

Supplementary Information

Endogenous vs Exogenous Allosteric Modulators in GPCRs:

A Dispute for shuttling CB1 among different membrane microenvironments

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SUPPLEMENTARY RESULTS

1. Homology models

To date, 400 class A GPCRs are known as potential drug target¹. However, only for 23 of them the structure was solved by X-ray crystallographic studies. In this regards, for structure-based purpose, aimed at discovering new GPCR ligands as drugs, the homology modeling approach is still essential. Several models of the CB₁ receptor have been proposed until now using rhodopsin, β_2 -adrenergic and adenosine receptor subtype 2A (A_{2A}) as template²⁻⁴. Among the possible CB₁ templates, recently, the human sphingosine 1-phosphate receptor (S1P₁) has been disclosed offering new possibility to build up more reliable 3D model for the CB₁ receptor^{5,6}. S1P₁ TM helices shows an average sequence identity of about 35%, with respect to CB₁ helices (Supplementary Table 1 and 2), making it a suitable template to build a 3D model of CB₁. Receptors having the highest sequence identity with respect to the CB₁, if the entire sequence are considered, are the S1P₁ and the A_{2A} {27% and 23% of sequence identity, respectively [(data obtained from the ClustalW identity matrix)⁷ see Supplementary Table 1 and 2]}. Moreover, S1P₁ orthosteric binding site was evolutionary selected to bind sphingosine (a lipid-derived ligand) and similarly CB₁ binds a lipid-derived ligand (anandamide) as transmitter^{8,9}. In addition, experimental evidences support the notion that CB₁ and S1P₁ share a common mechanism of binding, a common activation mechanism, and that sphingosine and its analogs interact with the CB₁ receptor^{6,9}. Therefore, the S1P₁ X-ray crystal structure (pdb code: 3V2Y) was choose and used as template to generate the 3D structure of CB₁. CB₁ and S1P₁ sequences were aligned using the ClustalW server⁷ (Supplementary Fig. 1) and the 3D model of CB₁ was generated using the Modeller9.11 software¹⁰.

Supplementary Table 1 | Identity Matrix. Percent Identity Matrix obtained from Clustal2.1.

	P21554 CNR1 HUMAN	P21453 S1PR1 HUMAN	P29274 AA2AR HUMAN	P07550 ADRB2 HUMAN	P02699 OPSD BOVIN
P21554 CNR1 HUMAN	100	27,03	23,41	18,37	17,35
P21453 S1PR1 HUMAN	27,03	100	25,29	18,33	17,77
P29274 AA2AR HUMAN	23,41	25,29	100	26,3	18,47
P07550 ADRB2 HUMAN	18,37	18,33	26,3	100	16,96
P02699 OPSD BOVIN	17,35	17,77	18,47	16,96	100

Supplementary Table 2. | TM Identity Matrix. Identity percentage for relevant CB₁ and S1P₁ domains.

Domain	% ID
TM1	24.24
TM2	42.31
TM3	34.38
TM4	33.33
TM5	35.48
TM6	34.29
TM7-H8	39.47
ECL2	40
Average	35.43

Supplementary Figure 1 | S1P₁-CB₁ sequences alignment. Sequence alignment between CB₁ and S1P₁ receptors. Highlighted in light grey residues included in TM domains, in red the cysteine residues forming disulphide bond, while in green and bold conserved residues in Class A GPCR. In yellow the initial and final residues considered to build the CB₁ model. Finally in cyan the IL3 portion substituted with the TL4 lysozyme in the X-ray structure of the S1P₁ receptor.

CNR1_HUMAN	MKSILDGLADTTFRITITDLLYVGSNDIQYEDIKGDMSKLGYPQKFPL	50
S1PR1_HUMAN	-----MG--PTSVPL	8
CNR1_HUMAN	TSFRGSPFQEKMTAGDNPQLVPADQVNITEFYNKSLSSFKENEENIQCGE	100
S1PR1_HUMAN	VKAHRS-----SVSDYVNYDIIVRHY	49
CNR1_HUMAN	NFMDIECFMVLNPSQQLAIAVLSLTGLTFTVLELLVLCVILHSRSLRCR	150
S1PR1_HUMAN	NYTGKLNISADKENSIKLTSVVFILICCFIILENIFVLLTIWKTKKF-HR	78
CNR1_HUMAN	PSYHFIGSLAVADLLGSVIFVYSFIDFHVHFKDSRNVFLFKLGGVTASF	200
S1PR1_HUMAN	PMYYFIGNLALSDLLAGVAYTANLLLSGATTYKLTPAQWFLREGSMFVAL	128
CNR1_HUMAN	TASVGSFLFLTAIDRYISIHRLPAYKRIVTRPKAVVAFCLMTTIAIVIAVL	250
S1PR1_HUMAN	SASVFSLLAIAIERIYITMLK-----NNFRLFLLISACNVISLILGGL	177
CNR1_HUMAN	PLLGWNCEKLQSVCSDFPHIDETYLFWIGVTSVLLLFIVYAYMYILWK	300
S1PR1_HUMAN	PIMGWNCISALSSCSTVLPLYHKHYILFCTTVFTLLLSIVILYCRIYSL	227
CNR1_HUMAN	AHSHAVRMIQRGTQKSIIHTSEDGKVQVTRPDQARMDIRLAKTLVLILV	350
S1PR1_HUMAN	VRT-----RSSENVALLKTVIIVLS	263
CNR1_HUMAN	VLIICWGEILLAIMVYDVFGKMN-KLIKTVFAFCSMCLLNSTVNFIIYAL	399
S1PR1_HUMAN	VFIACWAPLFILLLLDVGCKVKTCDILFRAEYFLVLAVLNSGTNFIITYTL	313
CNR1_HUMAN	RSKDLRHAFRSMFPSCEGTAQPLDNSMGDSCLHKHANNAASVHRAAESC	449
S1PR1_HUMAN	TNKEVRRAFIRIMSCCKCPSGDSAGKFKRPIIAGMEFSRSKSDNSSH---	360

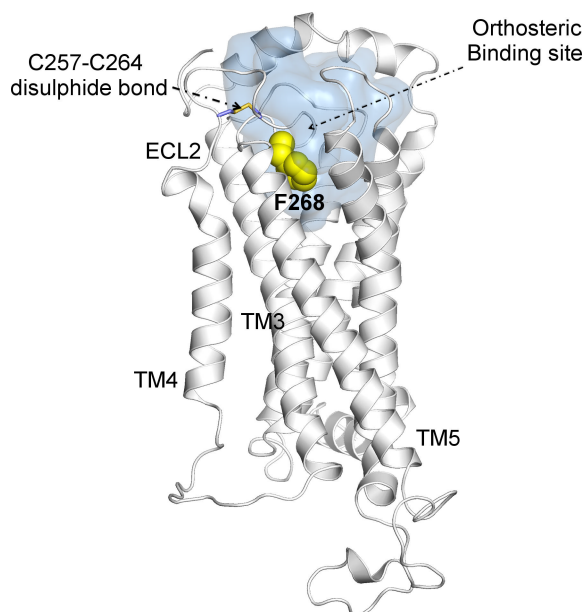
CNR1_HUMAN	IKSTVKIAKVTMSVSTDTSAEAL 472
S1PR1_HUMAN	PQKDEGDNPETIMSSGNVNSSS- 382

Twenty 3D models of CB₁ were generated using Modeller9.11 software¹⁰. The obtained models were scored using the available scoring functions in Modeller9.11 software¹⁰ (DOPE score), and through visual inspections. The best model had the conserved disulfide bond between C257 and C264 on extracellular loop 2 (ECL2). The protonation state of ionizable residues was fixed by using the Protein Preparation Wizard of the Maestro 9.1 graphical user interface,¹¹ and then checked by visual inspection. In this case the E^{1.49} and D^{2.50} residues were protonated, according to previous works.¹²

The geometry of the final model was optimized using several cycles of OPLS2005 force field (available in Maestro¹¹) minimization, until a gradient of 0.001 kcal/mol*Å² was reached.

The loop of the optimized 3D model of the CB₁ receptor were preliminary refined using the refinement loop tool available in Modeller 9.11 software¹⁰, and also in this case the obtained loop models were scored using the available scoring functions in Modeller9.11 software¹⁰, and through visual inspections. The final model was embedded in an explicit POPC/Cholesterol (2:1) bilayer and refined through 50ns of MD simulations (further details about the generation of the membrane-receptor complex, and MD simulation setting can be found in the next sections of the Supplementary Information). The resulting model is reported in Supplementary Figure 2, where it can be appreciate that the F268 residue on ECL2 is nicely accommodate inside the CB₁ binding pocket, according to the experimental evidences that this residue is relevant for ligand binding.¹³

Supplementary Figure 2 | Refined CB₁ homology models. 3D structure of the CB₁ homology model. Highlighted as surface and in blue the putative orthosteric binding pocket, while in yellow and spheres the F268 on ECL2 implicated in ligand binding.¹³ Finally, in stick and blue the cysteine residues forming the disulphide bond on ECL2.



2. Generation of the CB₁wt-membrane complex and MD simulations setting

A pre-equilibrated POPC phospholipids bilayers was generated using the membrane-builder tool of charm-gui.org (<http://www.charmm-gui.org>) 94x94 Å with a ratio POPC:cholesterol equal to 2:1. In order to place the receptor into the bilayer a hole was generated, and lipids in close contact (<1 Å distance from any protein atoms) were deleted. In this case POPC or cholesterol molecules were manually deleted in each layer in order to retain the same cholesterol concentration ratio. The membrane-receptor complexes thus obtained were solvated and neutralized using the solvation and autoionize modules of VMD1.8.9^{14,15}. The ionic strength was kept at 0.15 M by NaCl and we used TIP3 water model. Finally, the whole system was parametrized using the tleap tool of Amber12, and *Amber99SBildn*, *lipid11* and *gaff* as force field¹⁶.

The CB₁-C415-palmitoyl/membrane complex was submitted to a relaxation protocol based on six steps and a preliminary production phase of 50ns (Supplementary Table 3). The production phases was performed using the NPT ensemble and Langevin as thermostat. The cutoff for the non-bonded interactions was set equal to 10 Å and a smooth switching function was used to truncate the van der Waals potential energy smoothly at the cutoff distance; the parameters that specifies the distance at which the switching function should start was set to 8 Å, with a pairlistdist of 12 Å, updated every 2 steps. The PME grid spacing was set with a resolution of 1 Å and an interpolation order of 4. The time step was set equal to 2 fs and the RATTLE algorithm was applied. The initial periodic

boundary conditions were set equal to 94.5 x 94.5 x 110.5 Å, and frames were written every 50.000 steps.

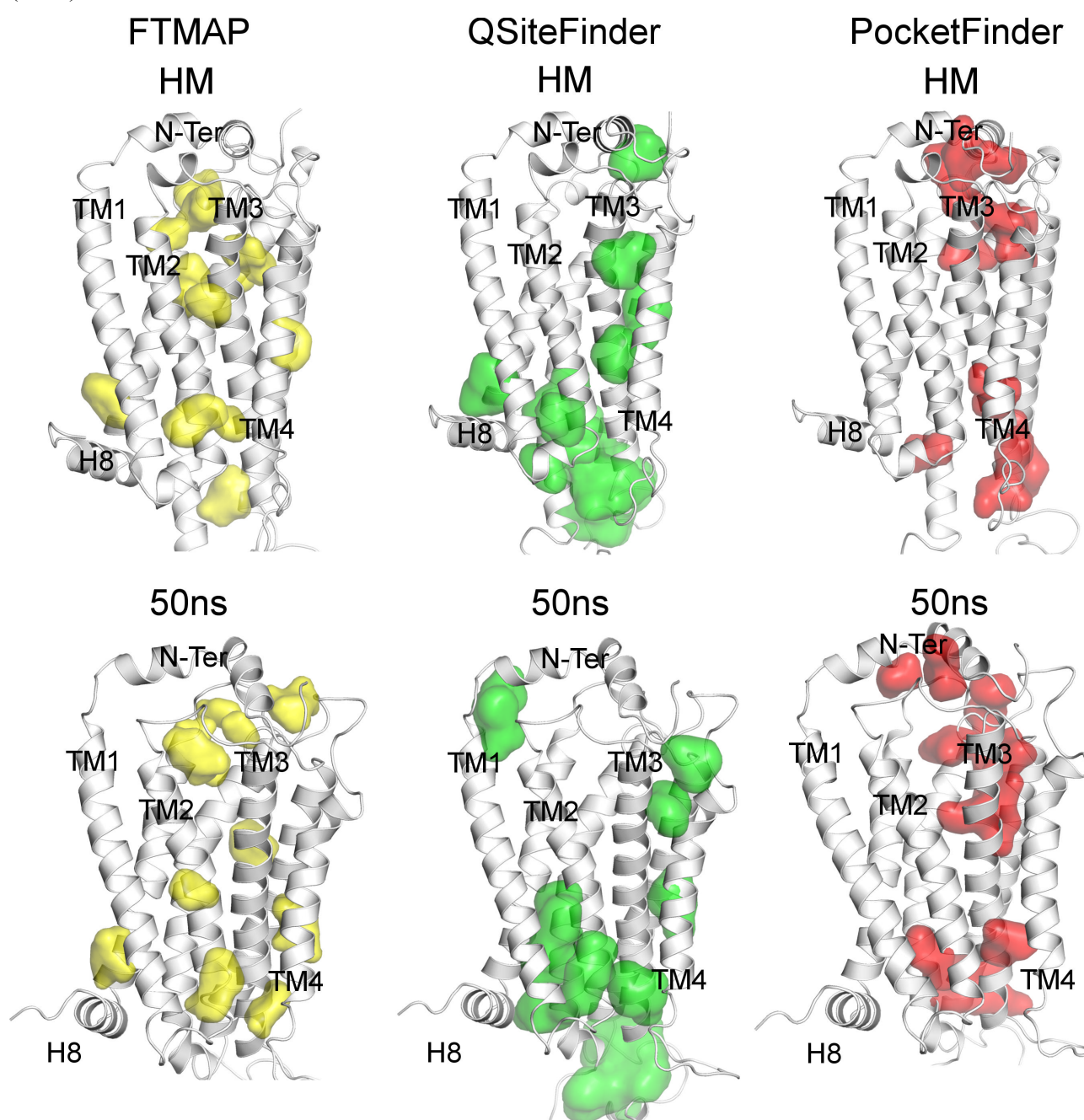
Supplementary Table 3 | MD setting. MD parameters setting.

Relaxation Phase								
	Phase	Steps/Time	Temp (K)	Harmonic Constraints kcal/mol*Å ²				Ensemble
				Backbone	Sidechains	Lipid	Solvent	
Step1	Mini	3000	0	50	50	25	25	NVT
Step2	Mini	3000	0	25	12.5	-	-	NVT
Step3	Mini	3000	0	10	5	-	-	NVT
NVT/NPT								
Step4	Heat/Pressure Equilibration	600ps	0→300	10 (Cα only)	5	-	-	Switch every 10ps
Step5	Equil1	500ps	300	5 (Cα only)	1	-	-	NPT
Step6	Equil2	10ns	300	-	-	-	-	NPT
Production Phase								
Step1	Production	50 ns	300	-	-	-	-	NPT

3. Consensus Pocket Prediction and Mutants Selection.

Reported in Supplementary Figure 3 the comparison of the 9 pockets identified by FTMAP^{17,18}, PocketFinder¹⁹, and Q-SiteFinder²⁰ in the refined homology model and in the relaxed structure of the CB₁ receptor model. In Supplementary Figure 4 the sequence alignment between CB₁ and CB₂ receptor, and highlighted the selected single point mutations.

Supplementary Figure 3 | Consensus Pocket Prediction. Comparison of the probes localization for each software used (yellow=FTMAP, green QSiteFinder, and red=PocketFinder), and for two different conformation, the refined homology model (HM), and after 50ns of MD simulations (50ns).



Supplementary Figure 4 | Mutant Selection. Sequence alignment between CB₁ and CB₂ receptors. Highlighted in gray the TM domains, in bolt and green the conserved residues of each TM domains for Class A GPCR, in bolt and red the cysteine residues forming the disulphide bond. Highlighted in green, cyan, yellow, blue and purple the sites where residue were mutated (According to the color code of Figure 1 Main Text).

sp	P21554	CNR1_HUMAN	MKSILDGLADTTFRITITDLLYVGSNDIQYEDIKGDMASKLGYFPQKFPLTSFRGSPFQE	60
sp	P34972	CNR2_HUMAN	-----	
sp	P21554	CNR1_HUMAN	KMTAGDNPQLVPADQVNITEFYNKSLSSFKENEENIQCGENFMDIECFMVLNPSQQLAIA	120
sp	P34972	CNR2_HUMAN	-----MEECWVTEIANGSKDGLDSNP-----MKDYMILSGPQKTAVA	37
sp	P21554	CNR1_HUMAN	VLSLTLTGTFVLELLVLCVILHSRSLRCRPSYHFIGSLAVALLGSAVIFVYSFIDFHVF	180
sp	P34972	CNR2_HUMAN	VLCTLLGLLSALEVAVLYLILSSHQLRRKPSYLFFIGSLAGAFSLASVVFACSFVNFHVF	97
sp	P21554	CNR1_HUMAN	HRKDSRNVFLFKLGGVTASFTASVGSFLTAIDYISIHRLPLAYKRIVTRPKAVVAFCLM	240
sp	P34972	CNR2_HUMAN	HGVDSKAVFLLKIGSVTMTFTASVGSLLLTAIDYLCRLRPPSYKALLTRGRALVTLGIM	157
sp	P21554	CNR1_HUMAN	VTIAIVLAVLPLLGNCEKLQSVCSDFPHIDETYLFWIGVTSVLLFIVYAYMYILWK	300
sp	P34972	CNR2_HUMAN	VLSALVSYLPLMGWTCPRP--CSELFPLIPNDYLLSWLLFIAFVFSGLIYTYGHVLWK	215
sp	P21554	CNR1_HUMAN	AHSHAVRMIQRGTQKSIIHTSEDGKVQVTRPDQARMDIRLAKTLVLILVVLIIICWGALL	360
sp	P34972	CNR2_HUMAN	AHQHVASLSGHQDR-----QVPGMARMRLDVRLAKTLGLVLAVLLICWFVVL	262
sp	P21554	CNR1_HUMAN	AIMVYDVFGKMNKLIKTVFAFCSMLCLLNSTVNIYIALRSKDRHAFRSMFSPCEGTAQ	420
sp	P34972	CNR2_HUMAN	ALMAHSLATTLSAQVKKAFACSMCLINSMVNIYIALRSGERSSAHHCLAHWKKCVR	322
sp	P21554	CNR1_HUMAN	PLDNSMGDSCLHKKHANNAASVHRAAESCIKSTVKIAKVTMSVSTDTSAEAL	472
sp	P34972	CNR2_HUMAN	GLG-----SEAKEEAPRSSVTETETADGKITPWPDSRDLDLSDC---	360

MUTATIONS SITE 1:

F129L
I395V
F408A

MUTATIONS SITE 2:

C139Y
H154L
F237L

MUTATIONS SITE 3:

F191L
A198M
I245A

MUTATIONS SITE 4:

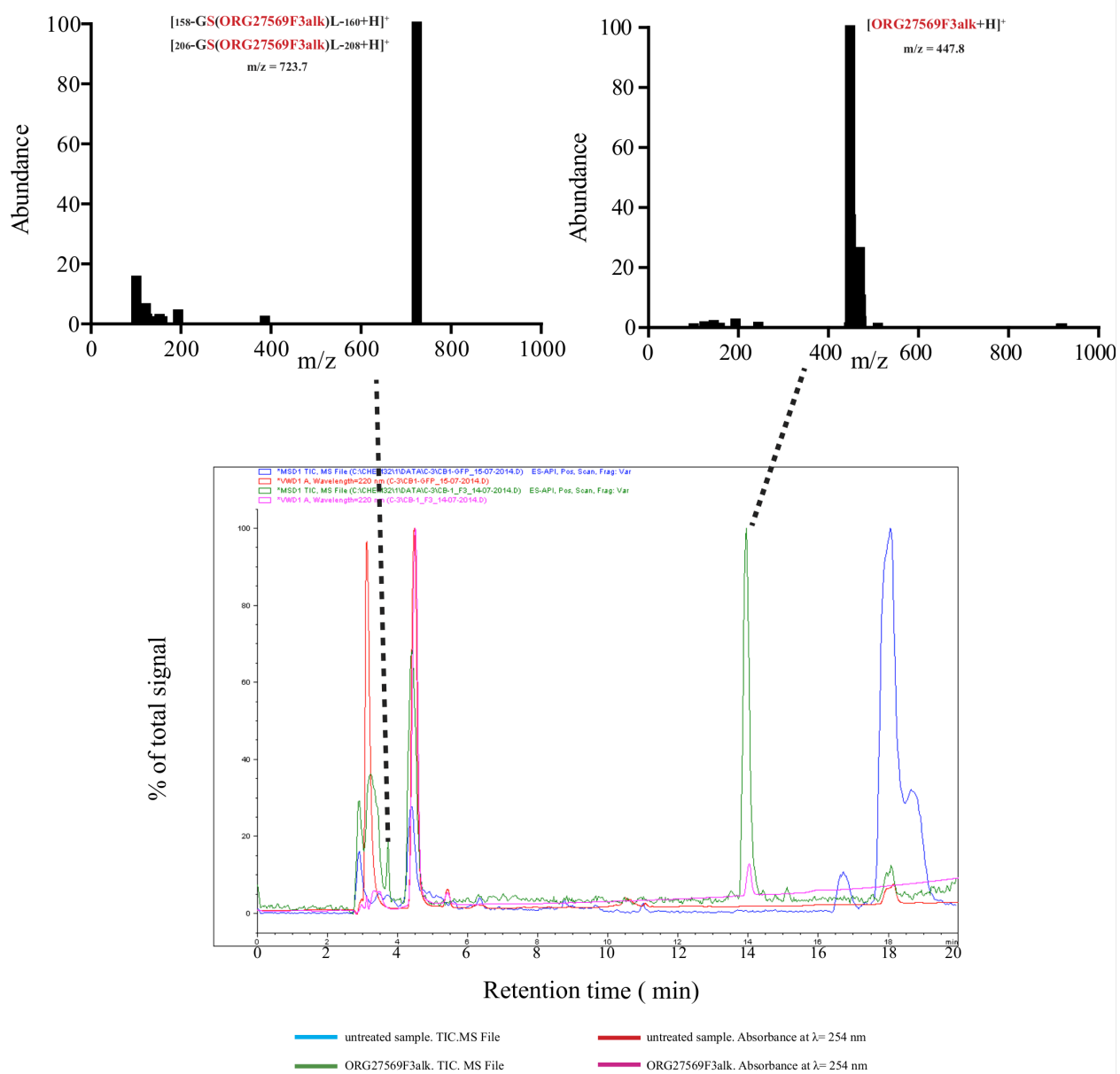
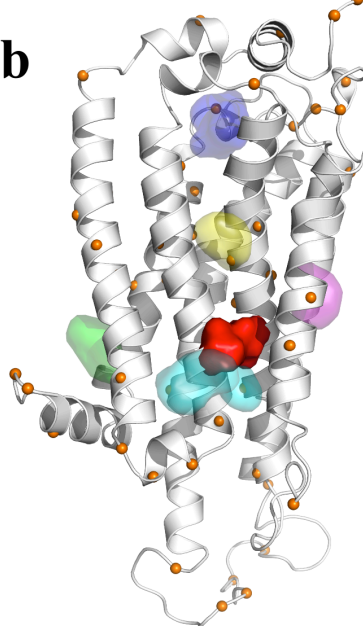
L193I
D366S
T377K

MUTATIONS SITE 5:

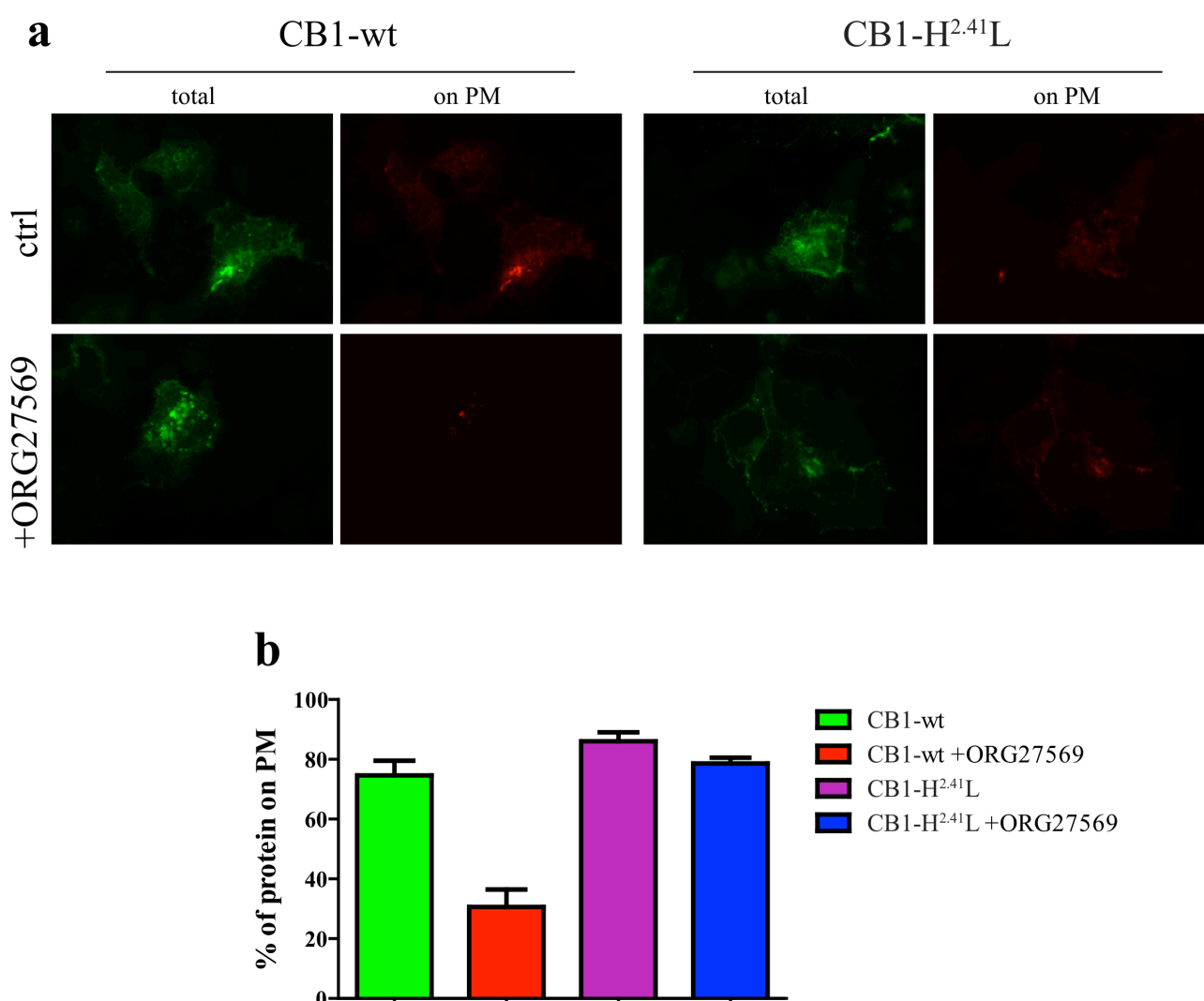
I243L
I247V
V282F

4. Liquid Chromatography/Mass Spectrometry (LC/MS) data

Supplementary Figure 5 | LC/MS analysis of ORG27569alkF3 treated CB₁. CB₁-wt HEK293 cells were treated with **ORG27569alkF3** (30 μ M) for 24 hours to be then lysed. After immunoisolation, CB₁ was digested with Proteinase K and analysed by LC/MS. Comparison between **ORG27569alkF3** treated vs. untreated CB₁ MS spectra enables to identify peptides presenting the probe covalently attached ($\Delta M = + 447.8$ Da). a) HPLC profiles of **ORG27569alkF3** treated (green curve) and untreated samples (blue curve). Absorbance at $\lambda = 254$ nm of the fractions (**ORG27569alkF3** (magenta curve) and untreated (red curve) samples) is reported. The dashed lines indicates the regions (retention time 3.8 and 14.0 min) where a difference in the HPLC profiles could be identified; the insets shows the MS spectra of sample fraction eluting at the two retention time and corresponding to CB₁ bound **ORG27569alkF3** (left panel) and unbound **ORG27569alkF3** (right panel); b) The 5 putative allosteric pockets mapped onto the CB₁ (accordingly to Fig.1a of the main text). The region addressed by **ORG27569alkF3** (S^{2.45} and S^{3.42}) is depicted with red surface superimposed with the P2 binding pocket (cyan, surface). The orange spheres highlights other possible nucleophile sites (Ser, Thr, Cys, Tyr).

a**b**

Supplementary Figure 6 | Measurement of PM localization of CB1. a) Setting used to quantitate PM localization of CB1 and CB1-H 2.41L. N-terminally 3x Flag-CB1-GFP wt and mutant were transiently expressed in HuH7 cells. These were incubated after fixation (without permeabilization) with a rabbit polyclonal anti-FLAG antibody followed by a Texas-Red coniugated secondary antibody. The immunofluorescence intensity in the Texas-Red channel (depending only on the PM localized CB1) was measured using NIH ImageJ Biophotonic programs and normalized to one of the GFP channel (depending on the total CB1-GFP expression, PM+intracellular). For each transfection, 20 cells were considered for quantification. b) Percentage of cells showing CB1 and CB1 (H2.41L) localized at PM upon treatment with ORG27569 for 4 hours (data depict the mean +/- s.e.m. and are representative of three or more independent experiments . One-way ANOVA was employed. $P < 0.05$).



5. Binding mode reliability and analysis of the trajectories

Binding mode reliability

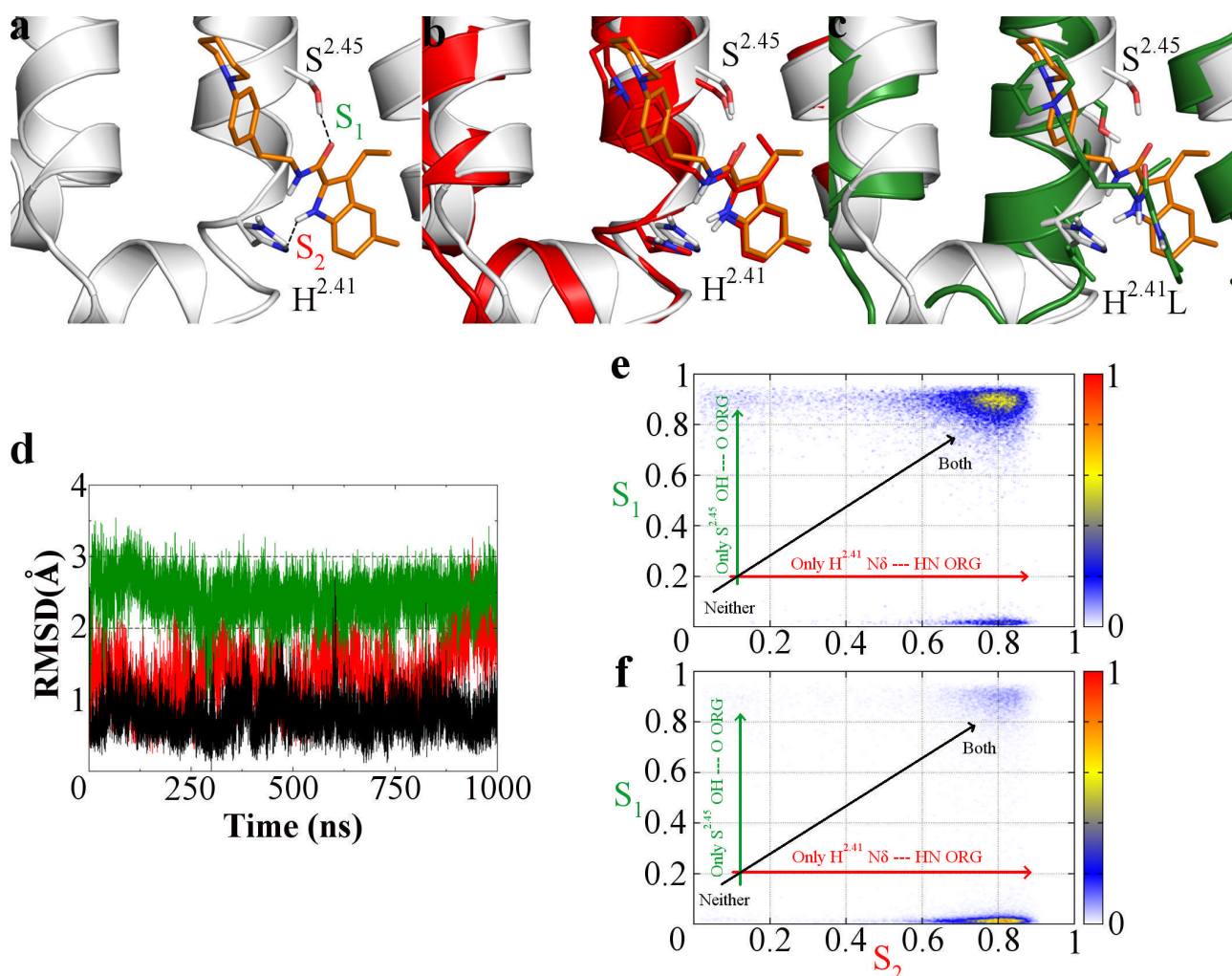
According to the binding studies on mutated CB₁ receptors, docking of ORG27569, by means of Glide software¹¹ was focused on P2 pocket. The resulting binding mode is depicted in Fig. 2a (main text), and the proposed pose fully explains the reported SAR²¹. In fact, the 3-ethyl chain, lying in a large apolar area, can be elongated without a loss of activity, the substitution of indole ring by a benzofuran moiety causes a reduced binding affinity due to the loss of the H-bond between the indole NH and the H^{2.41}N δ atom. The 1-(4-ethylphenyl)piperidine group protrudes toward the lipidic environment, establishing hydrophobic contacts with the receptor, accordingly to the reduced binding affinity observed for derivatives carrying hydrophilic substituents at this position.

Analysis of the trajectories

The PLUMED software²² was used, and H-bonds (s) between specific donor-acceptor pairs were defined by a switching functions (eq.1), with $r_0= 2.5$, $n=8$ and $m= 20$. In Supplementary Fig. 8 the probability of each H-bond is reported.

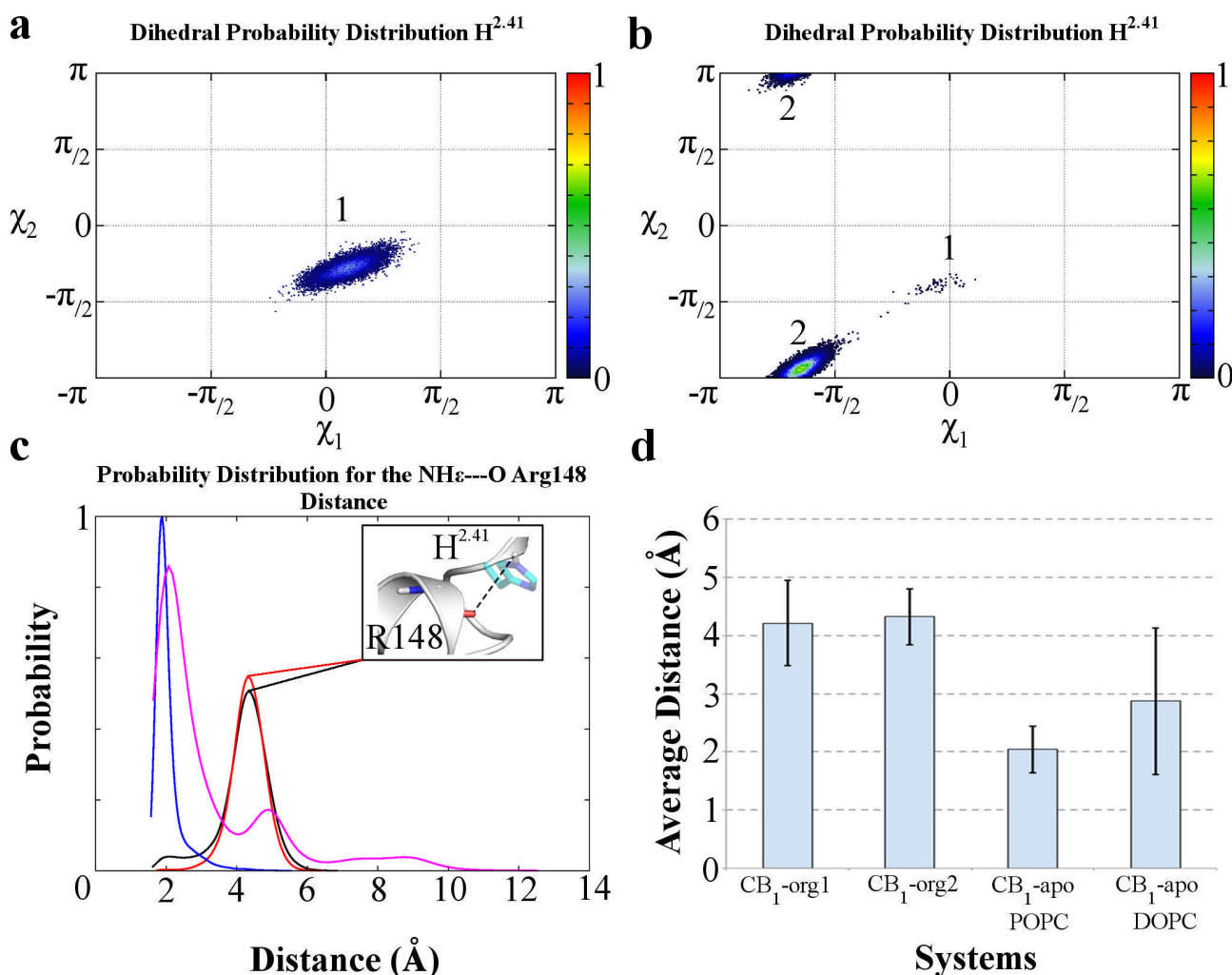
$$s = \sum_{ij} \frac{1 - \left(\frac{d_{ij}}{r_0}\right)^n}{1 - \left(\frac{d_{ij}}{r_0}\right)^m} \quad (\text{eq.1})$$

Supplementary Figure 7 | Statistical analysis of the binding mode stability. (a) ORG27569 Binding mode in the first MD simulations, highlighted the H-bond interactions with $S^{2.45}$ (S_1) and $H^{2.41}$ (S_2). (b) Comparison of the average binding modes of both run 1 and run 2 of CB_1 wt-ORG27569 simulations (See Appendix A for the selection of the structures). Noteworthy, even starting from different binding conformations, the two CB_1 wt-ORG27569 simulations seem to converge towards a unique binding mode. (c) Comparison of the average binding modes of both run 1 CB_1 wt-ORG27569 and CB_1 -($H^{2.41}L$)-ORG27569 simulations (See Appendix A for the selection of the structures). (d) Ligand RMSD for the run 1, run 2 CB_1 wt-ORG27569 (black and red lines, respectively) and CB_1 -($H^{2.41}L$)-ORG27569 (green line) simulations. (e, f) Probability distribution of the H-bond interactions between ORG27569 and $S^{2.45}$ and $H^{2.41}$ along the whole simulation of CB_1 wt-ORG27569 system (run 1 and run 2). The plot highlights the region where both H-bonds are present (upper-right corner), where only one H-bond is present (lower-right and upper-left corners), and the region where no H-bonds were detected (lower-left corner). The probability of each state was normalized. H-bond interactions were computed as indicated in eq. 1.



Supplementary Figure 8 | Statistical analysis of the structural effects induced by ORG27569 .

(a) $H^{2.41}$ χ_1 and χ_2 dihedral angles distribution for the CB₁-wt simulations. (b) $H^{2.41}$ χ_1 and χ_2 dihedral angles distribution for the run 2 of the CB₁wt-ORG27569 simulation. A highly represented conformer, in the CB₁wt-ORG27569 MD, for the $H^{2.41}$ residue (conformer 2), different from that observed in the CB₁ unbound state (conformer 1, see also Fig. 2b and 2d in the main text) was noticed. Remarkably, in the CB₁wt-ORG27569 simulations, the shift from $H^{2.41}$ conformer 1 to 2 causes the H-bond loss between the $H^{2.41}$ N ϵ (TM2) and the R148 backbone oxygen (ICL1, Fig. 2d). The probability of each state was normalized. (c) Probability distribution for the $H^{2.41}$ (NH ϵ)-R148(O) distance atoms, for CB₁wt in POPC:Chol 2:1 membrane environment (blue lines), the CB₁wt-ORG27569 simulations (black , and red lines), and CB₁wt in DOPC:Chol 2:1 membrane environment (purple line), respectively. The probability of each distance was normalized. When CB₁ is embedded in a more flexible environment (DOPC) the probability distribution shows a second, less populated, pick around 5 Å. Noteworthy, in both CB₁wt-ORG27569 simulations a clear and well defined pick around 4 Å is appreciable, supporting the statistical robustness of this observation. This behavior is also highlighted when the average values for the $H^{2.41}$ (NH ϵ)-R148(O) distance are plotted with their standard deviation (d).



Statistical Analysis of ICL1-H8 conformations

The ICL1 and H8 conformations reported in Fig. 2a,d and h were obtained as described in Appendix A (Structure Selection). To compute the RMSD reported in Supplementary Figure 9a and b we used as reference structures for CB₁wt-ORG27569 and CB₁wt those reported in Figure 2a and h, respectively. The systems were aligned on the C α atoms of the TM bundle, and the RMSD for the C α atoms of the ICL1 and H8 domains was computed for the two CB₁wt-ORG27569 simulations [org1 (black line) and org2 (red line) in Supplementary Figure 9a,b], and for the CB₁wt simulations (blue line). From the analysis of the plots it is clear that the selected conformations (reported in Figure 2a,d and h) are stable under the simulations conditions. This trend is also highlighted by the calculations of the average RMSD for both ICL1 and H8 domains (Supplementary Figure 9c and d, respectively). Since, for all systems the average RMSD, with respect to the reference structure, is around 1.7 Å, which correspond to the canonical value for thermal fluctuation in classical MD simulations. Moreover, the similar behavior showed by the two CB₁wt-ORG27569 simulations for both ICL1 and H8 domains highlights how both systems converged toward similar conformations. Nevertheless, the RMSD only is not a sufficient parameters to describe how different is the conformational space explored by MD simulations, therefore we decided to compare CB₁wt-ORG27569 and CB₁wt simulations by performing a modified version of the Essential Dynamics Analysis, as reported below.

Essential Dynamics Analysis

The trajectories were analyzed with VMD^{14,15}. An Essential Dynamics (ED) analysis²³ was performed by using the appropriate VMD plug-in in combination with the ptraj module of Amber11¹⁶. ED allows to identify a new essential subspace of coordinates by the diagonalisation of the covariance matrix of atomic fluctuations. For a X(t) multi-dimensioned system, where X could be a system of spatial coordinates, frame of molecular dynamics simulations etc., we could obtain the following covariance matrix:

$$C_{ij} = \left\langle \left(x_i - \langle x_i \rangle \right) \left(x_j - \langle x_j \rangle \right) \right\rangle$$

The symmetric matrix can be diagonalized by an orthonormal transformation matrix, which contains the eigenvectors or principal modes (columns of the matrix) and where the eigenvalues λ express the variance in the direction of the corresponding eigenvector. Every eigenvector is stored, in decreasing way, on the basis of the corresponding eigenvalue. Thus, for a dynamic process the

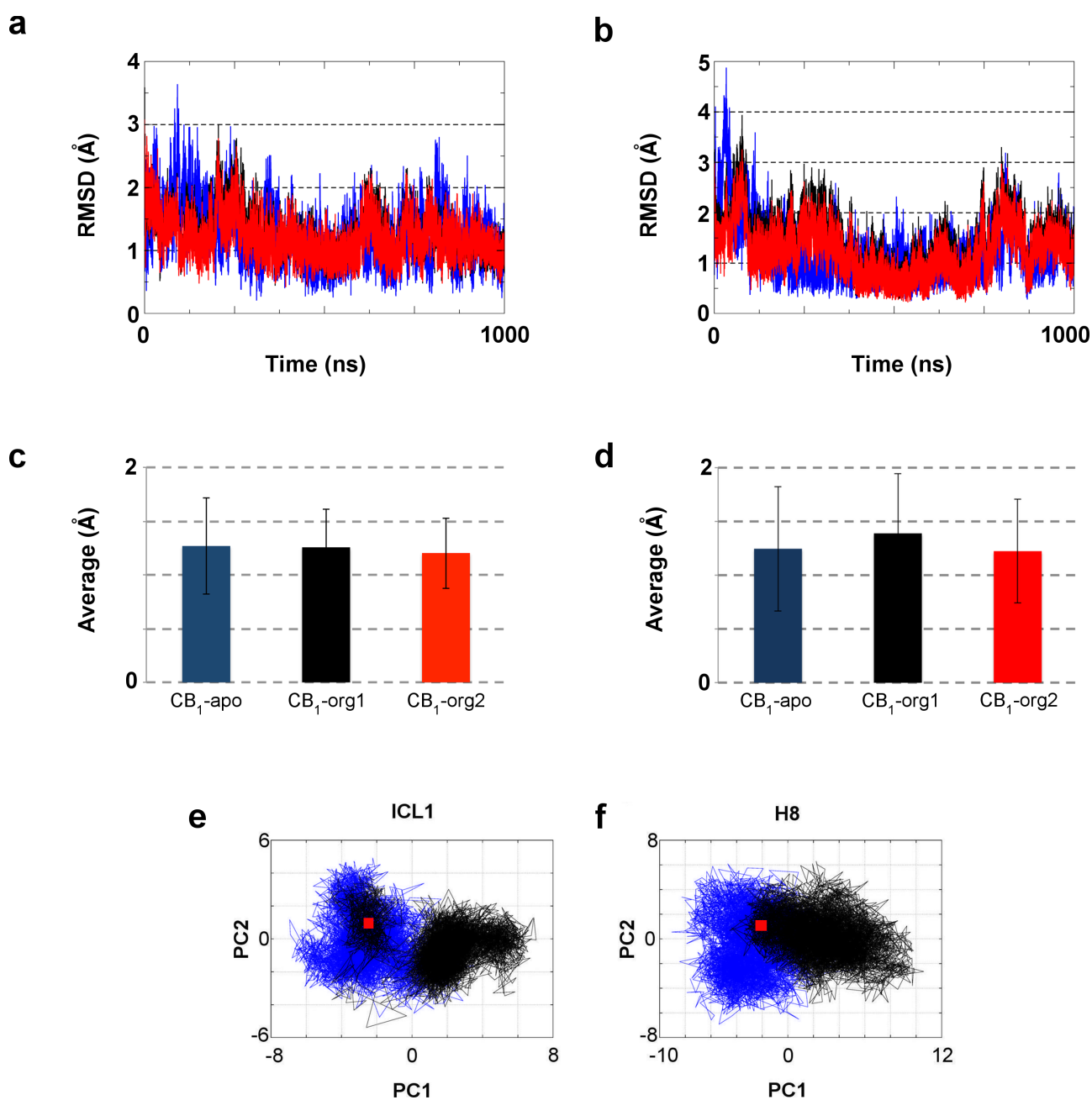
original data can be projected onto an eigenvector and the eigenvectors with larger eigenvalues match the more important displacement.

In our case a Combined Essential Dynamics (Comb-ED) analysis was performed²⁴. In the Comb-ED the covariance matrix is calculated for two or more concatenated trajectories, and the overall translational and rotational motion are eliminated fitting all the trajectories on the same reference structure. First of all, both simulations of CB₁wt unbound and CB₁wt-ORG27569 were concatenated and clustered on the RMSD of the C α atoms of the TM domains, using ptraj and the average-linkage cluster algorithm¹⁶. Ptraj turned out an average structure for the whole trajectories and a representative structure (the frame closest to the average structure in terms of RMSD). After that, both trajectories were finally fitted on the C α atoms of the TM domains of the representative structure and the principal modes were computed for the C α atoms of ICL1 (Supplementary Figure 9e) or H8 (Supplementary Figure 9e) only.

The analysis revealed, how ICL1 and H8 evolve in a different essential space (depending on the presence of ORG27569), and in turn, exploring different regions of the ICL1-H8 conformational space. The ICL1 and H8 starting conformation of all systems are the same (red box), since they were generated from the last steps of the initial 50ns simulations, for all systems.

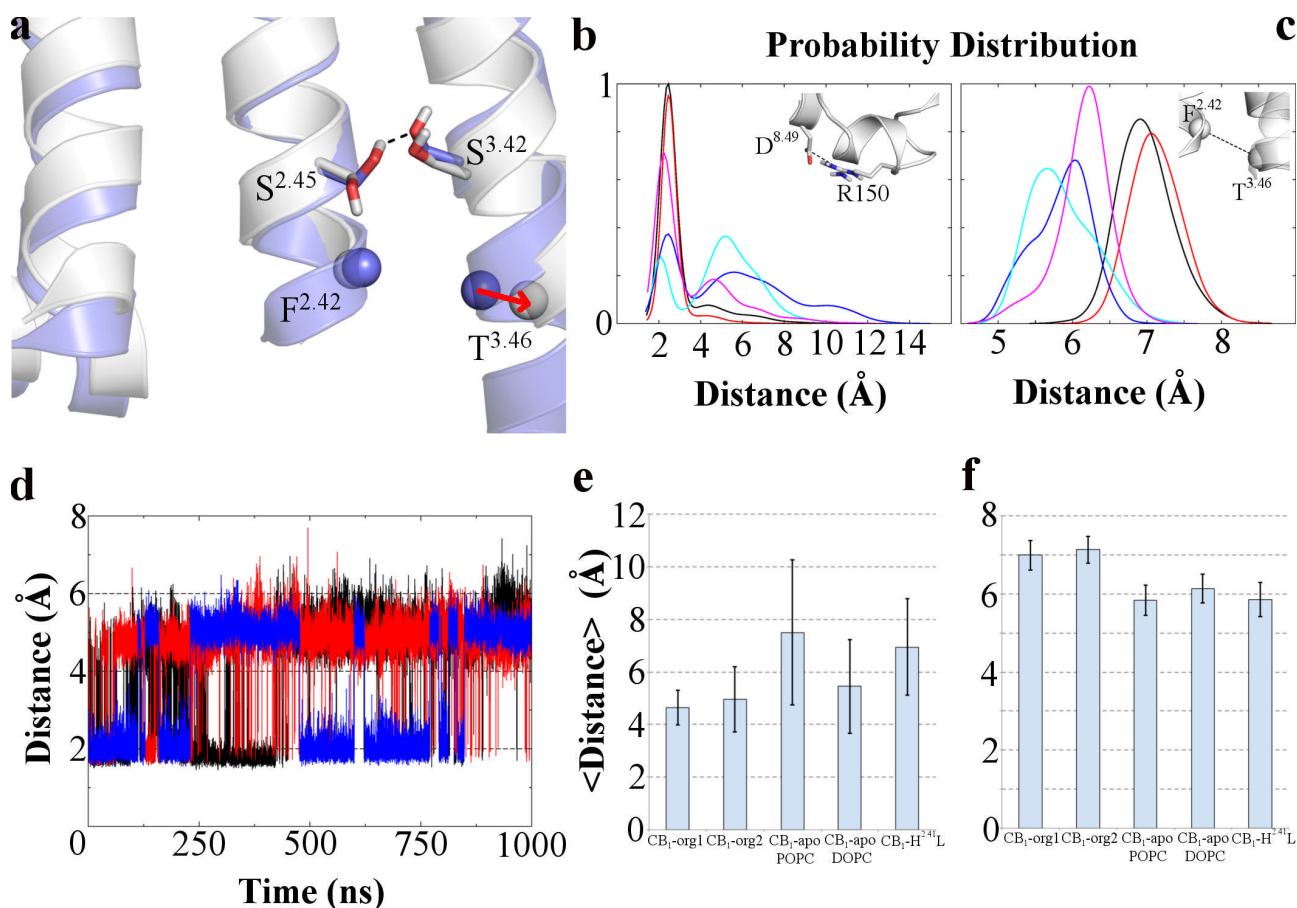
Supplementary Figure 9 | Conformational Analysis of ICL1-H8 in the different simulations.

(a) RMSD plot for ICL1 domain for the two CB₁wt-ORG27569 simulations [org1 (black line) and org2 (red line) in Supplementary Figure 9a,b], and for the CB₁wt simulations (blue line); (b) RMSD plot for H8 domain, color-coded as previously reported; (c) Average RMSD for ICL1 domain and H8 domain (d); (e) Comb-ED for ICL1 domain for CB₁wt-ORG27569 simulations (black line) and for the CB₁wt simulations (blue line); (f) Comb-ED for H8 domain for CB₁wt-ORG27569 simulations (black line) and for the CB₁wt simulations (blue line). Highlighted with a red box the location of the starting conformation of each domain and for each system.



Supplementary Figure 10 | Statistical analysis of the TM3 effects induced by ORG27569. The comparison of the unbound CB₁wt and CB₁wt-ORG27569 simulations reveals a pronounced displacement of the TM3 C-terminus region, at the T^{3.46} level (Supplementary Fig. S10a). This is another interesting result, since the region defined by S^{2.45}, S^{3.42}, T^{3.46}, F^{2.42} has been implicated in the so called Hydrophobic Hindering Mechanism (HHM), in CB₁ and other GPCRs^{25,26,27}, supporting the idea that the alteration of this region can affect the orthosteric ligand binding affinity. (a) Comparison of the representative structures of both run 1 of CB₁-ORG27569 (white cartoon) and CB₁wt simulations (blue cartoon). Highlighted in stick and with the same color code the S^{2.45} and S^{3.42} residues, while with the same color code and sphere the C α atoms of F^{2.42} and T^{3.46}. The red arrow highlights the T^{3.46} displacement. (b, c) Probability distribution for the R150(CZ)-D^{8.49}(CG) (b) and F^{2.42}(C α)-T^{3.46}(C α) (c) distance atoms, for CB₁wt (POPC:Chol 2:1) (blue lines), the CB₁wt-ORG27569 simulations (black, and red lines), the CB₁wt (DOPC:Chol 2:1) (purple line) and the CB₁-(H^{2.41}L)-ORG27569 simulations (cyan line), respectively. (d) Together with the TM3 displacement, the presence of ORG27569 alters the pattern of H-bonds between S^{2.45} and S^{3.42}. Indeed, in the CB₁wt simulations the S^{2.45} and S^{3.42} it breaks and it forms, periodically (blue line). In both run 1 and 2 of the CB₁wt-ORG27569 system the same interaction, after 450ns, is occasionally observed (black and red lines, respectively). (e) Average values for the R150(CZ)-D^{8.49}(CG) distance, with their standard deviation. (f) Average values for the F^{2.42}(C α)-T^{3.46}(C α) distances, with their standard deviation.

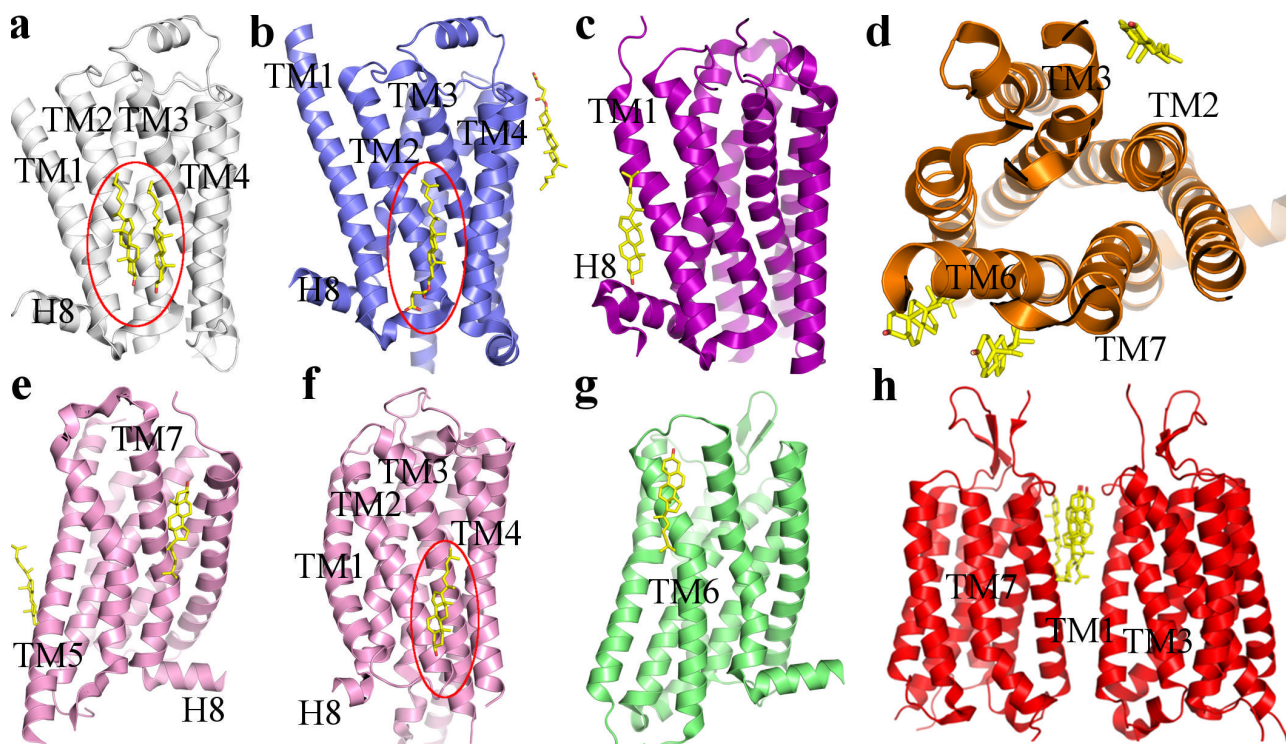
Of interest is the fact that in the CB₁-(H^{2.41}L)-ORG27569 simulations these effects were not observed, although the presence of ORG27569 inside the P2 pocket (Supplementary Fig. 10b and c). These observation support the notion that, only the proper disposition of ORG27569 inside the P2 pocket induces the observed conformational changes, which are not observed when the anchoring H^{2.41} is mutated in leucine (Supplementary Fig. 10b and c, cyan line).



6. Analysis of GPCR X-ray crystal structures in complex with cholesterol, and cholesterol interaction with CB₁wt

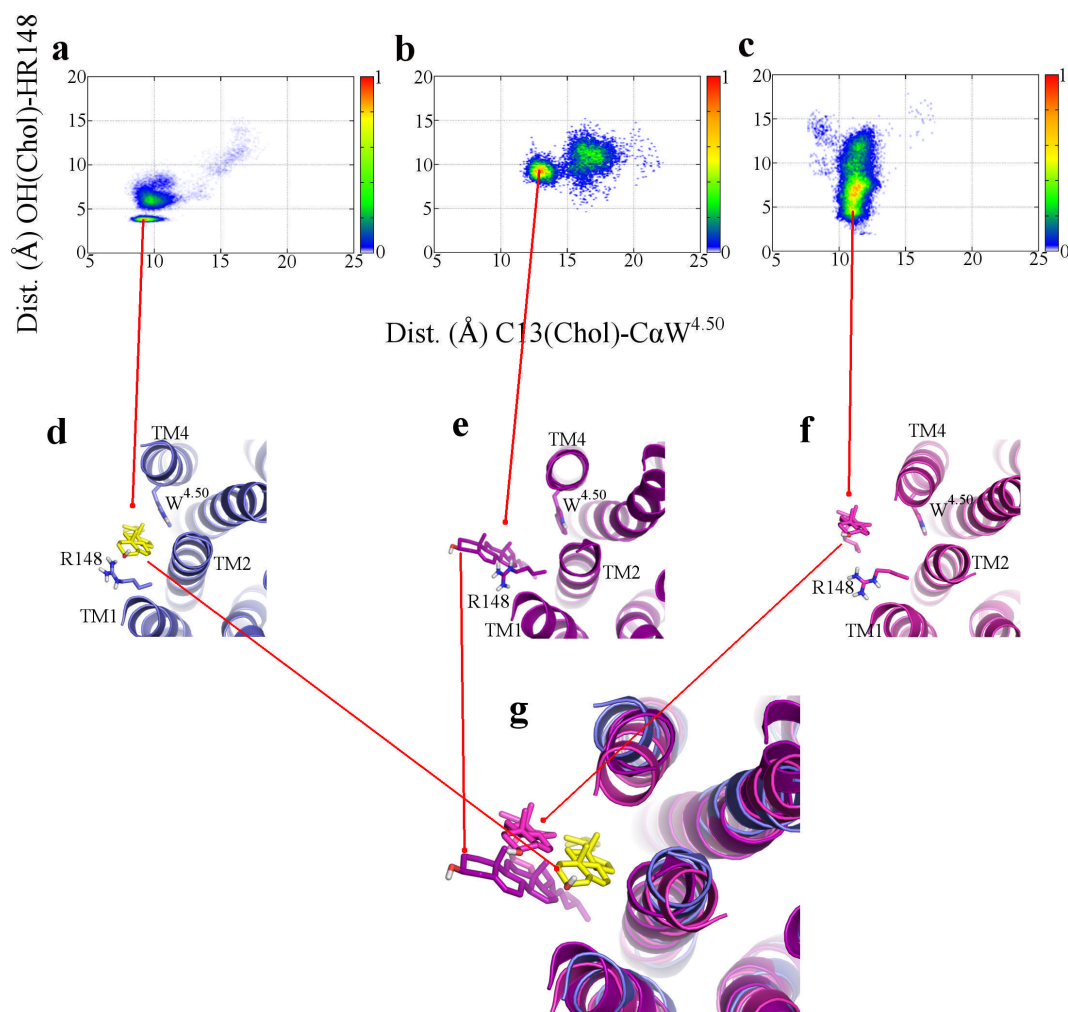
Up to date about 120 GPCR X-ray crystal structures were disclosed for both family A, B, C and F, from different research groups. Among them 21 shows cholesterol molecules directly bound the TM bundle. In Supplementary Figure 10 we reported the main GPCRs families in complex with cholesterol. Noteworthy, it has been reported that cholesterol negatively affects the agonist binding for the β_2 -adrenergic receptor. Indeed, cholesterol molecules bound to the receptor were found only in the inactive X-ray structures of such receptor (at the CCM level, PDB Code 2RH1 and 3D4S, Supplementary Figure 11a)²⁸, while no cholesterol molecules were found in the X-ray structures of the active state of the same receptor (PDB code 3PDS, 3P0G, 3SN6, 4LDO, 4LDL, 4LDE, 4QKX). On the contrary, it has been reported that platelet aggregation induced by P2Y₁₂ activation (agonist mediated) is reduced after cholesterol depletion.^{29,30} In the case of the P2Y₁₂ receptor, cholesterol molecules were found in both the inactive and inactive states (Supplementary Figure 11e and f), but only in the active state a cholesterol molecule bound to the P2Y₁₂-CCM was observed (Supplementary Figure 11f). On the basis of these experimental observation it can be hypothesized that CCM is one of the possible allosteric pockets, inside the GPCR families, through which cholesterol allosterically modulates GPCR functioning.

Supplementary Figure 11 | X-ray crystal structures analysis. GPCR X-ray crystal structures with cholesterol bound. **a.** β_2 -adrenergic receptor (PDB code 3D4S). **b.** β_1 -adrenergic receptor (PDB code 3ZPQ). **c.** 5-HT_{2B} receptor (PDB code 4IB4). **d.** A_{2A} receptor (PDB code 4EIY). **e.** Inactive P2Y₁₂ receptor (PDB code 4NTJ). **f.** Active P2Y₁₂ receptor (PDB code 4PXZ). **g.** μ -opioid receptor (PDB code 4DKL). **h.** mGlu1 receptor (PDB code 4OR2). Highlighted with a red circle cholesterol molecules bound to GPCR CCM.

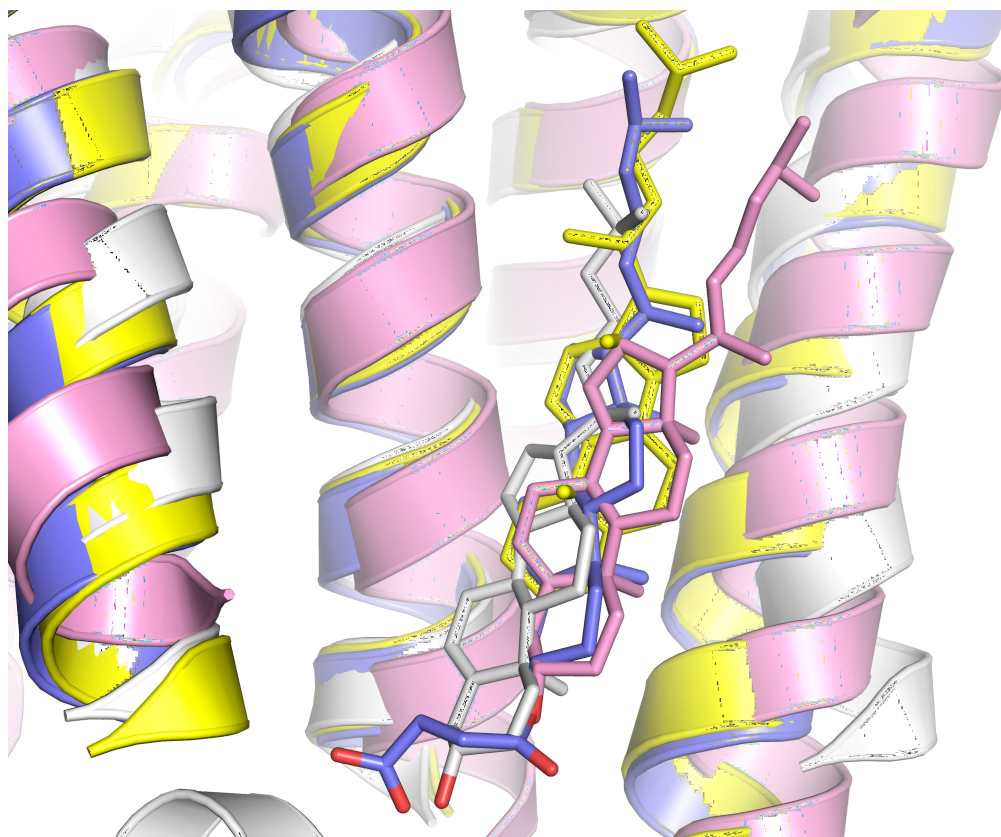


During the MD simulations of the CB₁wt systems cholesterol molecules interacts with different domains of the CB₁ receptor (Fig. 2f), including the P2 cleft (Fig. 2g and h and Supplementary Fig. S12), adopting a binding conformation similar to that observed in the CCM pocket of other GPCR X-ray structures (See Supplementary Fig. S11)²⁸.

Supplementary Figure 12 | Analysis of cholesterol binding in different membrane environment. **a.** Probability distribution of the cholesterol molecule, which binds to the CCM during the CB₁wt (POPC:Chol 2:1) simulations. **b.** Probability distribution of the cholesterol molecule in the CB₁wt (POPC:Chol 2:1 310K) simulations. **c.** Probability distribution of the cholesterol molecule in the CB₁wt (DOPC:Chol 2:1) simulations. The probability distribution distance for the W^{4.50}(C α)-CHL(C13) carbon atoms (x axis) is plotted vs. the probability distribution distance for the NH(Arg148) and the O atoms (y axis). **d.** Binding conformation in the CB₁wt (POPC:Chol 2:1) simulations. **e.** Binding conformation in the CB₁wt (POPC:Chol 2:1 310K) simulations. **f.** Binding conformation in the CB₁wt (DOPC:Chol 2:1) simulations. **g.** Superposition of the three binding conformations obtained, in the different systems.



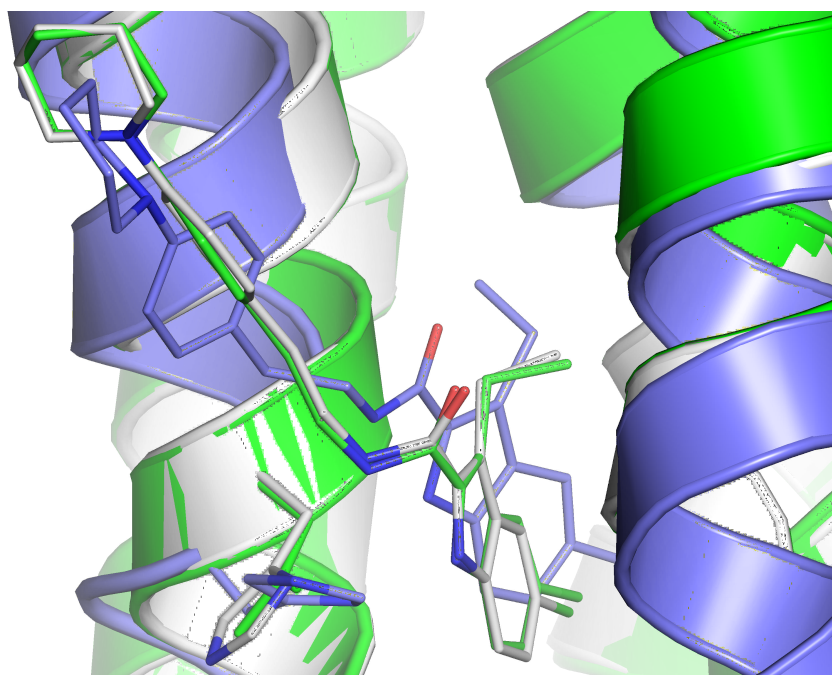
Supplementary Figure 13 | Cholesterol binding conformations. Superposition of the CB₁wt with a cholesterol molecule bound to the CCM (white cartoon and sticks), with the β_2 -adrenergic receptor (PDB code 3D4S, yellow), β_1 -adrenergic receptor (PDB code 3ZPQ, blue), and with the active P2Y₁₂ receptor (PDB code 4PXZ, pink). The CB₁wt-cholesterol structure was obtained from the CB₁wt (POPC:Chol 2:1) simulations.



7. Generation of the CB₁(H^{2.41}L)-ORG27569 system.

Starting from the refined CB₁wt-ORG27569 complex H^{2.41} was mutated in a leucine residue (Supplementary Fig. S14). The CB₁(H^{2.41}L)-ORG27569 complex, thus obtained, was submitted to the same MD protocol described for CB₁wt and CB₁wt-ORG27569 systems. A MD simulation 1 μ s long was performed. For the CB₁wt-ORG27569 run 2 simulation, a different binding mode was selected (the 2 binding pose ranked in the docking studies) as starting point, and the system was submitted to the same MD protocol described for CB₁wt and CB₁wt-ORG27569 systems, and a MD simulation 1 μ s long was performed.

Supplementary Figure 14 | Starting Conformations. Comparison of the CB₁wt-ORG27569 starting conformations (after minimizations) used for the MD simulations (cartoons and sticks are depicted in white and blue, respectively for run 1 and run 2) and CB₁-(H^{2.41}L)-ORG27569 (green) receptors in complex with ORG27569 (green sticks). In the figure ORG27569 and H/L^{2.41} are depicted in the same color code, depending on the system considered: CB₁wt-ORG27569 run 1 (white), CB₁wt-ORG27569 run 2 (blue), CB₁-(H^{2.41}L)-ORG27569 (green).



Supplementary Table 4 | MD simulations carried out. Sum up of the whole MD simulations carried out in this study.

System	Principal MD settings	Simulation Time
CB₁wt- ORG27569 run 1	Starting from the selected Docking pose	1 μ s
CB₁wt- ORG27569 run 2	Starting from a different binding pose as depicted in Supplementary Figure S14	1 μ s
CB₁wt-apo 1	POPC:Chol 2:1	1 μ s
CB₁wt-apo 2	DOPC:Chol 2:1	1 μ s
CB₁wt-apo 3	POPC:Chol 2:1 Cholesterol molecules with their Center Of Mass (COM) in close proximity to the CCM-COM (20 Å) were manually replaced with POPC. The MD simulations was conducted at 310K	1 μ s
CB₁-(H^{2.41}L)- ORG27569	H ^{2.41} L mutation as depicted in Figure S14	1 μ s

8. Appendix A.

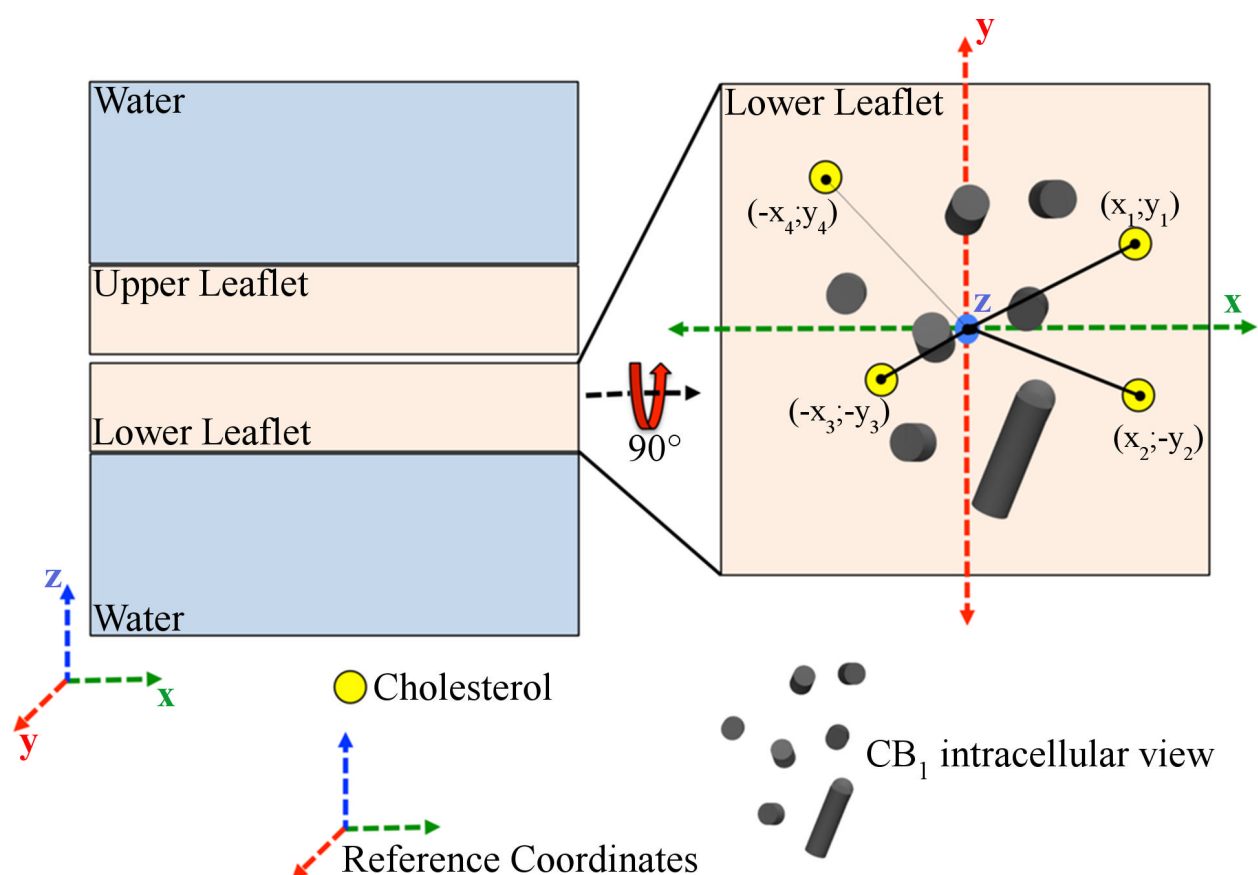
Structure selection reported in the Figures of the manuscript and Supplementary Informations.

- 1) The structures of CB₁wt-ORG27569 reported in Figure 2 and Supplementary Fig. 7 are the representative structures obtained from the cluster analysis performed on the frames where ORG27569 forms two H-bonds with H^{2.41} and S^{2.45} (upper-right corner of the plot reported in Supplementary Fig. 5). Frames were clustered on the RMSD of the ORG27569 heavy atoms, using ptraj and the average-linkage cluster algorithm¹⁶. Ptraj turned out an average structure for the whole trajectories and a representative structure (the frame closest to the average structure in terms of RMSD).
- 2) The structure of the CB₁wt reported in Supplementary Figure 7 is the representative structure obtained from the cluster analysis performed on the whole CB₁wt simulations (1 μ s). Frames were clustered on the RMSD of the C α atoms of the TM domains, using ptraj and the average-linkage cluster algorithm¹⁶. Ptraj turned out an average structure for the whole trajectories and a representative structure (the frame closest to the average structure in terms of RMSD).
- 3) The structures of the CB₁wt interacting with cholesterol molecules reported in Figure 2 and Supplementary Figure 12 and 13 were selected taking into account only the structures showing the closest distance between the receptor and the cholesterol molecule.

Calculation of the Probability Distribution of Cholesterol on Figure 2f of the main text

The probability distribution of the cholesterol molecules in the lower leaflet was computed applying a protocol similar to that reported in *J. Am. Chem. Soc.*, **134**, 16512–16515, (2012). In Particular, only cholesterol molecules into the lower leaflet were considered. For each cholesterol molecules, the relative position of the center of mass along the x and y axes was computed with respect to the protein main axis (along z axis), as reported in Supplementary Figure 15.

Supplementary Figure 15 | Calculation of the Cholesterol Probability Distribution. Protocol used to compute the probability distribution function reported in Fig. 2f of the main text.



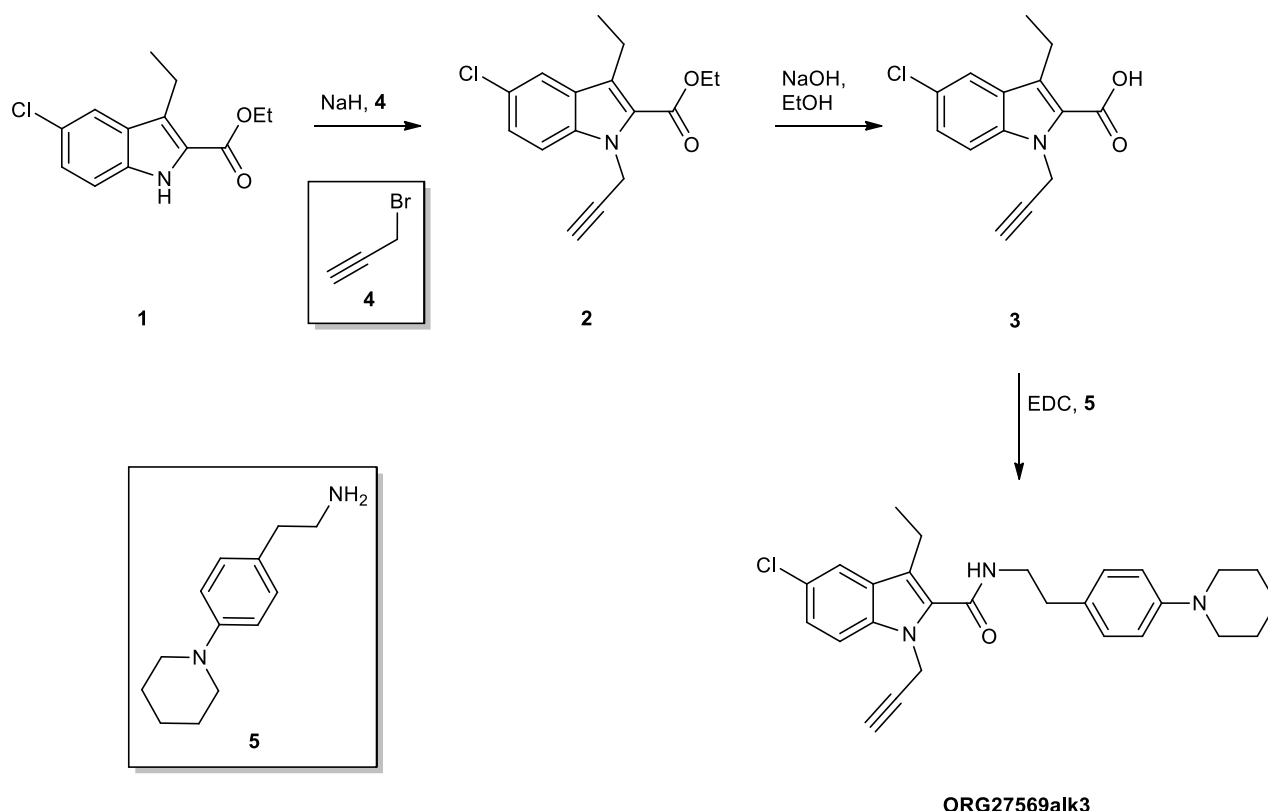
9. Chemistry section

General Methods. Commercially available reagents and solvents were used without further purification. Dichloromethane was dried by distillation from P_2O_5 and stored over activated molecular sieves (4 Å). When necessary the reactions were performed in oven-dried glassware under a positive pressure of dry argon. Melting points were determined in open glass capillaries and are uncorrected. All the compounds were characterized by IR. 1H and ^{13}C NMR were recorded on a 400 MHz. High-resolution ESI-MS spectra were performed on a Thermo LTQ Orbitrap XL mass spectrometer. The spectra were recorded by infusion into the ESI source using MeOH as the solvent. Chemical shifts (δ) are reported in part per million (ppm) relative to the residual solvent peak. Column chromatography was performed on silica gel (70–230 mesh ASTM) using the reported eluents. Thin layer chromatography (TLC) was carried out on 5 x 20 cm plates with a layer thickness of 0.25 mm (Silica gel 60 F254). When necessary they were developed with $KMnO_4$.

Compound	Name	Structure
1	ORG27569alk1	
2	ORG27569alk2	
3	ORG27569alk3	
4	ORG27569alk4	

Procedures for ORG27569alk3

Supplementary Scheme 1



Ester 2. To a solution of Ethyl 5-chloro-3-ethyl-1*H*-indole-2-carboxylate **1**³¹ (2.18 g, 8.7 mmol) in dry DMF (20 mL) at 0 °C, was introduced sodium hydride (60% dispersion in mineral oil, 0.7 g, 17.4 mmol) and the mixture was stirred for 30 min at 0 °C. Propargyl bromide (0.95 mL, 10.4 mmol) was then added to the reaction mixture and reaction was stirred for 16 h at room temperature³². The crude reaction mixture was treated with water (100 mL) and the aqueous phase was extracted with Diethyl Ether (3 × 100 mL). The combined organic layers were dried over anhydrous MgSO₄ and the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel (EtOAc/ Hexane 1:5) to yield the substituted product **2** as a white solid (2.04 g, 81 %).

Mp: 107-109 °C; TLC (EtOAc/ Hexane 1:3): *R_f* = 0.69; ¹H NMR (400MHz, CD₃OD): δ 7.67 (s, 1H), 7.50 (d, *J* = 8.96 Hz, 1H), 7.32 (dd, *J* = 6.96, 1.8 Hz, 1H), 5.36 (d, *J* = 2.16 Hz, 2H), 4.42 (q, *J* = 14.16, 7.04 Hz, 2H), 3.06 (q, *J* = 14.92, 7.48 Hz, 2H), 2.64-2.60 (m, 1H), 1.44 (t, *J* = 7.08 Hz, 3H), 1.24 (t, *J* = 7.44 Hz, 3H); ¹³C NMR (100 MHz, CDCl₃): δ 162.3, 136.4, 127.9, 127.7, 126.1, 126.0, 124.3, 78.7, 71.9, 60.9, 34.4, 18.7, 15.5, 14.2; IR (neat) *ν*_{max}: 3274, 2967, 1695, 1267, 1144 cm⁻¹; HRMS (ESI) [*M* + *H*]⁺ calculated for C₁₆H₁₇ClNO₂: 290.048, found: 290.0933.

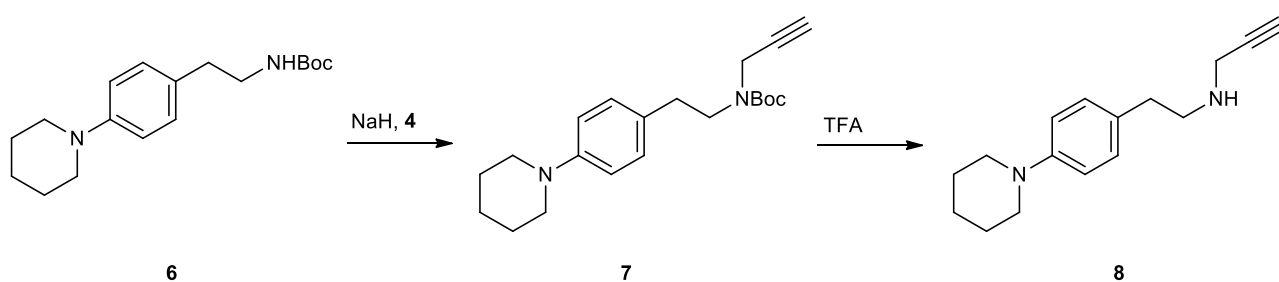
ORG27569alk3. A mixture of **2** (0.64 g, 2.22 mmol), 3 N sodium hydroxide (2.22 mL) and ethanol (5 mL) was heated to 60 °C for 1.5 h. After cooling, 1 N HCl was added until pH ~ 2. The mixture

was extracted with Ethyl Acetate (3×100 mL). The combined organic layers were dried over anhydrous MgSO_4 and the solvent was removed under reduced pressure. The carboxylic acid **3** was used for the next step without further purification.

EDC•HCl (0.065 g, 0.34 mmol) and 4-DMAP (0.041 g, 0.34 mmol) were added sequentially in single portions to a solution of carboxylic acid **3** (0.34 mmol) and amine **5**³¹ (0.34 mmol) in dry DMF (5 mL) at room temperature. The mixture was stirred at room temperature for 16 h. Upon completion, the reaction contents were diluted with EtOAc (30 mL), poured into water (40 mL), and extracted with EtOAc (2×50 mL). The combined organic layers were then washed with 1 M aqueous HCl (3×30 mL) and brine (30 mL), dried (MgSO_4), and concentrated. The residue was purified by silica gel column chromatography (EtOAc/ Hexane 1:3) to provide **ORG27569alk3** as a white solid (0.84 g, 55 %).

Mp: 178-180 °C dec.; TLC (EtOAc/ Hexane 1:3): $R_f = 0.29$; ^1H NMR (400MHz, CDCl_3): δ 7.55 (s, 1H), 7.33 (d, $J = 8.8$ Hz, 1H), 7.29-7.23 (m, 3H), 7.20-7.11 (m, 2H), 5.97-5.88 (m, 1H), 5.11 (d, $J = 1.76$ Hz, 2H), 3.77 (q, $J = 12.32, 6.12$ Hz, 2H), 3.24-3.07 (m, 4H), 2.90 (t, $J = 5.4$ Hz, 2H), 2.69 (q, $J = 15.28, 7.64$ Hz, 2H), 2.24 (s, 1H), 1.83-1.66 (m, 4H), 1.65-1.49 (m, 2H), 1.11 (t, $J = 7.52$ Hz, 3H); ^{13}C NMR (100 MHz, CDCl_3): δ 162.3, 135.4, 129.9, 129.8, 127.9, 125.9, 124.7, 119.6, 119.5, 78.6, 77.2, 72.4, 40.6, 34.6, 33.8, 18.03, 15.8; IR (neat) ν_{max} : 3285, 2928, 1621, 1544, 1454 cm^{-1} ; HRMS (ESI) $[\text{M} + \text{H}]^+$ calculated for $\text{C}_{27}\text{H}_{31}\text{ClN}_3\text{O}$: 448.2156, found: 448.2143.

Supplementary Scheme 2



Procedures for compound 8

Carbamate 7. Boc-protected amine **6**³¹ (0.216 g, 0.71 mmol) was dissolved in dry DMF (3 mL). The reaction mixture was cooled with an ice bath, and sodium hydride (60% in mineral oil, 0.035 g, 0.87 mmol) was added in small portions upon vigorous stirring. The reaction was allowed to proceed for 16 h at room temperature³³. Then the reaction mixture was quenched with water (5 mL)

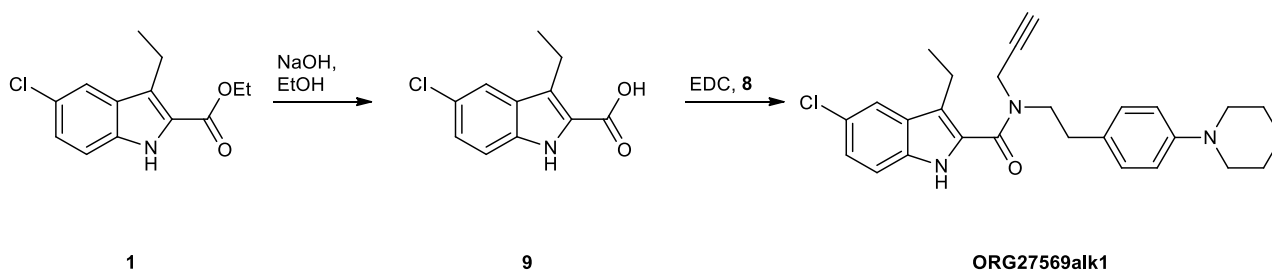
and extracted with Diethyl Ether (2×25 mL). The combined organic layers were dried over anhydrous MgSO_4 and the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel (EtOAc/ Hexane 1:7) to yield the substituted product **7** as a yellow oil (0.121 g, 47 %).

TLC (EtOAc/ Hexane 1:3): $R_f = 0.47$; ^1H NMR (400MHz, CDCl_3): δ 7.16-7.02 (m, 2H), 6.99-6.82 (m, 2H), 4.08-3.83 (m, 2H), 3.49 (t, $J = 7.4$ Hz, 2H), 3.19-3.04 (m, 4H), 2.79 (t, $J = 7.24$ Hz, 2H), 2.24-2.14 (m, 1H), 1.81-1.64 (m, 4H), 1.64-1.51 (m, 2H), 1.45 (s, 9H); ^{13}C NMR (100 MHz, CDCl_3): δ 154.6, 149.8, 129.4, 117.1, 80.1, 79.8, 77.2, 71.4, 51.2, 48.4, 36.1, 33.9, 33.6, 28.3, 25.7, 24.1; IR (neat) ν_{max} : 3307, 2928, 1695, 1511, 1160 cm^{-1} ; HRMS (ESI) $[\text{M} + \text{H}]^+$ calculated for $\text{C}_{21}\text{H}_{31}\text{N}_2\text{O}_2$: 343.2386, found: 343.2383.

Amine 8. A mixture of **7** (0.109 g, 0.3 mmol) and trifluoroacetic acid (0.3 mL) in dichlorometane (0.3 mL) was stirred at room temperature for 1 h. The solvent was evaporated and the residue was dried under high vacuum to provide crude **8** as bis- 2,2,2-trifluoroacetate which was used without further purification³¹.

Procedures for ORG27569alk1

Supplementary Scheme 3



ORG27569alk1. A mixture of **1** (0.64 g, 2.22 mmol), 3 N sodium hydroxide (2.22 mL) and ethanol (5 mL) was heated to 60 °C for 1.5 h. After cooling, 1 N HCl was added until pH ~ 2. The mixture was extracted with Ethyl Acetate (3×100 mL). The combined organic layers were dried over anhydrous MgSO_4 and the solvent was removed under reduced pressure. The carboxylic acid **3** was used for the next step without further purification.

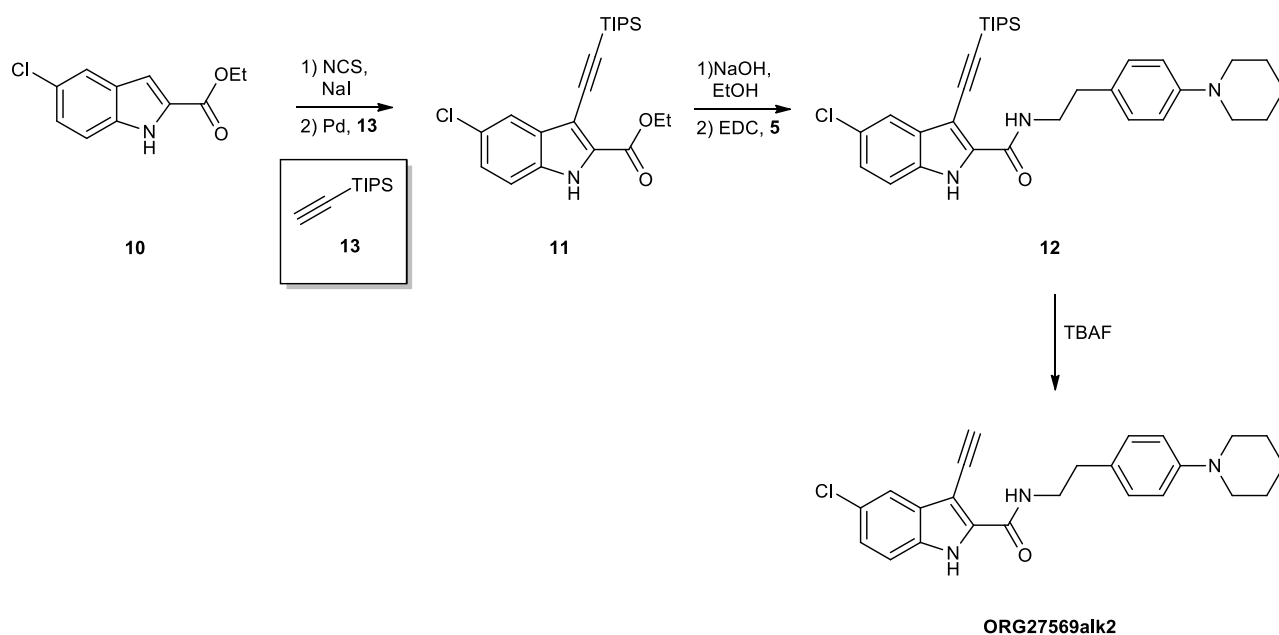
EDC•HCl (0.0658 g, 0.3 mmol) and 4-DMAP (0.036 g, 0.3 mmol) were added sequentially in single portions to a solution of the residue obtained above (0.3 mmol) and amine **8** (0.3 mmol) in dry DMF (5 mL) at room temperature. The mixture was stirred at room temperature for 16 h. Upon completion, the reaction contents were diluted with EtOAc (30 mL), poured into water (40 mL), and extracted with EtOAc (2×50 mL). The combined organic layers were then washed with 1 M

aqueous HCl (3×30 mL) and brine (30 mL), dried (MgSO_4), and concentrated. The residue was purified by silica gel column chromatography (EtOAc/ Hexane 1:3) to provide **ORG27569alk1** as a yellow oil (0.78 g, 58 %).

TLC (EtOAc/ Hexane 1:3): $R_f = 0.28$; ^1H NMR (400MHz, CDCl_3): δ 7.53 (s, 1H), 7.17-7.09 (m, 2H), 7.03-6.94 (m, 2H), 6.92-6.80 (m, 2H), 4.35-4.18 (m, 2H), 3.76 (t, $J = 5.88$ Hz, 2H), 3.21-3.07 (m, 4H), 2.68 (q, $J = 14.76, 7.12$ Hz, 2H), 2.35 (s, 1H), 1.79-1.66 (m, 4H), 1.62-1.51 (m, 2H), 1.20 (t, $J = 8.04$ Hz, 3H); ^{13}C NMR (100 MHz, CDCl_3): δ 164.9, 134.0, 129.9, 128.0, 127.5, 125.3, 123.9, 119.4, 112.5, 78.5, 77.2, 73.1, 32.8, 29.7, 17.9, 15.0; IR (neat) ν_{max} : 3291, 2928, 1626, 1443, 1234 cm^{-1} ; HRMS (ESI) $[\text{M} + \text{H}]^+$ calculated for $\text{C}_{27}\text{H}_{31}\text{ClN}_3\text{O}$: 448.2156, found: 448.2143.

Procedures for ORG27569alk2

Supplementary Scheme 4



Alkyne 11. To a solution of N-chlorosuccinimide (1.59 g, 12 mmol) in DMF (25 mL) was added sodium iodide (1.79 g, 12 mmol) in small portions. The resulting brown solution was stirred at room temperature for 1 h before the slow addition at 0 °C of 5-chloro-1H-indole-2-carboxylate **10** (1.89 g, 10 mmol) in DMF (20 mL). The reaction mixture was stirred for 1 h at room temperature. A solution of sodium thiosulfate (10 %; 5 mL) and water (10 mL) were added and the mixture was stirred for 1 h. The solid was filtered, washed with cold water and cold hexane and dried under high vacuum³⁴. The residue obtained was used for the next step without further purification.

To a mixture of the residue obtained above (0.525 g, 1.5 mmol), $\text{Pd}(\text{Ph}_3\text{P})_2\text{Cl}_2$ (0.053 g, 0.075 mmol, 5.0 mol %) and CuI (0.014 g, 0.075 mmol, 5.0 mol %) under argon atmosphere was added

triethylamine (7.5 mL) and stirred for 15 min. To this reaction mixture was added the terminal alkyne **13** (0.341 mL, 1.65 mmol) and stirred at room temperature for 16 h. After completion, the reaction mixture was quenched with water (50 mL) and extracted with EtOAc (3 X 15 mL). The combined organic layers were dried over anhydrous MgSO₄ and the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel (EtOAc/ Hexane 1:10) to yield the product **11** as a white solid (0.429 g, 71 %) ³⁵.

Mp: 136-138 °C, TLC (EtOAc/ Hexane 1:3): R_f = 0.52; ¹H NMR (400MHz, CDCl₃): δ 9.24 (s, 1H), 7.72 (s, 1H), 7.36-2.27 (m, 2H), 4.47 (q, *J* = 14.16, 7.08 Hz, 2H), 1.73-1.63 (m, 3H), 1.43 (t, *J* = 7.08 Hz, 3H), 1.19 (s, 18H); ¹³C NMR (100 MHz, CDCl₃): δ 161.1, 133.8, 130.3, 127.3, 126.7, 120.7, 113.4, 102.2, 102.0, 83.8, 75.7, 67.9, 61.7, 29.6, 25.5, 14.1 ; IR (neat) ν_{max}: 3313, 2939, 2587, 1684, 1253 cm⁻¹; HRMS (ESI) [M + H]⁺ calculated for C₂₂H₃₁ClNO₂Si: 404.1813, found: 404.1815.

TIPS Amide 12. A mixture of **11** (0.168 g, 0.42 mmol), 3 N sodium hydroxide (0.42 mL) and ethanol (1 mL) was heated to 60 °C for 1.5 h. After cooling, 1 N HCl was added until pH ~ 2. The mixture was extracted with Ethyl Acetate (3 × 25 mL). The combined organic layers were dried over anhydrous MgSO₄ and the solvent was removed under reduced pressure. The carboxylic acid **12** was used for the next step without further purification.

EDC•HCl (0.0658 g, 0.1 mmol) and 4-DMAP (0.036 g, 0.1 mmol) were added sequentially in single portions to a solution of the residue obtained above (0.1 mmol) and amine **5** (0.31 mmol) in dry DMF (2.8 mL) at room temperature. The mixture was stirred at room temperature for 16 h. Upon completion, the reaction contents were diluted with EtOAc (10 mL), poured into water (25 mL), and extracted with EtOAc (2 × 25 mL). The combined organic layers were then washed with 1 M aqueous HCl (3 × 10 mL) and brine (30 mL), dried (MgSO₄), and concentrated. The residue was purified by silica gel column chromatography (EtOAc/ Hexane 1:4) to provide **3** as a yellow solid (0.78 g, 40 %).

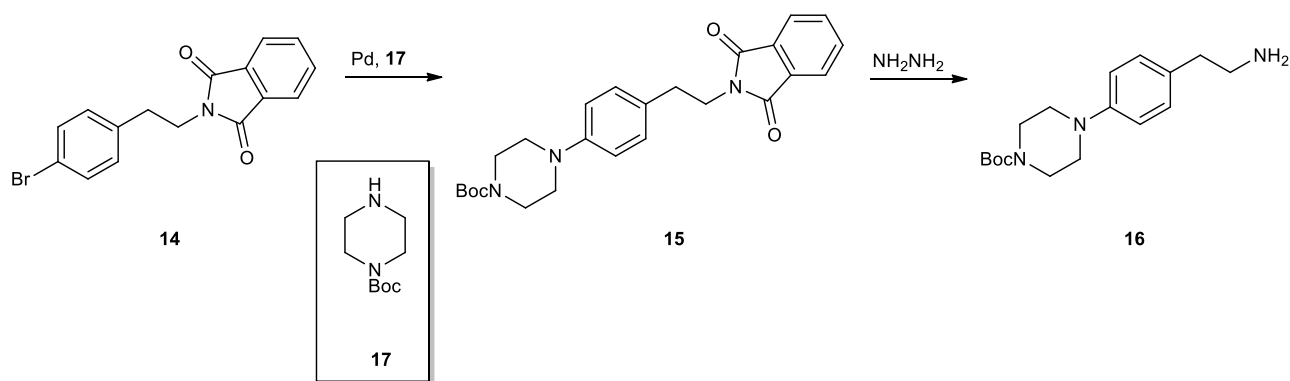
Mp: 183-185 °C dec.; TLC (EtOAc/ Hexane 1:3): R_f = 0.43; ¹H NMR (400MHz, CDCl₃): δ 7.97-7.89 (m, 1H), 7.64 (s, 1H), 7.38 (d, *J* = 8.68 Hz, 1 H), 7.29-7.27 (m, 1H), 7.26-7.24 (m, 2H), 7.19-7.09 (m, 2H), 3.68 (q, *J* = 14.00, 6.32 Hz, 2H), 3.22-3.05 (m, 4H), 2.88 (t, *J* = 8.52 Hz, 2H), 1.85-1.64 (m, 4H), 1.63-1.49 (m, 2H), 1.29-1.23 (m, 3H), 1.17 (s, 18H); ¹³C NMR (100 MHz, CDCl₃): δ 160.7, 133.8, 133.5, 130.2, 129.3, 127.2, 125.8, 120.3, 117.0, 113.8, 100.2, 99.5, 97.3, 44.8, 35.5, 25.7, 18.8, 11.3; IR (neat) ν_{max}: 3214, 2934, 1643, 1552, 1240 cm⁻¹; HRMS (ESI) [M + H]⁺ calculated for C₃₃H₄₅ClN₃OSi: 562.3020, found: 562.2985.

ORG27569alk2. Tetrabutylammonium fluoride (1 M in THF, 0.16 mL, 0.16 mmol) was added to a stirring solution of **12** (0.045 g, 0.08 mmol) in THF (1.2 mL). The reaction was stirred at room temperature for 16 h³⁴. The reaction was quenched with a saturated solution of NH₄Cl (10 mL) and extracted with Diethyl Ether (3 X 10 mL). The organic layers were combined, washed with saturated NaHCO₃ (20 mL), brine (20 mL), dried with MgSO₄ and concentrated under reduced pressure. The crude mixture was purified by flash chromatography (EtOAc/ Hexane 1:3) to afford **ORG27569alk2** as a white solid (0.029 g, 90%).

Mp: 188-190 °C dec.; TLC (EtOAc/ Hexane 1:3): R_f = 0.36; ¹H NMR (400MHz, CDCl₃): δ 7.68 (s, 1H), 7.61-7.53 (m, 1H), 7.38 (d, *J* = 8.68 Hz, 1H), 7.29-7.27 (m, 2H), 7.17 (d, *J* = 7.68 Hz, 1H), 7.06-6.91 (m, 2H), 3.81 (q, *J* = 12.72, 6.56 Hz, 2H), 3.39-3.33 (m, 1H), 3.21-3.10 (m, 4H), 2.89 (t, *J* = 6.64 Hz, 2 H), 1.83-1.70 (m, 4H), 1.68-1.51 (m, 5H); ¹³C NMR (100 MHz, CDCl₃): δ 160.4, 134.2, 133.2, 129.8, 129.5, 127.1, 125.7, 125.4, 119.9, 117.5, 113.6, 95.5, 85.8, 76.3, 40.7, 34.4, 30.2, 29.6, 25.5, 23.8; IR (neat) ν_{max}: 3214, 3159, 1637, 1547, 1437 cm⁻¹; HRMS (ESI) [M + H]⁺ calculated for C₂₄H₂₅ClN₃O: 406.1686, found: 406.1681.

Procedures for ORG27569alk4

Supplementary Scheme 5



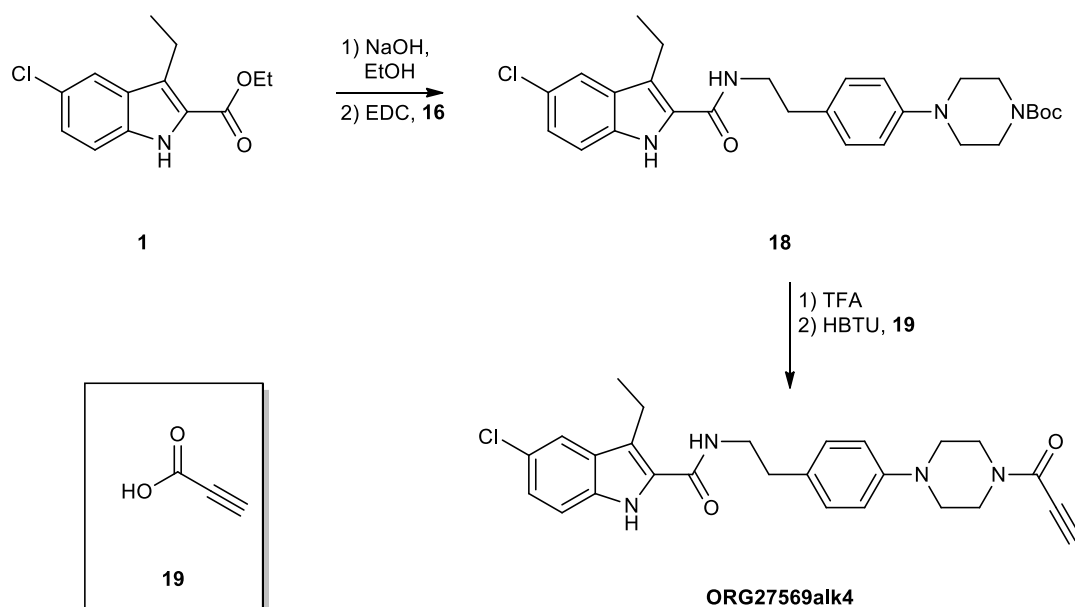
Phthalimide 17. A mixture of **14**³¹ (0.263 g, 0.8 mmol), tris(dibenzylideneacetone)dipalladium(0) (0.022 g, 3.0 mol %, 0.018 mmol), XPhos (0.045 g, 0.096 mmol), cesium carbonate (0.652 g, 2.0 mmol) and piperidine **17**³⁵ (0.372 g, 2.0 mmol) in degassed anhydrous toluene (7 mL) was heated at 110 °C for 16 h²⁹. After cooling, water and Diethyl Ether were added while shaking. The organic phase was washed with 3 N HCl (5 x 20 mL) and discarded. The acidic solution was neutralized with Na₂CO₃, and the mixture was extracted with Ethyl Acetate (3 X 25 mL). The combined organic layers were washed with brine, dried (MgSO₄) and evaporated. The crude mixture was

purified by flash chromatography (EtOAc/ Hexane 1:2) to afford **15** as a white solid (0.275 g, 79 %).

Mp: 166-168 °C; TLC (EtOAc/ Hexane 1:3): R_f = 0.36; ^1H NMR (400MHz, CDCl_3): δ 7.82 (dd, J = 3.08, 2.32 Hz, 2H), 7.69 (dd, J = 3.04, 2.36 Hz, 2H), 7.16 (d, J = 8.4 Hz, 2H), 6.85 (d, J = 7.38 Hz, 2H), 3.87 (t, J = 7.68 Hz, 2H), 3.61-3.52 (m, 4H), 3.12-3.04 (m, 4H), 2.91 (t, J = 8.0 Hz, 2H), 1.47 (s, 9H); ^{13}C NMR (100 MHz, CDCl_3): δ 168.1, 154.7, 149.9, 133.8, 132.1, 129.7, 129.6, 123.1, 120.2, 116.8, 79.8, 49.5, 39.3, 33.7, 30.78, 28.4, ; IR (neat) ν_{max} : 2934, 2851, 1711, 1681, 1544, 1454 cm^{-1} ; HRMS (ESI) $[\text{M} + \text{H}]^+$ calculated for $\text{C}_{25}\text{H}_{30}\text{N}_3\text{O}_4$: 436.2236, found: 436.2215.

Amine 16. A mixture of **17** (0.1 g, 0.3 mmol) and hydrazine monohydrate (0.075 g, 0.073 mL, 1.5 mmol) in ethanol (3 mL) was heated at reflux for 2 h³¹. After cooling, the reaction mixture treated with Diethyl Ether and 3 N HCl (5 x 20 mL) while shaking. The combined aqueous extracts were neutralized with Na_2CO_3 and extracted with Ethyl Acetate. The organic layers were washed with brine, dried (MgSO_4) and evaporated. The amine **18** was used for the next step without further purification.

Supplementary Scheme 6



Amide 17. A mixture of **1** (0.64 g, 2.22 mmol), 3 N sodium hydroxide (2.22 mL) and ethanol (5 mL) was heated to 60 °C for 1.5 h. After cooling, 1 N HCl was added until pH ~ 2. The mixture was extracted with Ethyl Acetate (3 x 100 mL). The combined organic layers were dried over

anhydrous MgSO_4 and the solvent was removed under reduced pressure. The carboxylic acid was used for the next step without further purification.

EDC•HCl (0.0658 g, 0.3 mmol) and 4-DMAP (0.036 g, 0.3 mmol) were added sequentially in single portions to a solution of the residue obtained above (0.3 mmol) and amine **16** (0.3 mmol) in dry DMF (5 mL) at room temperature. The mixture was stirred at room temperature for 16 h. Upon completion, the reaction contents were diluted with EtOAc (30 mL), poured into water (40 mL), and extracted with EtOAc (2×50 mL). The combined organic layers were then washed with 1 M aqueous HCl (3×30 mL) and brine (30 mL), dried (MgSO_4), and concentrated. The residue was purified by silica gel column chromatography (EtOAc/ Hexane 1:3) to provide **3** as a yellow solid (0.78 g, 58 %).

Mp: 205-207 °C dec.; TLC (EtOAc/ Hexane 1:3): $R_f = 0.28$; ^1H NMR (400MHz, CDCl_3): δ 9.22-9.14 (m, 1H), 7.54 (s, 1H), 7.29 (d, $J = 8.68$ Hz, 1H), 7.21 (d, $J = 1.88$ Hz, 1H), 7.20-7.14 (m, 2H), 6.99-6.90 (m, 2H), 6.03-5.95 (m, 1H), 3.78 (q, $J = 12.32, 6.28$ Hz, 2H), 3.67-3.54 (m, 4H), 3.17-3.06 (m, 4H), 2.90, (t, $J = 6.56$ Hz, 3H), 2.71 (q, $J = 15.28, 7.64$ Hz, 2H), 1.48 (s, 9H), 1.09 (t, $J = 7.64$ Hz, 3H); ^{13}C NMR (100 MHz, CDCl_3): δ 162.1, 135.8, 133.4, 129.6, 128.9, 128.2, 125.5, 124.9, 119.3, 112.9, 67.9, 40.8, 34.5, 34.2, 30.3, 29.7, 28.4, 21.2, 18.2, 15.3; IR (neat) ν_{max} : 3285, 2967, 1677, 1632, 1429 cm^{-1} ; HRMS (ESI) $[\text{M} + \text{H}]^+$ calculated for $\text{C}_{28}\text{H}_{36}\text{ClN}_4\text{O}_3$: 511.2476, found: 511.2441.

ORG27569alk4. A mixture of **18** (0.109 g, 0.3 mmol) and trifluoroacetic acid (0.3 mL) in dichloromethane (0.3 mL) was stirred at room temperature for 1 h. The solvent was evaporated and the residue was dried under high vacuum to provide the amine as bis- 2,2,2-trifluoroacetate which was used without further purification.

A mixture of the amine obtained above (0.005 mL, 0.08 mmol), Hunig base (0.021 mL, 0.12 mmol), **19** (0.08 mmol) and HBTU (0.036 g, 0.096 mmol) in dry DMF (0.220 mL) was stirred overnight at room temperature³⁸. The reaction mixture was treated with H_2O (10 mL) and extracted with CH_2Cl_2 (2 X 25 mL). The combined organic layers was washed with brine, dried (MgSO_4) and concentrated. The residue was purified by silica gel column chromatography (EtOAc/ Hexane 1:3) to provide **23** as a yellow solid (0.015 g, 41 %).

Mp: 174-176 °C dec.; TLC (EtOAc/ Hexane 1:3): $R_f = 0.28$; ^1H NMR (400MHz, CDCl_3): δ 9.14-9.07 (m, 1H), 7.55 (s, 1H), 7.29 (d, $J = 8.72$ Hz, 1H), 7.25-7.19 (m, 3H), 7.19-7.11 (m, 2H), 6.07-5.98 (m, 1H), 4.14-4.05 (m, 2H), 4.02-3.90 (m, 2H), 3.77 (q, $J = 12.60, 6.56$ Hz, 2H), 3.32-3.26 (m, 2H), 3.25-3.20 (m, 2H), 3.18 (s, 1H), 2.94 (t, $J = 6.72$ Hz, 2 H), 2.75 (q, $J = 15.00, 7.40$ Hz, 2H), 1.11 (t, $J = 7.60$ Hz, 3 H); ^{13}C NMR (100 MHz, CDCl_3): δ 160.4, 134.2, 133.2, 129.8, 129.5, 127.1,

125.7, 119.9, 117.5, 113.6, 95.6, 85.8, 76.1, 40.7, 34.4, 30.2, 29.6, 25.5, 23.9; IR (neat) ν_{max} : 3274, 2923, 1626, 1539, 1432 cm^{-1} ; HRMS (ESI) $[\text{M} + \text{H}]^+$ calculated for $\text{C}_{26}\text{H}_{28}\text{ClN}_4\text{O}_2$: 463.1901, found: 463.1904.

SUPPLEMENTARY METHODS

Mutagenesis. The following upstream (up) and downstream (dw) primers were used to introduce the indicated single aminoacidic substitutions:

V282F-up gatgttctggatcgggTTCaccagcgtactgcttc
V282F-dw gaagcagtagctggtGAAccgatccagaacatc
T377K-up gaacaagctattaagAAGgtgtttgcattctgc
T377K-dw gcagaatgcaaacacCTTcttaatgagcttgctc
L193I-up gtgtttctgttcaaaATCgggtggggtcacggcctc
L193I-dw gaggccgtgaccccaccGATtttgaacagaaacac
I395V-up ctccaccgtgaaccccGTCatctatgctctgagg
I395V-dw cctcagagcatagatGACgggggttcacgggtggag
I247V-up gaccatagccattgtgGTCgccgtgctgcctctcc
I247V-dw ggagaggcagcacggcGACcacaatggctatggtc
I245A-up gatgtggaccatagccGCTgtgatcgccgtgctgc
I245A-dw gcagcacggcgatcacAGCggctatggtccacatc
I243L-up gcctgatgtggaccCTAgccattgtgatcgccgtg
I243L-dw cacggcgatcacaatggcTAGggtccacatcaggc
H154L-up gcaggccttctacCTCttcatcggcagcctggcg
H154L-dw cgccaggctgccgatgaaGAGgtaggaaggcctgc
H154L-up (rat) gcaggccttctacCTCttcatcggcagcctggcg
H154L-dw (rat) gccaggctgccgatgaaGAGgtaggaaggcctgc
F408A-up gacctgcgacacgctGCCcggagcatgttccctc
F408A-dw gagggaaacatgctccgGGCagcgtgtcgcaggtc
F237L-up caaggccgtggtggcgCTTtgcctgatgtggacc
F237L-dw ggtccacatcaggcaAAGcggccaccacggccttg
A198M-up ctgggtgggggtcacgATGtccttactgcctccg
A198M-dw cggaggcagtgaaaggaCATcgtgacccacccag
C139Y-up gaacctctggtgctgTACgtcatcctccactccc
C139Y-dw gggagtgaggatgacGTAcagcaccaggaggttc

D366S-up gcaatcatggtgtatAGTgtctttggaagatgaac
 D366S-dw gttcatcttcccaaagacACTatacaccatgattgc
 F129L-up cacgctgggcaccTTAacggtctctggagaacctcc
 F129L-dw ggaggttctccaggaccgtTAAGgtgccagcgtg
 F191L-up cgcaacgtgttctgCTCaaactgggtgggg
 F191L-dw cccacccagtttGAGcagaaacacgttgcg

T1117 Fluorescent measurement. 50 μ l of membrane suspension (15 to 30 μ g/ μ l of total proteins) in PBS were incubated in 96 well black Optiplate (Perkin Elmer) with 500 nM T1117 (T1117 affinity binding) or with 3 μ M T1117 (sensitivity to ORG27569). 15 minutes after the incubation the plates were inserted in a 2104 Envision Multi-label plate reader (Perkin Elmer). Non-specific binding was measured by adding AM251 2,5 μ M to the sample and measuring change in T1117 fluorescence (specific quenching). Each sample was excited with Envision filter 206 (535 ± 25 nm ; 50% T) (Perkin Elmer) and fluorescence filtered with an emission filter 203 (615 ± 8.5 nm ; 80% T) (Perkin Elmer), using a normal top mirror. Plots were fitted in Prism5 (**GraphPad Software Inc.**, La Jolla, CA) using inhibition sigmoidal curve to calculate IC₅₀.

Docking studies. The compound was geometrically optimized by means of Macromodel¹¹, using MMFFs as force field, water as implicit solvent until a convergence value of 0.05kcal/mol*Å². The computational protocol applied consist of the application of 500 steps of the Polak-Ribière conjugate gradient (PRCG) for structure minimizations. The CB₁ protein structure was prepared through the Protein Preparation Wizard of the Maestro9.1¹¹. Docking was accomplished through the Glide induced fit docking (IFD) tool available in Maestro9.1¹¹. The grid was centered on the residues shaping the allosteric binding pocket defined by mutagenesis data reported in this work (H^{2.41} and F^{4.46} residues according to Ballesteros–Weinstein numbering). The flexible region of the protein was fixed until 8 Å around the center of the grid. Each docking run was carried out with the standard precision (SP) method, and the van der Waals scaling factor of non polar atoms was set to 0.8. Fifteen docking poses were obtained and among these poses we selected the best pose in accordance with the mutagenesis data, and structure-activity relationship studies previously

reported for this class of CB₁ allosteric modulators²¹. The selected docking pose were minimized using OPLSA2005 as force field, the PRCG methods until a gradient of 0.001 kcal/mol*Å² applying a stepwise relaxation protocol for which harmonic constraints were progressively reduced for backbone, side chains and ligand atoms.

MD Simulations of CB1 unbound, CB1wt-ORG27569 bound and CB1-(H^{2.41}L)-ORG27569 bound systems. The first CB1wt-ORG27569 bound form was built starting from the refined complex selected from the docking studies. ORG27569 atomic single charges were computed using Gaussian03, HF/6-31** as theory level (http://www.gaussian.com/g_misc/g03/g03_rel.htm#e1), and fitted with restrained electrostatic potential (RESP). The second CB1wt-ORG27569 bound form was built starting from a different binding conformation (the second ranked in the docking studies) and parametrized as described above (For details see Supplementary Fig. S14). Both CB1wt-ORG27569 complexes were modified as already described for the CB1wt unbound form (Protonation state, and palmitoylation site were considered as previously reported). The resulting complexes (CB1wt-ORG27569 x 2) were embedded in explicit POPC/Chol (2:1) membrane environment, while the CB1wt form was embedded in three different explicit membrane environments [POPC/Chol (2:1), DOPC/Chol(2:1), and POPC/Chol(2:1)-310K] as previously described, and the first N-terminal (S87) and the last C-terminal (E416) residues were capped with ACE and NME respectively. Only for the second POPC/Chol(2:1) system cholesterol molecules, having their center of mass (COM), in close proximity to the CCM-COM (20 Å) were replaced by POPC molecules, and the simulations was conducted at 310K for 1μs. Molecular dynamics simulations (relaxation and production phase) of the CB1wt-ORG27569 complexes were conducted as previously described for the CB1 unbound form. The final production phase was conducted for 1μs for each system.

The CB1wt-ORG27569 complex was used to generate the CB1-(H^{2.41}L)-ORG27569 complex (Supplementary Fig. S14), and MD simulation 1μs long was conducted using the same MD setting reported previously. Computational and structural details about the generation of the different CB1 complexes and MD simulations setting are deeply discussed in Supplemental Material. Supplementary Table 4 lists all the MD simulations carried out.

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