation levels of CB2 receptor, thereby altering the pharmacological classification of novel synthetic cannabinoid ligands (Fig. 11). While in membranes devoid of cholesterol the MRI ligands act as partial agonist of CB2, in cholesterol-enriched membranes that same ligands became either inverse agonists or neutral antagonists of this receptor.

Some of us have previously reported the stabilizing effect of anionic lipids such as PG and PS on purified CB2 protein reconstituted in liposomes. It was also shown that the negatively charged cholesterol derivative, cholesteryl hemisuccinate (CHS), stabilizes the recombinant CB2 protein in detergent micelles and liposomes. At the same time, it was reported that cholesterol did not affect the activation of CB2 by the full agonist CP-55,940. Since these observations were made on a CB2 receptor activated by the high affinity full agonist CP-55,940, the relatively moderate effects of the lipid matrix on receptor activation may have been masked.

Cholesterol is the major sterol found in higher eukaryotes. The rigid planar structure of cholesterol modulates fluidity, thickness, curvature and permeability of membranes. The presence of cholesterol in membranes increases ordering of lipid acyl chains. Physicochemical parameters of membranes have been implicated in the regulation of function of integral membrane proteins. Lateral compression and hydrophobic matching between the lipid bilayer and transmembrane domains of the protein are affected by cholesterol content. These parameters are important for the structural stability of embedded membrane proteins.
It was previously reported that cholesterol rafts in human immune cell membranes modulate the activity of CB1 receptor but do not affect the activation behavior of CB2 receptor. That conclusion was reached by quantifying several signaling pathways in cells treated with methyl-β-cyclodextrin (MβCD). However, the effects of cholesterol/ MβCD were assessed in the presence of the full agonist of CB2, CP-55,940 that may have masked moderate effects on activation of CB2 receptor by the lipid bilayer. Here, by using a novel synthetic ligand MRI-2646 we demonstrated that the content of cholesterol in lipid bilayers modulates CB2 activation.

We have demonstrated that cholesterol increases the constitutive activity of the CB2 receptor. This effect was first shown in membrane preparations of several types of cells expressing CB2. One can argue that the difference between the membranes of mammalian cells (HEK, CHO) on the one hand, and insect and bacterial cells— on the other hand is not only in the content of cholesterol but in many other parameters including composition of phospholipids. Indeed, it has been reported that the content of phosphocholine (PC) lipids in HEK cell membranes is about 33% of total lipids, about two-fold higher compared to Sp9 membranes which have higher phosphoethanolamine (PE) (almost 40% of total lipids). The difference in lipid composition is even more pronounced when E. coli membranes are considered: they consist predominantly (almost 75%) of zwitterionic PE with the remainder consisting of anionic lipids (PG) and cardiolipin. To prove that the presence or absence of cholesterol plays a major role in modulating the spontaneous signaling by CB2, we reconstituted the purified receptor into lipid bilayers of defined composition, containing POPC/POPG, 3/1, mol/mol and cholesterol in the range of 0–40% of total lipids. Indeed, the presence of cholesterol in these artificial bilayers resulted in an increase of spontaneous signaling by CB2. This effect was observed for the recombinant receptor isolated from E. coli cells as well as from the HEK Expi293F cells, providing additional evidence for an increase of basal activity of the receptor by cholesterol.

What are the mechanisms by which cholesterol modulates function of GPCR? There are several examples of GPCR that exhibit a certain affinity for cholesterol, and whose activities are regulated in response to the content of cholesterol in membranes. This includes the β2-adrenergic receptor and the μ-opioid receptor for which specific high affinity cholesterol binding sites have been reported near the transmembrane helices.

A putative cholesterol binding sequence (CRAC) was reported for transmembrane helix 7 of human CB1 receptor. This sequence was proposed to be involved in directing the interaction of CB1 receptor with cholesterol-rich microdomains of cell membranes. Moreover, the presence of a cholesterol molecule was recently reported in a crystal structure and a cryo-EM structure of CB1 receptor. At the same time, there was no evidence of a specific retention of cholesterol in a recently published CB2 crystal structure. There is cholesterol included in PDB 6PT0, a cryo-EM structure of CB2, although its origin is unclear since the protein was expressed in Sp9 cells that produce very little if any cholesterol. Also, any specific interaction of cholesterol with certain sites on the receptor may not explain why modulation of receptor function occurs at relatively high cholesterol concentrations in the lipid matrix surrounding the receptor.

Using MD simulations, we searched for structural and dynamical correlates of the experimental results. In simulations of free CB2, the ICL3-TM6 region, known to interact with Gαi, deviated significantly in RMSD/RMSF from the antagonist-bound conformation in the presence of cholesterol. Since the antagonist-bound conformation would be less likely to recruit G protein by definition, deviating from it is consistent with the large experimentally observed increase in constitutive activity. By contrast, the MRI-2659-bound structure showed relatively little deviation in this region, consistent with the strong antagonist activity of MRI-2659 in both cholesterol conditions.

The situation is less clear with the other two simulated ligands. We observed that the size of the ligand is inversely correlated with the effect of cholesterol on both Trp258 and ICL3-TM6. Yet this correlation does not map precisely to the corresponding changes in categorizing the ligands’ actions. When cholesterol is included in the membrane, the strong agonist MRI-2594 remains a (less-strong) agonist, the weaker agonist MRI-2646 becomes an inverse agonist, and the strong inverse agonist MRI-2659 remains as such (Fig. 3). The ligands are categorized relative to the baseline of ligand-free constitutive activity. The relative activity rank order of the MRI ligand series ligands (Fig. 3, y-axes) is preserved in both cholesterol conditions. We consider that the simulation-observed changes in the binding and ICL3-TM6 (i.e. G-protein binding) sites may be independent.
components contributing to the overall experimentally-observed effects, rather than ligand-specific effects that directly correlate with the pharmaceutical categories of ligands, since the simulation results correlated with the ligand sterics rather than their pharmaceutical categories. This is consistent with the hypothesis that the primary effect of cholesterol is to modify the baseline constitutive activity that defines how the tested ligands are categorized.

Our simulation cannot elucidate the allosteric pathway from binding pocket to G protein that would be responsible for the data—such an endeavor is well outside the scope of this work. While the chemical identity of the ligand is presumably important, we have not specifically addressed the effects of specific ligands beyond sterics. Future work might include simulating the CB2-G-protein complex and estimating the binding energy difference as a function of cholesterol and ligand, but this is an exceedingly large task.

The data do not explain how the cholesterol substituent at the 6-position of the benzo[b]thiazole arm may regulate effects of cholesterol on CB2 activation. While the toggle switch Trp2586.48 functions as an important molecular determinant in activation or deactivation of the receptor, it sheds limited light on differential interactions leading to neutral vs. inverse agonism. That the compound MRI-2654 with a bromo-substituent still behaves as an inverse agonist attests to the subtle difference in size and electronegativity of the chloro-group in influencing the molecular dynamics and signaling processes resulting in modulation of receptor function.

By using MRI-2646 ligand we demonstrate that cholesterol increases the constitutive activity of CB2 receptor. The content of cholesterol in preparations of cell membranes expressing CB2 correlates with an increase in basal signaling through CB2, which could be reversed by depletion of cholesterol using cyclodextrin. These results suggest that the pharmacological properties of synthetic ligands can be influenced by the cholesterol composition of cell membranes harboring cannabinoid receptor. Such a regulatory mechanism may contribute for well documented tissue- and cell-specific differences in the efficacy of partial CB2 agonists, such as the endocannabinoid anandamide or the plant-derived cannabinoid Δ9-tetrahydrocannabinol. For example, THC acted as a full CB2 agonist in suppressing interferon-γ-induced activation of microglia, whereas it had no CB2 agonist activity and acted as a CB2 antagonist by blocking 2-AG-induced migration of natural killer cells, which have high levels of membrane lipid, including cholesterol.

**Methods**

**Materials.** Chromatographic resin Ni-NTA was purchased from Qiagen (Germantown, MD). Streptactin XT was from IBA Life Sciences (Goettingen, Germany). The detergents CHAPS (3-[(3-Cholamidopropyl)-Dimethylammonio]-1-Propane Sulfonate) • N,N-Dimethyl-3-Sulfo-N-[3-[(3a,5β,7a,12a)-3,7,12-Trihydroxy-24-Oxocholan-24-y][Amino]propyl]-1-Propanaminium Hydroxide, Inner Salt), LMNG (Lauryl Maltose Neopentyl Glycol) and DDM (Dodecyl-β-D-Maltoside) were from Anatrace (Maumwee, OH). CHS-Tris salt was from Anatrace. The detergent Façade-TEG (3a,7a,12a-tri-((O-b-D-glucopyranosyl)ethyl)-cholane) and lipids POPC, POPG, brain lipid extract and cholesterol were from Avanti Polar Lipids (Alabaster, AL).

The potent non-selective CB2 agonist CP-55,940 ((-)cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl) cyclohexanol, the high affinity selective CB2 inverse agonist SR-144,528 5-(4-Chloro-3-methoxyphenyl)-1-[(4-methylphenyl)methyl]-N-[1S,2S,4R]-1,3,3-trimethylbicyclo[2.2.1]hept-2-yl]-1H-pyrazole-3-carboxamide were from Cayman Chemical (Ann Arbor, MI). 

Habeled CP-35,940 was from Perkin Elmer Life Sciences (Akrhon, OH). All other reagents were from Sigma-Aldrich (USA).

Preparations of CHO cell membranes expressing CB2 were obtained from the following sources: PerkinElmer (Cat. No ES111-M400UA, Billerica, MA), Millipore ChemiScreen (Cat. No P34972, Burlington, MA) and Applied Cell Sciences (Cat. No A318, Rockville, MD). Preparations of S9 cell membranes expressing CB2 were from Signal Screen (Cat. No 6110130, Rockville, MD).

Expression of HEK cells were obtained from ThermoFisher Scientific (Cat. No: A14527), and CB2 expressed and membranes obtained in house were used as described elsewhere. The E. coli cells BL21 (DE3) were obtained from EMD Millipore-Sigma (Cat. No. 69450), and CB2 expressed and membranes were obtained in house as described elsew34.

**Chemistry.** Commercially available regents were purchased and used as is. Proton (1H NMR) spectra were recorded on a Varian 400 or Bruker 500 MHz spectrometer in solvents indicated with the values given in ppm (TMS as internal standard) and J (Hz) assignments of proton resonance coupling. Mass spectra (HRMS) were recorded on a JEOL SX102a mass spectrometer. Thin layer chromatography (TLC) was carried out on 5 cm × 10 cm silica gel GHLF 0.25 mm plates using various gradients of EtOAc:

**Synthesis and characterization of MRI-2687** and MRI-2594. The data do not explain how the chloro- substituent at the 6-position of the benzo[b]thiazole arm may regulate effects of cholesterol on CB2 activation. While the toggle switch Trp2586.48 functions as an important molecular determinant in activation or deactivation of the receptor, it sheds limited light on differential interactions leading to neutral vs. inverse agonism. That the compound MRI-2654 with a bromo-substituent still behaves as an inverse agonist attests to the subtle difference in size and electronegativity of the chloro-group in influencing the molecular dynamics and signaling processes resulting in modulation of receptor function.

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**Synthesis and characterization of MRI-2687** and MRI-2594. Synthesis and characterization of MRI-2646, MRI-2654, MRI-2653 and MRI-2659 were carried as outlined in Li et al. N-(6-methyl-3-(2-hydroxyethyl)benzo[d]thiazol-2(3H)-ylidene)-2,2,3,3-tetramethylcyclopropane-1-carboxamide (4a).

N-(6-chloro-3-(2-hydroxyethyl)benzo[d]thiazol-2(3H)-ylidene)-2,2,3,3-tetramethylcyclopropane-1-carboxamide (4b).
2-amino-6-chlorobenzothiazole (1.84 g, 10.0 mmol) gave compound 4b (700 mg, 20%) as a white powder over two steps. Mp 147–149 °C; ¹H-NMR (400 MHz, CDCl₃): δ 7.56 (s, 1H), 7.36 (d, J = 8.7 Hz, 1H), 7.22 (d, J = 8.7 Hz, 1H), 4.49 (t, J = 4.6 Hz, 3H), 4.06 (t, J = 4.6 Hz, 3H), 1.58 (s, 2H), 1.32 (s, 8H), 1.22 (s, 8H). LCMS [M + H]+: 353.2.

N-(6-bromo-3-(2-hydroxyethyl)benzo[d]thiazol-2(3H)-ylidene)-2,2,3,3-tetramethylcyclopropane-1-carboxamide (4c).

2-amino-6-bromobenzothiazole (2.29 g, 10.0 mmol) gave compound 4c (950 mg, 24%) as a white powder over two steps. Mp 173–175 °C; ¹H-NMR (400 MHz, CDCl₃): δ 7.70 (s, 1H), 7.50 (d, J = 8.7 Hz, 1H), 7.17 (d, J = 8.7 Hz, 1H), 4.49 (t, J = 4.5 Hz, 2H), 4.17 (s, 1H), 4.06 (s, 2H), 1.58 (s, 1H), 1.32 (s, 6H), 1.22 (s, 6H). LCMS [M + H]+: 397.1.

N-(3-(2-hydroxyethyl)-6-methoxybenzo[d]thiazol-2(3H)-ylidene)-2,2,3,3-tetramethylcyclopropane-1-carboxamide (4d).

2-amino-6-methoxybenzothiazole (1.8 g, 10.0 mmol) gave compound 4d (920 mg, 26%) as a white powder over two steps. Mp 164–166 °C; ¹H-NMR (400 MHz, CDCl₃): δ 7.18 (d, J = 8.9 Hz, 1H), 7.11 (s, 1H), 6.99–6.96 (m, 1H), 4.82 (s, 1H), 4.48 (t, J = 4.5 Hz, 2H), 4.05 (d, J = 3.5 Hz, 2H), 3.83 (s, 3H), 1.55 (s, 4H), 1.32 (s, 6H), 1.21 (s, 6H). LCMS [M + H]+: 349.2.

N-(3-(2-hydroxyethyl)-6-(trifluoromethoxy)benzo[d]thiazol-2(3H)-ylidene)-2,2,3,3-tetramethylcyclopropane-1-carboxamide (4e).

2-amino-6-trifluoromethoxybenzothiazole (1.5 g, 6.4 mmol) gave compound 4 (610 mg, 24%) as a white powder over two steps. Mp 149–151 °C; ¹H-NMR (400 MHz, CDCl₃): δ 7.46 (s, 1H), 7.30 (s, J = 7.6 Hz, 2H), 4.51 (s, 2H), 4.07 (s, 3H), 1.59 (s, 1H), 1.32 (s, 6H), 1.22 (s, 6H). LCMS [M + H]+: 403.2.

2-(6-methyl-2-((2,2,3,3-tetramethylcyclopropane-1-carbonyl)imino)benzo[d]thiazol-3(2H)-yl)ethyl acetate (MRI-2687) (2a).

2-(6-Chloro-2-((2,2,3,3-tetramethylcyclopropane-1-carbonyl)imino)benzo[d]thiazol-3(2H)-yl)ethyl acetate (MRI-2646) (2b).
Compound 4b (300 mg, 0.85 mmol) was used as a starting material to give compound 2b (195 mg, 58%) as a white powder. Mp 123–125 °C; ¹H-NMR (400 MHz, CDCl₃): δ 7.54 (s, 1H), 7.34 (d, J = 8.8 Hz, 1H), 7.23 (s, 1H), 4.56 (t, J = 5.2 Hz, 2H), 4.46 (t, J = 5.3 Hz, 2H), 1.95 (s, 3H), 1.64 (s, 1H), 1.33 (s, 6H), 1.23 (s, 6H). LCMS [M + H]⁺: 395.2.

2-(6-Bromo-2-(((2,2,3,3-tetramethylcyclopropane-1-carbonyl)imino)benzo[d]thiazol-3(2H)-yl)ethyl acetate (MRI-2654) (2c).

Compound 4c (300 mg, 0.75 mmol) was used as a starting material to give compound 2c (140 mg, 42%) as a white powder. Mp 127–129 °C; ¹H-NMR (400 MHz, CDCl₃): δ 7.68 (s, 1H), 7.48 (d, J = 8.3 Hz, 1H), 7.19 (d, J = 8.6 Hz, 1H), 4.55 (d, J = 5.3 Hz, 2H), 4.47 (d, J = 5.2 Hz, 2H), 1.95 (s, 3H), 1.64 (s, 1H), 1.57–1.49 (m, 7H), 1.33 (s, 6H), 1.23 (s, 6H). LCMS [M + H]⁺: 439.2.

2-(6-Methoxy-2-(((2,2,3,3-tetramethylcyclopropane-1-carbonyl)imino)benzo[d]thiazol-3(2H)-yl)ethyl acetate (MRI-2653) (2d).

Compound 4d (400 mg, 1.1 mmol) was used as a starting material to give compound 2d (230 mg, 51%) as a white powder. Mp 154–156 °C; ¹H-NMR (400 MHz, CDCl₃): δ 7.21 (s, 1H), 7.09 (s, 1H), 6.96 (d, J = 9.3 Hz, 1H), 4.55 (d, J = 5.3 Hz, 2H), 4.47 (d, J = 5.2 Hz, 2H), 7.24–1.22 (m, 82H), 3.83 (s, 3H), 1.95 (s, 3H), 1.62 (s, 1H), 1.34 (s, 6H), 1.22 (s, 6H). LCMS [M + H]⁺: 391.2.

2-(2-(((2,2,3,3-tetramethylcyclopropane-1-carbonyl)imino)-6-(trifluoromethoxy)benzo[d]thiazol-3(2H)-yl)ethyl acetate (MRI-2659) (2e).

Compound 4e (300 mg, 0.75 mmol) was used as a starting material to give compound 2e (190 mg, 57%) as a white powder. Mp 81–83 °C; ¹H-NMR (400 MHz, CDCl₃): δ 7.44 (s, 1H), 7.32 (d, J = 8.8 Hz, 1H), 7.26 (s, 2H), 4.57 (d, J = 5.4 Hz, 2H), 4.47 (t, J = 5.4 Hz, 2H), 1.95 (s, 3H), 1.64 (s, 1H), 1.33 (s, 6H), 1.23 (s, 6H). LCMS [M + H]⁺: 445.3.

**CB2 expression in E. coli and purification.** CB2 was expressed as a fusion with the maltose binding protein (MBP) in BL21 (DE3) E. coli culture and purified on milligram-scale as previously described.14,63 In brief, 10
L of 2xYT media containing 0.2% glucose supplemented with ampicillin was inoculated with an overnight culture of *E. coli*. After reaching an optical density of 0.4, CB2 expression was induced by addition of 0.5 mM IPTG and 2.5 μM CB2 agonist CP-55,490. Expression was conducted for additional 42 h at 20 °C. After expression, cells were harvested by centrifugation, washed with cold PBS, and lysed in a cell homogenizer (Avestin). Receptor was solubilized for 1 h at 4 °C under continuous stirring by addition of concentrated detergent to final concentrations of (0.1% CHS, 1.0% DDM, 0.5% CHAPS, all w/v). The insoluble material was removed by centrifugation at 170,000 × g for 1 h and the solubilized receptor was then purified by the affinity chromatography in 50 mM Tris pH 7.5, 150 mM NaCl, 0.1% CHS, 0.1% DDM, 0.5% CHAPS, all w/v; 30% glycerol (v/v) and 10 μM CP-55,490 (buffer A) on Ni–NTA (Qiagen). MBP fusion partner was then removed upon incubation with the tobacco etch virus (TEV) protease for 4 h at 4 °C, and the released receptor was further purified by chromatography on StrepTactin XT (IBA Biosciences) and eluted in buffer A supplemented with 50 mM biotin as described previously.

**CB2 expression in HEK cells and purification.** Biomass from 3 L of Exp293F GNTI cells expressing CB2 containing N-terminal twin-Streptag and C-terminal His10 tag was obtained according to manufacturer’s protocol (ThermoFisher Scientific). Protein was solubilized in detergents and purified by the two successive rounds of affinity chromatography on Ni–NTA resin and StrepTactin XT resin as described.

**Removal of the CHS and ligand from purified CB2.** An amount of 2 mg of purified CB2 was bound to 1200 μL of HisPur Co2+ (ThermoFisher) resin in buffer A and incubated under shaking for 2 h at 4 °C. The protein sample was then transferred to a disposable gravity column and washed with 40 column volumes (CV) of 0.5% CHAPS/0.1% DDM in 50 mM Tris–HCl, pH 7.5, 150 mM NaCl. Protein was then eluted with 6xCV of the same buffer supplemented with 250 mM imidazole; combined eluates concentrated on 30 kDa MWCO spin concentrator and washed 3 times to remove imidazole. Concentrated protein was supplemented with 15% glycerol and aliquots stored at −80 °C until further use. To confirm that both ligand and CHS have been removed from the sample, 20 μL aliquot was mixed with 300 μL of chloroform–methanol mixture (1:1 v/v) and 1H-NMR spectra acquired.

**Reconstitution of CB2 into liposomes.** Reconstitution of the purified CB2 into liposomes was performed as described earlier. Briefly, 200 μg of the purified protein was mixed with 2 mg of lipid mixture (POPC:POPG, 3:1, mol/mol without or with addition or 20 mol% or 40 mol% of cholesterol) solubilized in 1% CHAPS at a concentration of 5 mg lipid/mL, and incubated on ice for 30 min. The detergents were then removed on 4 mL Detergent Removal spin column (Pierce), according to manufacturer’s instructions. The combined filtrate containing proteoliposomes was collected, and aliquots frozen in liquid nitrogen. Frozen liposomes were stored at −80 °C until further use. Content of protein in proteoliposomes was determined by BioRad DC assay.

**Ligand-binding assay in hCB2-CHO-K1 cell membranes.** The assay was performed as described previously with slight modifications. Briefly, hCB2-CHO-K1 cell membranes (4 μg) were incubated with 0.05 nM [3H] CP-55,490 as the radioligand. Plasma membranes were from cultured CHO-K1 cells stably transfected with human CB2R (Perkin Elmer). Two microgram plasma membrane protein was used in a 1 mL reaction mixture. Ki values were derived by computerized curve fitting and using the Cheng-Prusoff equation to account for the affinity of the radioligand, using the GraphPad Prism 8 program (GraphPad Prism Software Inc.).

**[35S] GTPyS binding assay in hCB2-CHO-K1 cell membranes.** [35S] GTPyS binding was assayed as described earlier with slight modifications. Briefly, hCB2-CHO-K1 cell membranes (4 μg) were incubated with 0.05 nM [35S] GTPyS, and the indicated concentrations of ligands in TEM buffer (50 mM Tris–HCl, 0.2 mM EGTA, and 9 mM MgCl2, pH 7.4) containing 100 μM GDP, 150 mM NaCl, and 0.1% (w/v) bovine serum albumin in a total volume of 1 mL for 60 min at 30 °C.

**[35S] GTP nucleotide exchange (GEF) assays.** The subunits of G protein were expressed and purified as described previously. The nucleotide exchange assay was performed as previously described.

**Molecular dynamics simulations.** The CHARMM36 force field was used. Before ligand parameterization, each ligand was geometry optimized using the B3LYP/6-31G** quantum mechanics level of theory and basis set using Gaussian09. Ligand parameters were derived from CGenFF; these high affinity ligands were not expected to explore the extremes of their conformational space. The CB2 structure starting point was the cryo-EM structure previously described (Protein Data Bank: 6PT0). This was oriented and placed in a lipid membrane using the Orientations of Proteins in Membranes (OPM) database using the CHARMM-GUI input generator. In the wt–cholesterol condition, the lipid membrane consisted of 40% cholesterol, and POPC:POPG in a 3:1 ratio. Sodium and chloride atoms were added to 0.15 M with excess for electroneutrality. The cholesterol molecules present in the cryo-EM structure were retained, particularly since the extracellular cholesterol conformations were analogous to those observed in the structurally similar mu opioid receptor. The system was minimized and equilibrated with side chain and backbone restraints which were subsequently released, and production simulations were run in the isothermic-isobaric ensemble at 303.15 K using NAMD 2.13 with GPU extensions. Particle Mesh Ewald summation of long-range interactions was used, as were the Langevin barostat and thermostat.
References

1. Jastrzebska, B., Debinski, A., Filipek, S. & Palczewski, K. Role of membrane integrity on G protein-coupled receptors: Rhodopsin stability and function. *Prog. Lipid Res.* 50, 267–277. https://doi.org/10.1016/j.plipres.2011.03.002 (2011).

2. Marino, K. A., Prada-Gracia, D., Provasi, D. & Filizola, M. Impact of lipid composition and receptor conformation on the spatio-temporal organization of mu-opioid receptors in a multi-component plasma membrane model. *PLoS Comput. Biol.* 12, e1005240. https://doi.org/10.1371/journal.pcbi.1005240 (2016).

3. Salas-Estrada, L. A., Leioats, N., Romo, T. D. & Grossfield, A. Lipids alter receptor function via ligand-like and solvent-like interactions. *Biophys. J.* 114, 355–367. https://doi.org/10.1016/j.bpj.2017.11.021 (2018).

4. Escriba, P. V. et al. Role of lipid polymorphism in G protein-membrane interactions: Nonlamellar-prone phospholipids and peripheral protein binding to membranes. *Proc. Natl. Acad. Sci. U.S.A.* 94, 11375–11380. https://doi.org/10.1073/pnas.94.21.11375 (1997).

5. Maxfield, F. R. & van Meer, G. Cholesterol, the central lipid of mammalian cells. *Curr. Opin. Cell Biol.* 22, 422–429. https://doi.org/10.1016/j.celrep.2010.05.004 (2010).

6. Chini, B. & Parenti, M. G-protein-coupled receptors, cholesterol and palmitoylation: Facts about fats. *J. Mol. Endocrinol.* 42, 371–379. https://doi.org/10.1677/JME-08-0114 (2009).

7. Manna, M. et al. Mechanism of allosteric regulation of beta2-adrenergic receptor by cholesterol. *Elife* https://doi.org/10.7554/ eLife.18432 (2016).

8. Guixa-Gonzalez, R. et al. Membrane cholesterol access into a G-protein-coupled receptor. *Nat. Commun.* 8, 14505. https://doi.org/10.1038/ncomms14505 (2017).

9. Hefizi, F. F. Requirements for a lead compound to become a clinical candidate. *BMC Neurosci.* 9, S7. https://doi.org/10.1186/1471-2202-9-S3-S7 (2008).

10. D
35. Parathath, S. et al. Changes in plasma membrane properties and phosphatidylcholine subtypes of insect SF9 cells due to expression of scavenger receptor class B, type I, and CD36. J. Biol. Chem. 279, 41310–41318. https://doi.org/10.1074/jbc.M404952200 (2004).

36. Gimpl, G., Klein, U., Reilander, H. & Fahrenholz, F. Expression of the human oxytocin receptor in baculovirus-infected insect cells: High affinity binding is induced by a cholesterol-cycloexetrin complex. Biochemistry 34, 13794–13801. https://doi.org/10.1016/S0006-2952(95)00042-010 (1995).

37. Huang, Z. & London, E. Cholesterol lipids and cholesterol-containing lipid rafts in bacteria. Chem. Phys. Lipids 199, 11–16. https://doi.org/10.1016/j.chemphyslip.2016.03.002 (2016).

38. Liu, S. L. et al. Orthogonal lipid sensors identify transbilayer asymmetry of plasma membrane cholesterol. Nat. Chem. Biol. 13, 268–274. https://doi.org/10.1038/nchembio.2268 (2017).

39. Mahammad, S. & Parmryd, I. Cholesterol depletion using methyl-beta-cyclodextrin. Amino Acids 51, 105–113. https://doi.org/10.1007/s00726-016-2364-7 (2016).

40. Yeliseev, A. A. & Vukoti, K. in Methods and Protocols. Humana Press, New York (2015).

41. Yeliseev, A. A., Zoubak, L. & Schmidt, T. G. M. Application of Strep-Tactin XT for affinity purification of Twin-Strep-tagged CB2, a G protein-coupled cannabinoid receptor. Protein Expr. Purif. 131, 109–118. https://doi.org/10.1016/j.pep.2016.11.006 (2017).

42. Yeliseev, A., Xu, J. et al. Thermostability of a recombinant G protein-coupled receptor expressed at high level in mammalian cell culture. Sci. Rep. 10, 16805. https://doi.org/10.1038/s41598-020-73813-7 (2020).

43. Vukoti, K., Kimura, T., Macke, L., Gawrisch, K. & Yeliseev, A. Stabilization of functional recombinant cannabinoid receptor CB2 in detergent micelles and lipid bilayers. PLoS ONE 7, e66290. https://doi.org/10.1371/journal.pone.0066290 (2012).

44. Xing, C. et al. Cryo-EM structure of the human cannabinoid receptor CB2-Gi signaling complex. Cell https://doi.org/10.1016/j.cell.2020.01.007 (2020).

45. Huang, W. et al. Structural insights into micro-opioid receptor activation. Nature 524, 315–321. https://doi.org/10.1038/nature14886 (2015).

46. Salari, R., Joseph, T., Lohia, R., Henin, J. & Brannigan, G. A streamlined, general approach for computing lipid binding free energies and its application to GPCR-bound cholesterol. J. Chem. Theory Comput. 14, 6560–6573. https://doi.org/10.1021/acs.jctc.8b00447 (2018).

47. Kimura, T. et al. Role of cholesterol in the function and organization of G protein-coupled receptors. J. Biol. Chem. 292, 12354–12367. https://doi.org/10.1074/jbc.M116.769324 (2017).

48. Andersen, O. S. & Koeppe, R. E. Bilayer thickness and membrane protein function: An energetic perspective. Biochim. Biophys. Acta 1768, 1311–1324. https://doi.org/10.1016/j.bbabmb.2007.03.026 (2007).

49. Sohlenkamp, C. & Geiger, O. Bacterial membrane lipids: Diversity in structures and pathways. FEMS Microbiol. Rev. 40, 133–159. https://doi.org/10.1093/femsre/fvu008 (2016).

50. Hua, T. et al. Crystal structure of the human cannabinoid receptor CB1. Cell 167, 750–762. https://doi.org/10.1016/j.cell.2016.10.004 (2016).

51. Krishnan, K. V. & Kuo, T. Structural and functional characterization of recombinant cannabinoid receptor 1. Mol. Pharmacol. 88, 488–498. https://doi.org/10.1124/mol.117.107830 (2015).

52. Yeagle, P. L. Modulation of membrane function by cholesterol. Biochim. Biophys. Acta 733, 1303–1310. https://doi.org/10.1016/0005-2728(85)90093-9 (1985).

53. Pucadyil, T. J. & Chattopadhyay, A. Role of cholesterol in the function and organization of G-protein coupled receptors. Prog. Lipid Res. 45, 293–333. https://doi.org/10.1016/j.plipres.2006.02.002 (2006).

54. Salari, R., Joseph, T., Lohia, R., Henin, J. & Brannigan, G. A streamlined, general approach for computing lipid binding free energies and its application to GPCR-bound cholesterol. J. Chem. Theory Comput. 14, 6560–6573. https://doi.org/10.1021/acs.jctc.8b00447 (2018).

55. Ohnishi, C. et al. Diversity in structures and pathways. FEMS Microbiol. Rev. 40, 133–159. https://doi.org/10.1093/femsre/fvu008 (2016).

56. Hua, T. et al. Crystal structure of the human cannabinoid receptor CB1. Cell 167, 750–762. https://doi.org/10.1016/j.cell.2016.10.004 (2016).

57. Krishnan, K. V. & Kuo, T. Structural and functional characterization of recombinant cannabinoid receptor 1. Mol. Pharmacol. 88, 488–498. https://doi.org/10.1124/mol.117.107830 (2015).

58. Yeagle, P. L. Modulation of membrane function by cholesterol. Biochim. Biophys. Acta 733, 1303–1310. https://doi.org/10.1016/0005-2728(85)90093-9 (1985).

59. Pucadyil, T. J. & Chattopadhyay, A. Role of cholesterol in the function and organization of G-protein coupled receptors. Prog. Lipid Res. 45, 293–333. https://doi.org/10.1016/j.plipres.2006.02.002 (2006).

60. Ohnishi, C. et al. Diversity in structures and pathways. FEMS Microbiol. Rev. 40, 133–159. https://doi.org/10.1093/femsre/fvu008 (2016).

61. Hua, T. et al. Crystal structure of the human cannabinoid receptor CB1. Cell 167, 750–762. https://doi.org/10.1016/j.cell.2016.10.004 (2016).

62. Krishnan, K. V. & Kuo, T. Structural and functional characterization of recombinant cannabinoid receptor 1. Mol. Pharmacol. 88, 488–498. https://doi.org/10.1124/mol.117.107830 (2015).

63. Yeagle, P. L. Modulation of membrane function by cholesterol. Biochim. Biophys. Acta 733, 1303–1310. https://doi.org/10.1016/0005-2728(85)90093-9 (1985).

64. Pucadyil, T. J. & Chattopadhyay, A. Role of cholesterol in the function and organization of G-protein coupled receptors. Prog. Lipid Res. 45, 293–333. https://doi.org/10.1016/j.plipres.2006.02.002 (2006).

65. Ohnishi, C. et al. Diversity in structures and pathways. FEMS Microbiol. Rev. 40, 133–159. https://doi.org/10.1093/femsre/fvu008 (2016).

66. Hua, T. et al. Crystal structure of the human cannabinoid receptor CB1. Cell 167, 750–762. https://doi.org/10.1016/j.cell.2016.10.004 (2016).

67. Krishnan, K. V. & Kuo, T. Structural and functional characterization of recombinant cannabinoid receptor 1. Mol. Pharmacol. 88, 488–498. https://doi.org/10.1124/mol.117.107830 (2015).

68. Yeagle, P. L. Modulation of membrane function by cholesterol. Biochim. Biophys. Acta 733, 1303–1310. https://doi.org/10.1016/0005-2728(85)90093-9 (1985).
71. Lee, J. et al. CHARMM-GUI input generator for NAMD, GROMACS, AMBER, OpenMM, and CHARMM/OpenMM simulations using the CHARMM36 additive force field. *J. Chem. Theory Comput.* **12**, 405–413. https://doi.org/10.1021/acs.jctc.5b00935 (2016).

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**Author contributions**

A.Y., M.R.I., R.C. conceived the project and designed the experimental procedures. M.R.I. performed synthesis of cannabinoid ligands; L.Z. and A.Y. purified the protein and performed GEF experiments; R.C. and N.J.C. performed ligand binding and GTP-binding experiments; A.Y. prepared the proteoliposomes; T.T.J. performed the MD simulation; K.G. and G.K. supervised the work and provided funding. A.Y. and M.R.I. drafted the paper with contributions from R.C., T.T.J., K.G. and G.K. All authors reviewed the final paper.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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