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STRUCTURE OF THE AGONIST 12-HHT IN ITS BLT2 RECEPTOR-BOUND STATE

STRUCTURE OF THE AGONIST 12–HHT IN ITS BLT2 RECEPTOR-BOUND STATE

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ABSTRACT

G Protein-Coupled receptors represent the main communicating pathway for signals from the outside to the inside of most of eukaryotic cells. They define the largest family of integral membrane receptors at the surface of the cells and constitute the main target of the current drugs on the market. The low affinity leukotriene receptor BLT2 is a receptor involved in pro- and anti-inflammatory pathways and can be activated by various unsaturated fatty acid compounds. We present here the NMR structure of the agonist 12-HHT in its BLT2-bound state and a model of interaction of the ligand with the receptor based on a conformational homology modeling associated with docking simulations. Put into perspective with the data obtained with leukotriene B4, our results illuminate the ligand selectivity of BLT2 and may help define new molecules to modulate the activity of this receptor.

KEYWORDS: GPCR NMR conformational homology docking BLT2 12-HHT LTB4

1 INTRODUCTION

2
3 G protein-coupled receptors (GPCRs) are integral membrane proteins that allow the sig-
4 nal transduction from the outside to the inside of most of eukaryotic cells.¹ These receptors
5 consist in a large family of proteins whose activities can be related to various ligands, from
6 small organic compounds like neurotransmitters or hormones, to lipids, peptides or proteins.
7 As such, they are key players in many biological processes and represent one of the most com-
8 mon target of clinical drugs.^{2,3} Signal transduction through GPCRs concentrates a cascade
9 of biological events that, if we exclude the constitutive activity, starts with the interaction of
10 an extracellular signaling molecule with these membrane proteins, and triggers, at the end,
11 a cellular response. This binding of a ligand onto its cognate receptor represents a funda-
12 mental stage in the activation process. To get a picture of this interaction at the atomic
13 scale is not trivial as the number of high-quality crystals in the presence of natural agonists
14 is limited.⁴⁻⁶

15 In addition to X-ray diffraction and cryo-electronic microscopy (cryo-EM),⁷⁻¹⁰ NMR
16 spectroscopy can bring important information regarding conformational and energy land-
17 scapes¹¹⁻¹⁶ or, as shown here, on the structure of natural GPCR ligands in their receptor
18 bound-states. This technique can indeed provide a detailed description of the ligand in its
19 bound-state, at physiological temperature and with a native protein.¹⁷⁻²⁵ Especially with
20 very flexible ligands, like those described in this study, NMR data can constitute the basic
21 input to subsequent X-ray- or cryo-EM-based molecular modeling of ligand/GPCR com-
22 plexes.

23 The leukotriene receptors 1²⁶ (BLT1) and 2²⁷⁻³⁰ (BLT2) are cell surface GPCRs that
24 share 45% amino acid sequence identity in human and are involved in pro- and anti-inflammatory
25 pathways.³¹⁻³⁴ They were initially named high (BLT1) and low leukotriene B4 (LTB4)
26 (BLT2) receptors as the equilibrium dissociation constant (K_d) values of LTB4 in the pres-
27 ence of membrane fractions transfected by either BLT1 or BLT2 is 20-fold weaker in the

28 case of BLT2 compared to BLT1 transfected HEK 293 cells (*i.e.* ~ 1 nM and ~ 20 nM for
29 BLT1 and BLT2, respectively).³⁰ BLT1 receptor is essentially expressed in leukocytes and
30 lymphocytes and is mainly activated by the LTB4³⁵ which is a strong potent lipid inflam-
31 matory mediator. By contrast, BLT2 is expressed in various tissues and has been shown to
32 bind to different arachidonic acid metabolites with moderate affinities, including LTB4.³⁶

33 In 2008, the heptadecanoid 12S-hydroxyheptadeca-5Z,8E,10E-trienoic acid³⁷ (12-HHT)
34 was suggested to be the endogenous ligand of BLT2.³⁸ In membrane fractions of Chinese
35 Hamster Ovary cells (CHO) transfected by BLT2, the half maximal inhibitory concentration
36 (IC50) and the half maximal effective concentration (EC50) values of 12-HHT are about one
37 order of magnitude lower than LTB4 while it does not bind to BLT1.³⁸ The main source of
38 12-HHT comes as a reaction product of the conversion of prostaglandin H2 to thromboxane
39 A2 and malonyldialdehyde by the thromboxane synthase.³⁹ Recent studies highlighted an
40 important activity of the 12-HHT/BLT2 axis in various pathologies, including inflammatory
41 and allergic diseases,^{38,40-42} wound healing⁴³ and cancers.⁴⁴⁻⁴⁸

42 Here, we determined by NMR spectroscopy the three-dimensional (3D) structure of the
43 agonist 12-HHT associated with human BLT2. As observed with the LTB4 in the presence
44 of the same receptor,¹⁸ 12-HHT adopts also a non-extended conformation. We propose
45 also a tentative model of interaction of 12-HHT with BLT2 based on X-ray crystal structure
46 conformational homology modeling and docking simulations, with the support of unequivocal
47 experimental data.

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METHODS

SAMPLE PREPARATIONS. The heptadecanoid 12-HHT and the eicosanoid LTB4 were obtained from Cayman Chemical, Ann Arbor, USA, as ethanolic solutions. The ethanol was extensively evaporated under vacuum. Then the eicosanoids in excess respectively to the receptor were directly dissolved by the NMR sample containing the receptor associated with perDAPol in a 100%-D₂O solution (20 mM Tris/HCl buffer pH 8, 100 mM NaCl) at final concentrations of ~ 120 and ~ 140 μM of 12-HHT and LTB4, respectively. The receptor concentration was $\sim 15\mu\text{M}$ which gives rise to ligand/BLT2 molar ratio of 8 and ~ 9 for 12-HHT/BLT2 and LTB4/BLT2. Considering a percentage of properly folded receptor ranging from 50 to 70%,⁴⁹ this means an effective ligand/receptor ratios of 15 ± 4 . Synthesis of perDAPol was performed as already described⁵⁰ and the overexpression, purification and folding of perdeuterated human BLT2 receptor is detailed in Catoire *et al.* 2010.¹⁸

SITE-DIRECTED MUTAGENESIS. All mutations were introduced in the wild-type BLT2 receptor by PCR-mediated mutagenesis using the QuickChange multisite-directed mutagenesis kit (Stratagene) and the wild-type BLT2 construct as a template. Mutations were confirmed by nucleotide sequencing.

LIGAND BINDING ASSAYS. Agonist binding to the isolated BLT2 receptor was monitored through ligand-dependent receptor-catalyzed G protein activation, as described in Arcemisb  h  re et al (two different types of experiments were carried out to demonstrate 2010). $G_{\alpha i2}$ and $G_{\beta 1\gamma 2}$ were prepared as previously described.⁵¹ Briefly, agonist-dependent functional coupling of the purified receptor to $G_{\alpha i2\beta 1\gamma 2}$ was assessed through the rate of GTP γ S binding at increasing agonist concentrations determined by monitoring the relative increase in the intrinsic fluorescence ($\lambda_{excitation} = 300$ nm, $\lambda_{emission} = 345$ nm) of $G_{\alpha i2}$ (200 nM of purified G protein) in the presence of BLT2 (20 nM) in buffer containing 10 mM MOPS,

78 pH 7.2, 130 mM NaCl, and 2 mM MgCl₂ at 15°C after the addition of 10 μM GTPγS. The
79 data were normalized to the fluorescence maximum obtained in the presence of saturating
80 concentrations in 12-HHT (10 μM).

81

82 NMR SPECTROSCOPY. All NMR experiments were conducted at 25°C and 700 MHz on
83 a Bruker Avance spectrometer equipped with a cryoprobe. The dipolar interactions were
84 detected and collected in a transferred mode, *i.e.* in the presence of an excess of ligand over
85 the receptor thanks to a electrostatically-driven fast association and the perdeuteration of
86 the receptor which allow detection of transferred cross-relaxation for GPCR ligands with
87 equilibrium dissociation constants in the high-to-low nanomolar range (see the theoretical
88 and experimental demonstration in Catoire *et al.* 2011¹⁹). The following parameters were
89 used for 2D NOESY experiments: 4 different mixing times ($\tau_m = 0.1$ s, 0.2 s, 0.35 s, 0.5 s
90 in the study of 12-HHT and $\tau_m = 0.05$ s, 0.1 s, 0.2 s, 0.5 s with the LTB4); data size =
91 $256(t_1) \times 8,192(t_2)$ complex points, $t_{1_{max}} = 36.5$ ms, $t_{2_{max}} = 585$ ms, 128 acquisitions per
92 increment, experiment time = 11.5 to 15.7 hours. Water suppression was conducted by using
93 an excitation sculpting scheme with gradients.⁵² Prior to Fourier Transform, the time do-
94 main signal was apodized by a square cosine in both dimensions. No baseline correction was
95 applied. ¹H chemical shifts are referenced to H₂O (calibrated at 4.7 ppm at 25°C). Chemical
96 shift assignments are based on COSY spectra from Catoire *et al.* 2010¹⁸ and 2011.¹⁹ Data
97 processing and analyzing were performed with TOPSPIN software.

98

99 STRUCTURE CALCULATIONS. 12-HHT and LTB4 pdb files were produced with PRO-
100 DRG.⁵³ Parameter and topology files were generated with XPLO2D (version 3.3.2).⁵⁴ Struc-
101 ture calculations were performed with the program ARIA (Ambiguous Restraints for Itera-
102 tive Assignment) (version 2.3)⁵⁵ associated with CNS⁵⁶ using standard protocols. For each
103 ligand, calculations were based on four sets of NOE data corresponding to four distinct τ_m
104 (see [Tables S1 to S4](#) and [S6 to S9](#) for 12-HHT and LTB4, respectively). A full relaxation

105 matrix treatment of NOE data has been applied in ARIA/CNS to take into account indirect
106 ^1H - ^1H cross-relaxation pathways.^{57,58} In the case of 12-HHT, only dipolar contacts involv-
107 ing H2, H3, and H17 with the other ligand protons were taken into account as these dipolar
108 restraints display the lowest level of non-specific binding contribution to the peak volumes
109 (unstructured parts in the ligand in the absence of the receptor). For LTB4, only protons at
110 both ends interacting with the other protons in the ligands, *i.e.* H2, H3, H4 and H16, H17,
111 H18, H19 and H20, were taken into account in the structure calculation. The structures
112 were drawn using the software PyMOL.

113

114 HOMOLOGY MODELLING OF RECEPTORS AND LIGAND DOCKING SIMULATIONS. Homol-
115 ogy modelling of BLT2 based on X-ray crystal structures was performed with the software
116 Modeller (version 9.2).⁵⁹⁻⁶¹ Several structures were tested, including the two mentioned in
117 this manuscript: BLT1 (pdb code 5x33⁶²) and β 2AR (pdb code 3p0g⁴). Docking simulations
118 of 12-HHT in human BLT2 receptor were subsequently performed with HADDOCK (version
119 2.2) taking as *active residues* S174^{ECL2} and R270^{7,35} only.

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123 RESULTS

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125 STRUCTURE OF 12-HHT ASSOCIATED WITH BLT2 RECEPTOR.

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127 The NMR study of 12-HHT in its receptor-bound state was realized *in vitro* in a
128 detergent-free solution⁶³ following a method that has been already applied with the LTB4 in
129 the presence of the same receptor.^{18,19} Briefly, the heterologous human BLT2 receptor was
130 expressed in *Escherichia coli* in a 100%-D₂O solution to inclusion bodies^{64,65} and was sub-
131 sequently folded to its native state using amphipols.^{49,66} The NMR structure of 12-HHT is

132 based on the detection of dipolar interactions in the ligand through two-dimensional homonu-
133 clear ^1H Nuclear Overhauser Effect Spectroscopy (NOESY) experiments.⁶⁷ The dipolar in-
134 teractions were collected in a transferred mode in the presence of an excess of ligand over the
135 receptor. Indeed, it has been demonstrated that solution-state NMR can detect transferred
136 NOEs even with equilibrium dissociation constants below the micromolar range because of
137 *i*) an inherent ultra-fast diffusive association of these negatively charged agonists onto a
138 highly positively charged extracellular surface, and *ii*) the slowing down of the ^1H - ^1H cross-
139 relaxation thanks to receptor perdeuteration.¹⁹ In order to improve the number and quality
140 of intra-ligand ^1H - ^1H dipolar contacts, BLT2 was maintained soluble and stable in solution
141 associated with a perdeuterated amphipol named perDAPol.⁵⁰ Compared to the pioneer
142 study of LTB4 associated with BLT2, perDAPol offered the possibility to observe intra-
143 aliphatic ^1H dipolar interactions in the ligand (Figure 1) (for a comparative observation, see
144 Figure S1).

145 In the presence of BLT2 associated with either amphipols or nanodiscs, 12-HHT displays
146 a higher proportion of non-specific binding compared to LTB4 (see for instance Supplemen-
147 tary Figure S8 in Casiraghi *et al.* 2016¹¹). This is presumably due to a more hydrophobic
148 character which favours the interaction of the ligand with the belt of surfactant molecules
149 or lipids.¹¹ To correctly assess the presence of specific intra-ligand dipolar interactions, the
150 NMR collection of constraints was based on a rigorous observation of specific intra-ligand ^1H -
151 ^1H dipolar interactions in the bound-state in the presence of a perdeuterated receptor.^{18,19} In
152 the absence of the receptor, *i.e.* in the presence of perDAPol only, we observed the absence
153 and/or the presence of weak ^1H - ^1H dipolar interactions between aliphatic protons located at
154 both ends, *i.e.* from protons H2 to H4 on one side, and from H13 to H17 on the other side,
155 with the other ^1H in the ligand (Figures S2 and S3). This indicates that both ligand ends are
156 not structured in the absence of the receptor. Moreover, non-specific ^1H to ^1H interactions
157 between the ligand and the surfactant can be observed in a 2D NOESY spectrum (*e.g.* the
158 regions squared with a green dashed line in Figure S2).

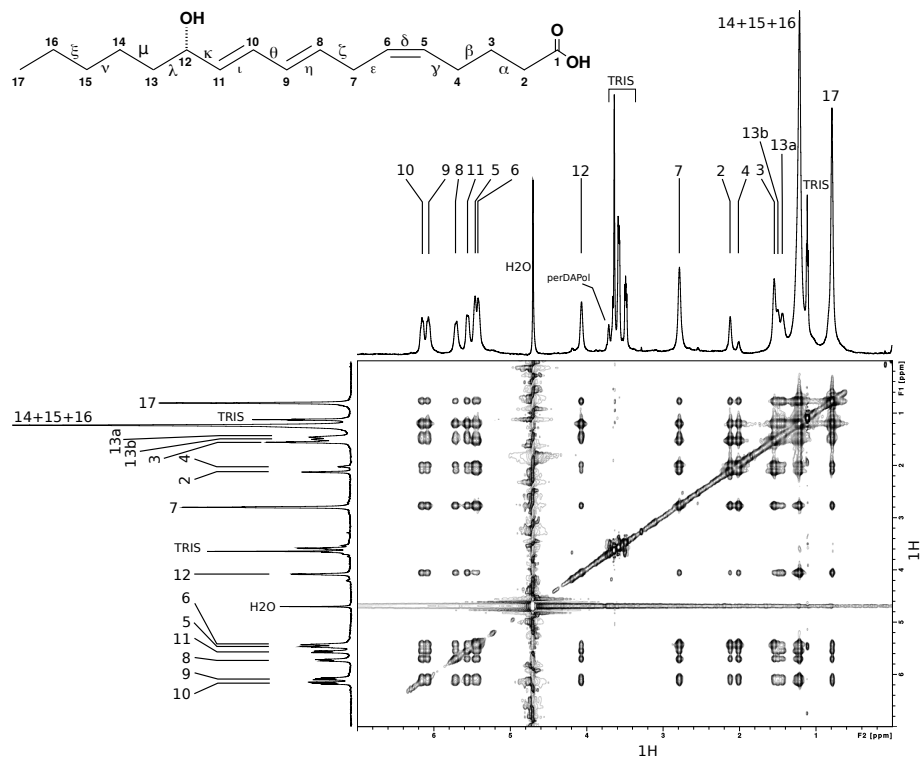


Figure 1: Dipolar interactions in the 12-HHT/ $u\text{-}^2\text{H}$ -wtBLT2/perDAPol sample observed in a 2D NOESY spectrum ($\tau_m = 0.5$ s, $\nu_H = 700$ MHz, 25°C , $[12\text{-HHT}] = 120$ μM , $[\text{BLT2}] = 15$ μM). The corresponding 1D ^1H spectrum is shown above the 2D spectrum and a 1D spectrum of free 12-HHT in solution is displayed on the left side. Numbers refer to the protons annotated on the 12-HHT chemical structure indicated above the spectrum.

159 The NMR data corresponding to only specific interactions were collected at four different
160 Nuclear Overhauser Effect (NOE) mixing times τ_m , *i.e.* 0.1, 0.2, 0.35 and 0.5 s and integrated
161 for structure calculations (Tables S1 to S4). Dipolar interactions between H2, H3 and H17
162 with the other protons in the ligand were actually enough to obtain a converged set of
163 structures (Tables S1 to S4 and Figure S4). This set of low energy conformers of 12-HHT in
164 its BLT2 bound-state is depicted in Figure 2 (with associated structural statistics gathered
165 in Table 1). In order to describe any conformational rearrangement of the structure of the
166 ligand upon binding to its receptor, a structure analysis of 12-HHT free in solution was
167 performed (Table S5) and is also indicated in Figure 2. As expected, in the free state,
168 structure calculation indicates that both aliphatic ends of the molecule are flexible with
169 the coexistence of various rotamers. However, compared to calculations conducted without
170 any experimental restraints, 12-HHT free in solution adopts preferential conformations at
171 the pentyl-end (carbon atoms 13 to 17), precluding any extended conformation along the
172 axis defined by the unsaturated bonds. Compared to the free-state in solution, the ligand
173 describes a well-constrained conformation in the presence of the receptor. In particular,
174 both ends, the carboxyl-end (1-carboxy-pent-4-ene-5-yl chain, carbons C1 to C6) and the
175 pentyl-end (carbon atoms 13 to 17), adopt a unique orientation respectively to the rigid core
176 of the molecule (dihedral angles ζ and κ in Figure 2).

177

178

179 DOCKING MODEL OF 12-HHT ASSOCIATED WITH BLT2 RECEPTOR.

180

181 The set of 20 low energy conformers depicted in Figure 2 was further integrated in a model
182 of the BLT2 receptor to perform docking simulations using the software HADDOCK.^{68,69}
183 The model of the BLT2 receptor using Modeller⁵⁹⁻⁶¹ was based on the active state of the
184 β 2 adrenergic receptor (β 2AR) (pdb code 3p0g⁴) in spite a structure of BLT1 associated
185 with an inverse agonist being available in the protein databank (pdb code 5x33⁶²). Using

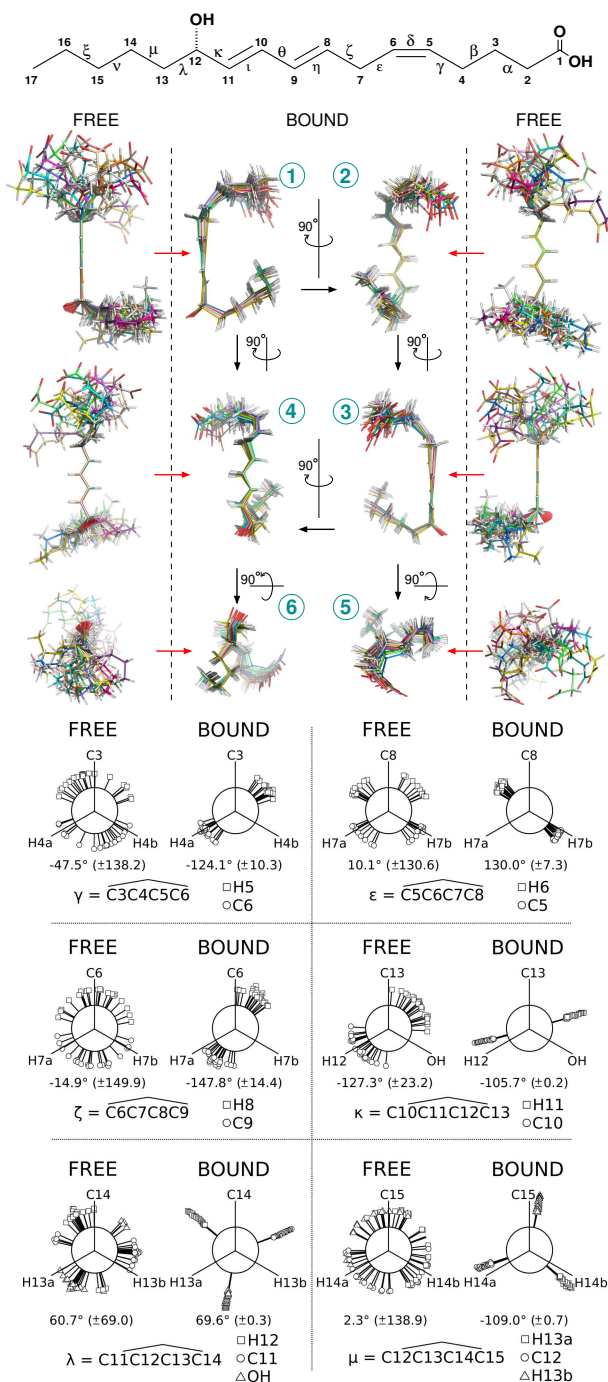


Figure 2: Three-dimensional structure of 12-HHT free in solution or bound to BLT2. (Top) Primary chemical structure of 12-HHT. The carbons are numbered from the carboxyl function to the methyl group. Greek letters refer to some dihedral angles displayed at the bottom of the figure. (Middle) Six different views of two ensembles of 20 energy-minimized conformers (in *white*, hydrogen atoms; in *red*, oxygen atoms; carbon atoms are assigned a different color for each conformer). The *red* arrows indicate the transition from the *free* to the *bound* state for a same orientation of the diene located at the center of the molecule (carbons C8 to C11). (Bottom) Comparison of dihedral angles between the free and the bound states for the set of 20 conformers displayed above.

Table 1: Summary of structural constraints and structure statistics for a set of 20 structures of 12-HHT in the presence of BLT2 receptor. (in the case where the inter-protons distance indicated is not an integer, this means that magnetically not equivalent protons could not be distinguished in the NOESY spectrum. For instance, between H5 and H6, a dipolar interaction with H7 corresponds to an average inter-proton restraints of 2.5).

NOE-based distance restraints Inter-protons i,j at $\tau_m = 0.1$ s		NOE-based distance restraints Inter-protons i,j at $\tau_m = 0.2$ s	
$ i-j =1$	3	$ i-j =1$	3
$ i-j =2$	2	$ i-j =2$	3
$ i-j =2.5$	1	$ i-j =2.5$	1
$ i-j =3$	3	$ i-j =3$	2
$ i-j =4$	4	$ i-j =4$	3
$ i-j =5$	3	$ i-j =5$	4
$ i-j =6$	3	$ i-j =6$	3
$ i-j =7$	3	$ i-j =7$	3
$ i-j =8$	2	$ i-j =8$	3
$ i-j =9$	1	$ i-j =9$	2
$ i-j =10$	1	$ i-j =10$	2
$ i-j =11$	2	$ i-j =11$	2
$ i-j =12$	1	$ i-j =12$	2
$ i-j =13$	2	$ i-j =13$	2
$ i-j =15$	1	$ i-j =15$	1
Total	32	Total	36
Inter-protons i,j at $\tau_m = 0.35$ s		Inter-protons i,j at $\tau_m = 0.5$ s	
$ i-j =1$	3	$ i-j =1$	2
$ i-j =2$	3	$ i-j =2$	1
$ i-j =2.5$	1	$ i-j =2.5$	1
$ i-j =3$	2	$ i-j =3$	1
$ i-j =4$	4	$ i-j =4$	3
$ i-j =5$	4	$ i-j =5$	3
$ i-j =6$	3	$ i-j =6$	2
$ i-j =7$	3	$ i-j =6.5$	1
$ i-j =8$	3	$ i-j =7$	2
$ i-j =9$	2	$ i-j =8$	2
$ i-j =10$	2	$ i-j =9$	3
$ i-j =11$	2	$ i-j =10$	2
$ i-j =12$	1	$ i-j =11$	1
$ i-j =13$	2	$ i-j =12$	2
$ i-j =14$	1	$ i-j =13$	2
$ i-j =15$	1	$ i-j =14$	1
		$ i-j =15$	1
Total	37	Total	30
Structural statistics			
Number of NOE violations > 0.5 Å	1 ± 0		
Number of NOE violations > 0.2 Å	1 ± 0		
Number of NOE violations > 0.1 Å	3.4 ± 0.49		
Mean global rms	0.22 ± 0.20 (Å)		
Deviation from idealized geometry			
Mean rms bond	$4.8 \times 10^{-3} \pm 5.1 \times 10^{-5}$ (Å)		
Mean rms angle	$0.78 \pm 5.7 \times 10^{-3}$ (degrees)		
Mean rms improper	$2.16 \pm 8.2 \times 10^{-3}$ (degrees)		
Mean rms dihedral	$0.37 \pm 7.0 \times 10^{-3}$ (degrees)		
Mean energies (kcal.mol ⁻¹)			
E_{bonds}	$1.05 \pm 2.2 \times 10^{-2}$		
E_{angles}	7.53 ± 0.11		
$E_{impropers}$	18.12 ± 0.14		
$E_{dihedrals}$	$0.83 \pm 3.2 \times 10^{-2}$		
E_{vdw}	-9.48 ± 0.16		
E_{total}	18.06 ± 0.16		

186 BLT1 crystal structure in an inactive state does not allow the ligand 12-HHT to interact
187 with important amino acids in the BLT2 receptor that have been identified by site-directed
188 mutagenesis experiments associated with ligand binding assays (Figure 3A and Table 2).
189 In particular, residue S174 in the extra-cellular loop 2 (ECL2) BLT1-based BLT2 model is
190 located too far from the top of the ligand orthosteric pocket as the loop is in an open-lid con-
191 formation in the inactive state (Figure S5). To reproduce contacts between the ligand and
192 the receptor based on our binding studies, a conformational homology model was built. In
193 addition, the identity in amino acid sequence between BLT1 and BLT2 is only 45%, which
194 is mostly in the 7TM. Docking simulations were performed for each of the 20 NMR con-
195 formers based on two active residues identified by mutagenesis, S174 and R270 (Figure 3A).
196 Simulations with HADDOCK were started with 12-HHT NMR conformers well away from
197 the orthosteric site of β 2AR-active-based BLT2 model, *i.e.* not partly positioned in the
198 orthosteric site.

199 All these simulations gave rise to a single cluster or a predominant cluster of structures
200 representing 97 to 100% of the water-refined models generated by HADDOCK. The simula-
201 tions proposed various possible orientations of the ligand in the orthosteric pocket, but only
202 one orientation depicted in Figure 4 is compatible with site-directed mutagenesis experiments
203 associated with ligand binding assays (Figure 3A and Table 2) with a particular focus on the
204 two residues that establish hydrogen bonds with 12-HHT, *i.e.* S174^{ECL2} and R270^{7.35} (su-
205 perscripts indicate residue numbering following the Ballesteros-Weinstein nomenclature⁷⁰).
206 Indeed, that orientation shows an excellent agreement with these two single mutations, *i.e.*
207 S174^{ECL2}A and R270^{7.35}A, with EC50 values shifted from 21 nM (wild-type) to 295 nM for
208 S174^{ECL2}A and 235 nM for R270^{7.35}A. In that position, S174^{ECL2} establishes hydrogen bonds
209 with the carboxylate group of 12-HHT and R270^{7.35} interacts with the hydroxyl moiety of
210 the ligand through hydrogen bonds as well (Figure 4). A similar position of the ligand that
211 came out from the simulations involves an additional hydrogen bond between the hydroxyl
212 group of the ligand and Q267^{7.32}, but as no significant change in ligand binding could be

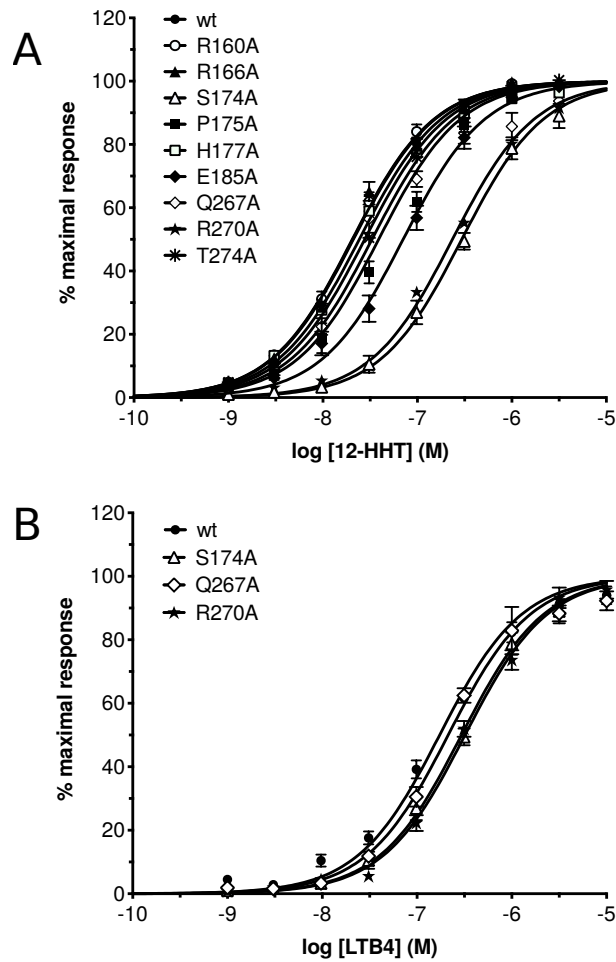


Figure 3: G_i protein activation catalyzed by the wild-type BLT2 receptor and its mutants in the presence of increasing 12-HHT (A) or LTB4 (B) concentrations. Data are presented as the mean \pm SEM of three experiments.

Table 2: EC50 values inferred from G_i protein activation catalyzed by the wild-type BLT2 receptor and its mutants in the presence of 12-HHT.

CONSTRUCTS	LOGEC50	EC50
WT	-7.677	2.102×10^{-8}
R160 ^{4.64} A	-7.689	2.046×10^{-8}
R166 ^{ECL2} A	-7.684	2.071×10^{-8}
S174 ^{ECL2} A	-6.539	2.948×10^{-7}
P175 ^{ECL2} A	-7.410	3.889×10^{-8}
H177 ^{ECL2} A	-7.615	2.427×10^{-8}
E185 ^{5.42} A	-7.151	7.070×10^{-8}
Q267 ^{7.32} A	-7.481	3.307×10^{-8}
R270 ^{7.35} A	-6.629	2.350×10^{-7}
T274 ^{7.39} A	-7.557	2.771×10^{-8}

213 observed by introducing the mutation Q267^{7.32}A (Figure 3A and Table 2), that orientation
214 was discarded.

215 The model of the interaction of 12-HHT NMR structure with a model of BLT2 based
216 on an active conformation of β 2AR displays interactions with five secondary elements in
217 the receptor: 4 helices (II, III, VI and VII), which delineate the contours of the orthosteric
218 pocket, and one extra-cellular loop (ECL2) which plays a role of lid above the ligand pocket
219 (Figure 4). In addition to the two amino acids that establish hydrogen bonds with the ligand
220 (S174^{ECL2} and R270^{7.35}), 10 other amino acids located at a distance $\leq 4\text{\AA}$ from the ligand
221 (depicted in Figure 4) show various weak interactions, including CH-to- π , CH-to-O, NH-to-
222 π , S-to-CH or N-to-CH proximities. The model of interaction depicted in Figure 4 is also
223 in accordance with some other neutral mutations that have been tested: first, residues that
224 are important for LTB4 binding to BLT1. R160^{4.64}, which is highly conserved in both BLT1
225 and BLT2 receptors (Figure S6), has been identified to be crucial for LTB4 binding on BLT1
226 (residue R156 in BLT1) by potentially making a direct hydrogen bond with the carboxylate
227 head group.⁷¹ Mutation of this residue to alanine results in a complete loss of LTB4 binding.⁷¹
228 Accordingly to our model, in which R160^{4.64} is located very far from 12-HHT (Figure S7),
229 R160^{4.64}A mutant has no effect on 12-HHT binding (Figure 3A and Table 2). In the same
230 way, the E185A mutation did not significantly affect 12-HHT binding (Figure 3A) whereas
231 mutating this residue had a noticeable impact on LTB4 binding onto BLT1.⁷¹ Second, some
232 neutral mutations have been conducted. Just beside S174^{ECL2} in ECL2, but not establishing
233 any interaction with the ligand, P175^{ECL2} and H177^{ECL2}, which mutations to alanine do not
234 display a significant impact on ligand binding compared to the wild-type receptor. Another
235 residue in ECL2, which could possibly interact with the carboxyl function of the ligand,
236 R166^{ECL2}, and an additional neutral mutation close to R270^{7.35}, T274^{7.39}A, do not impact
237 receptor ligand properties (Figure 3A and Table 2) in accordance with our model.

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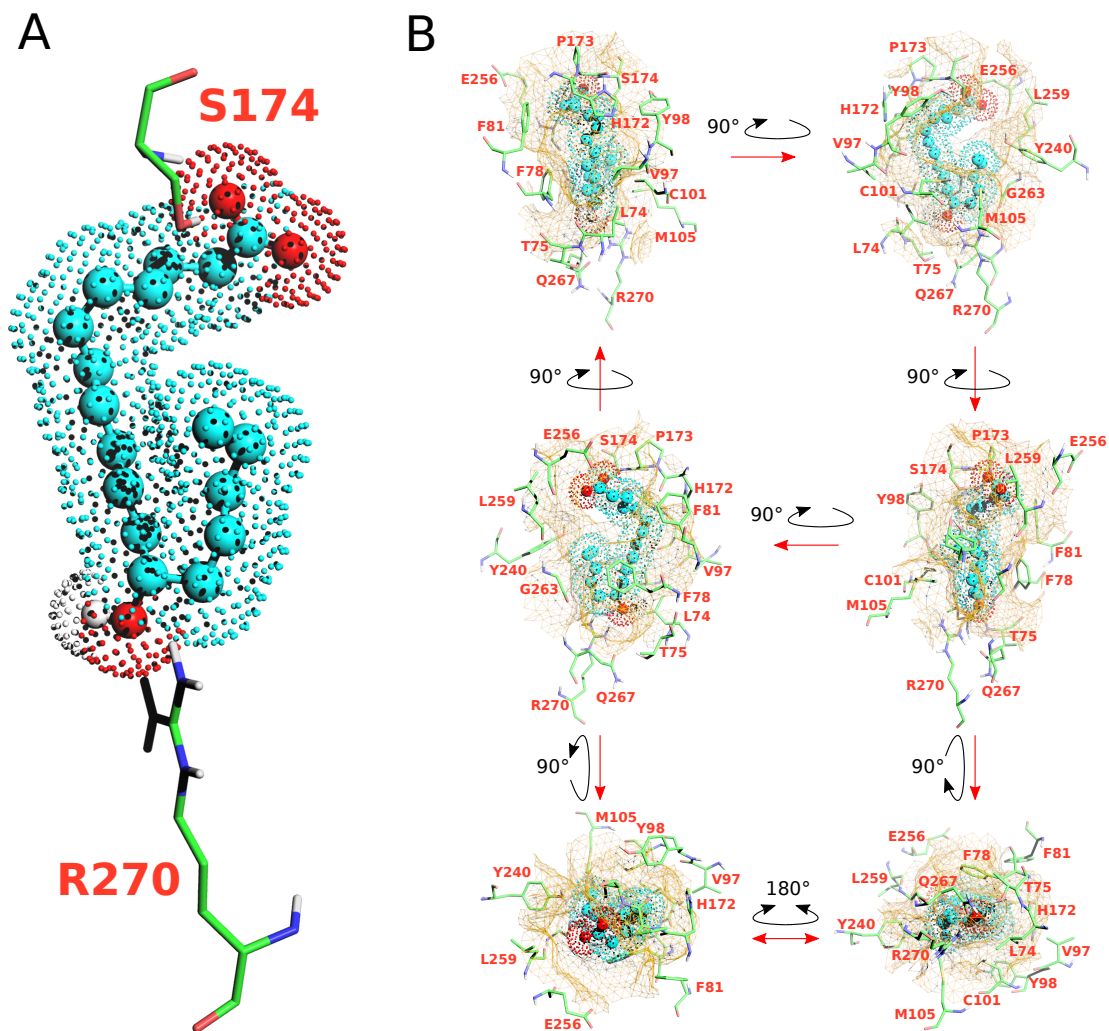


Figure 4: Docking model of the NMR structure of 12-HHT in human BLT2 receptor (see HADDOCK structural statistics in [Table S11](#)). (A) represents the ligand in spheres and dots (hydrocarbon skeleton in *cyan*, oxygen atoms in *red* and the proton of the hydroxyle group in position 12 in *white*) double-locked at the top and the bottom of the orthosteric pocket by two hydrogen bonds with S174 and R270 residues. (B) represents six different views of the ligand in the orthosteric pocket of the receptor. The cavity of the ligand binding pocket is represented with a brown mesh surface at a maximum distance of 5Å from the ligand. Amino acids delineating the pocket are indicated in *orange*.

We present also in this study a new set of converged structures of LTB4 associated with BLT2 in order to compare the bound structures of 12-HHT and LTB4 obtained under identical conditions and procedures. Compared to the first calculation published in 2010,¹⁸ NMR data were collected at 700 MHz with a receptor associated with perDAPol⁵⁰ instead of DAPol and by using a softer methodology to remove the ¹H signal of H₂O to not affect signal intensities from the ligand (see Material and Methods). As observed with 12-HHT, in the absence of the receptor, both ends of the ligand are not structured, based on the observation of intra-ligand ¹H-to-¹H dipolar interactions (Figure S8). Calculation based on NMR data collected in the presence of BLT2 gives rise to a folded structure (Figure S9 and Table S10) similar to the previous published structure,¹⁸ but with an orientation of the carboxyl-end (carbons 1 to 5) more loosely defined if we take into account an ensemble of 15 or 20 NMR structures (Figure S9 and see dihedral ζ in Figure S10). If we try to coincide the lowest energy conformers of LTB4 with the 12-HHT structure ensemble by superimposing the most rigid part of the hydrocarbon skeletons, *i.e.* carbons 7 to 12, we find that, globally, the fold of LTB4 is close to the 12-HHT structure in the presence of the same receptor (Figure 5): the orientation of the carboxyl-end is similar, but not identical, the hydroxyl group in position 12 points towards the same direction despite an opposite chirality of the asymmetric carbon, and the methyl end for both ligands are quite close despite the LTB4 chain containing three more carbons. However, the two chains from carbon 12 –bearing the hydroxyl group– to the methyl end display different orientations (see views 1 and 3 in Figure 5). This region of these ligands is supposed to be located at the bottom of the pocket of the receptor, based on the grafting of fluorescent probes at the carboxyl-end on the LTB4 for instance that does not affect the binding properties to BLT2.⁷²

Attempts to get a model of LTB4 associated with BLT2 failed because we could not identify clear mutants that impact significantly on the binding of LTB4 onto BLT2, and this

267 prevented us from getting a reasonable model of the ligand:receptor complex. Furthermore,
268 in contrast to 12-HHT, LTB4 is a very low-affinity ligand for BLT2, and this certainly
269 contributes to the fact that we could not get any satisfying model for this ligand.

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273 DISCUSSION

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275 Historically BLT2 was designated as the low-affinity LTB4 receptor, in contrast to BLT1,
276 with *in cellulo* K_d of ~ 20 nM compared with ~ 1 nM for BLT1.³⁰ More recently, strong ev-
277 idences led to the discovery of BLT2 endogenous agonist, 12-HHT,^{33,38,41} a non-eicosanoid
278 fatty acid compound which essentially comes from the conversion of prostaglandin H2 to
279 thromboxane A2. *In cellulo* measurements indicate a higher affinity of 12-HHT for BLT2
280 compared to LTB4, by about one order of magnitude.³⁸ This was also observed by *in vitro*
281 binding measurements of LTB4 and 12-HHT onto a purified BLT2 receptor associated with
282 amphipols in solution, with K_d of ~ 200 and ~ 60 nM, respectively.¹⁹ To be noted, the affinity
283 of the isolated receptor for its agonists is lower than that measured in cell systems. However,
284 high affinity can be recovered by associating the isolated receptor with its cognate G pro-
285 teins.⁵¹ Hence, the structures obtained here with the isolated BLT2 are likely signatures of
286 the low-affinity, uncoupled state of the receptor. A qualitative comparison of NMR NOESY
287 spectra clearly indicate an organization of both ends of 12-HHT in the presence of BLT2
288 (Figure S2 and S3). Structure calculation confirmed that observation and led to a single set
289 of converged structures (Figure 2). The model proposed herein describes a ligand that is
290 double-locked in the receptor by two hydrogen bonds which are in accordance with single-
291 directed mutagenesis associated with ligand binding experiments (Figure 3A): one $\text{NH} \cdots \text{O}$
292 hydrogen bond between the OH moiety of the ligand and R270^{7,35} at the bottom of the
293 orthosteric pocket, and a stronger $\text{OH} \cdots \text{O}$ hydrogen bond involving the COOH group of

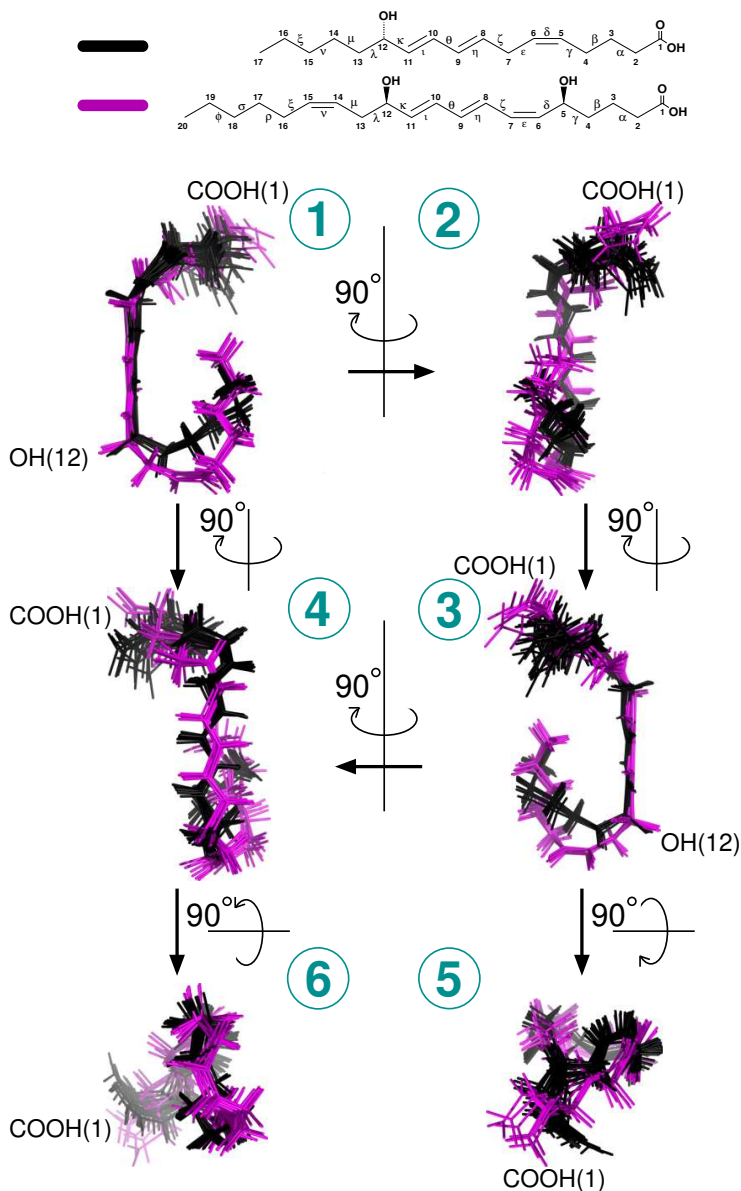


Figure 5: Comparison of 12-HHT and LTB4 3D NMR structures associated with human BLT2. Six different views of superimposed ensembles of 20 energy-minimized conformers of 12-HHT (in *black*, from Figure 2), and the 7 lowest energy conformers of LTB4 (in *purple*, from Figure S9). On *Top* are represented the chemical structures of the ligands.

294 12-HHT with S274^{ECL2}, *i.e.* on the opposite side of the ligand pocket (Figure 4). These two
295 residues are highly conserved in BLT receptors (Figure S6), but interestingly, while BLT1
296 is almost activated by the LTB4 only, and not by 12-HHT, mutation of these two residues
297 does not affect the binding of LTB4 onto BLT2 (Figure 3B and Table 3). In complement to
298 measurements at equilibrium, *in vitro* off-rate constant measurements led to a bound time
299 3.6 times longer for 12-HHT than LTB4.¹⁹ This also tends to suggest additional short range
300 interactions for 12-HHT compared with LTB4. A tentative superimposition of 12-HHT
301 and LTB4 in their BLT2-bound states described a similar fold, especially if we take into
302 account the 7 lowest energy structures obtained in the converged ensemble of structures of
303 LTB4 (Figure 5 and Table S10). However, several main features distinguish 12-HHT and
304 LTB4 than could explain these different binding properties: a shorter hydrocarbon chain for
305 12-HHT, with a double bond less, the absence of a hydroxyl group on position 5, and an
306 opposite chirality for the asymmetric carbone 12 (see top of Figure 5). In addition, super-
307 imposing the rigid core of these two ligands indicates noticeable differences, especially from
308 the asymmetric carbon 12 to the methyl end (Figure 5), a region which should interact with
309 the bottom of the orthosteric pocket.

310 Structures have revealed that a high percentage of identity between sub-families of class A
311 GPCRs can be observed for amino acids sculpting the orthosteric binding pocket in contrast
312 with the extracellular domains and membrane interface, which comprise the N-terminus end
313 and three extracellular loops and the top of the TMs. These regions display a higher di-
314 versity in both sequence and length.⁷³ Experimental data indicate that ECLs are intimately
315 implicated in GPCR activation.⁷⁴ Compilation of that information suggests a role of these
316 extracellular regions in GPCR signaling, including ligand binding and selectivity⁷⁵ in addi-
317 tion to ligand efficacy,⁷⁶ allosteric modulations,*e.g.*⁷⁷ and constitutive activation.⁷⁸ Our
318 model based on the crystallographic active state of the β 2AR highlights the importance of
319 ECL2 as a lock above the orthosteric site which one residue, S174, display the strongest in-
320 teraction above all residues that interact with the ligand. Two additional residues, *i.e.* H172

Table 3: EC50 values inferred from G_i protein activation catalyzed by the wild-type BLT2 receptor and its mutants in the presence of LTB4.

CONSTRUCTS	LOGEC50	EC50
WT	-7.677	2.102×10^{-8}
R160 ^{4.64} A	-7.689	2.046×10^{-8}
R166 ^{ECL2} A	-7.684	2.071×10^{-8}
S174 ^{ECL2} A	-6.539	2.948×10^{-7}
P175 ^{ECL2} A	-7.410	3.889×10^{-8}
H177 ^{ECL2} A	-7.615	2.427×10^{-8}
E185 ^{5.42} A	-7.151	7.070×10^{-8}
Q267 ^{7.32} A	-7.481	3.307×10^{-8}
R270 ^{7.35} A	-6.629	2.350×10^{-7}
T274 ^{7.39} A	-7.557	2.771×10^{-8}

321 and P173, associated with S174 define a hood above the ligand that probably contribute to
322 improve the residence time of the ligand to promote the binding of an intracellular part-
323 ner as equilibrium binding properties may not totally govern the activation of GPCRs. In
324 other words, non-equilibrium kinetics of the ligand binding event may also play an important
325 role.⁷⁹

326 The method proposed in this study deserves to be improved as some imperfections could
327 introduce biases in both the structure calculation and also in the model. First of all, the
328 definition of parameter and topology files for organic compounds is not so trivial despite
329 the development of very efficient and convenient programs like PRODRG⁵³ and XPLO2D⁵⁴
330 that have been used in the present study. Structure calculations were based on the program
331 ARIA⁵⁵ associated with CNS⁵⁶ which contains a full relaxation matrix treatment of NOE
332 data to take into account indirect ^1H - ^1H cross-relaxation pathways^{57,58} but does not take
333 into account the contribution of the chemical exchange of the ligand from the receptor in
334 the calculation. It would be interesting to include a matrix of exchange to properly gauge
335 the impact of the k_{off} –or conversely the residence time– of the ligand in the structure cal-
336 culation. In the present study, the receptor is perdeuterated (98%) in order to limit the spin
337 diffusion into the ligand only, *i.e.* not relayed by protons of the protein, but the remaining
338 2% of protons in the receptor may slightly impact also the intra-ligand dipolar restraints
339 observed by NMR. We also tried to be as cautious as possible to use specific intra-dipolar
340 interactions only in the structure calculations, but this does not exclude some imperfections
341 in the approach. For all these reasons, we cannot exclude that both flexible ends of 12-HHT
342 (carbons 1 to 4 and 13 to 17) may display slightly different orientations compared to the set of
343 structures described herein. It should be noted that in the recent published structure of the
344 prostaglandin E2 bound to EP3 receptor,⁸⁰ the ligand displays also a non-extended conforma-
345 tion in accordance with our results (Figure S11). In addition, docking simulations with the
346 set of conformers of free 12-HHT in solution (see Figure 2) could not reproduce the contacts
347 observed between 12-HHT BLT2-bound structures and S174 and R270 (Figure S12). How-

348 ever, to help us to improve the method, NOE peak volumes for both 12-HHT and LTB4 are
349 available to the community in [Tables S1 to S4](#) and [Tables S6 to S9](#), respectively. Ideally, the
350 experimental determination of a high-resolution structure of BLT2 receptor associated with
351 12-HHT would greatly help to adjust the approach detailed here. Other biophysical methods
352 like NMR chemical shift perturbation experiments with a specifically isotope-labeled BLT2
353 receptor, crosslinking and/or hydrogen/deuterium exchange associated with mass spectrom-
354 etry, and also molecular dynamics simulations could help to improve the model proposed in
355 the present study by determining some contact between the ligand and some amino acids of
356 the receptor. **These methods have been used in the GPCR field to delineate ligand:receptor**
357 **contacts⁸¹⁻⁸³ and probe the changes in receptor conformation induced by the interaction with**
358 **the ligands.⁸⁴** Overall, our data bring a first description of 12-HHT in its receptor-bound
359 state. This demonstrates the interest of a NMR-based approach to provide a description of
360 the structure and dynamics of natural ligands bound to unmodified receptors at physiological
361 temperatures, in complement to X-ray crystallography and cryoEM methods.

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AUTHOR CONTRIBUTIONS

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379 F.G. synthesized the perdeuterated amphipol; M.C., E.P., A.P. and L.J.C. performed the
380 production and purification of the receptor; M.D. and J.L.B performed single mutagenesis
381 experiments and ligand binding assays; F.G. and J.R. characterized the perdeuterated am-
382 phipol; C.L.B managed the NMR spectrometer and K.M. controlled structure calculations;
383 L.J.C. designed and supervised the project, performed the NMR sample preparations and
384 experiments; conducted structure calculations, molecular modeling and docking simulations,
385 and wrote the manuscript with editorial input from all authors.

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CONFLICT OF INTEREST

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390 The authors declare that they have no conflict of interest.

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References

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(1) Bockaert, J., & Pin, J.P. Molecular tinkering of G protein-coupled receptors: an evo-
393 lutionary success. *EMBO J.* **18**, 1723-1729 (1999).

394

(2) Santos, R., Ursu, O., Gaulton, A., Bento, A.P., Donadi, R.S., Bologa, C.G., Karlsson,
395 A., Al-Lazikani, B., Hersey, A., Oprea, T.I., & Overington J.P. A comprehensive map
396 of molecular drug targets. *Nat. Rev. Drug. Discov.* **16**, 19-34 (2017).

397

(3) Hauser, A.S., Attwood, M.M., Rask-Andersen, M., Schiöth, H.B., & Gloriam, D.E.
398 Trends in GPCR drug discovery : New agents, targets and indications. *Nat. Rev.*
399 *Drug. Discov.* **16**, 829-842 (2017).

- 400 (4) Rasmussen, S.G., Choi, H.J., Fung, J.J., Pardon, E., Casarosa, P., Chae, P.S., De-
401 vree, B.T., Rosenbaum, D.M., Thian, F.S., Kobilka, T.S., Schnapp, A., Konetzki, I.,
402 Sunahara, R.K., Gellman, S.H., Pautsch, A., Steyaert, J., Weis, W.I., & Kobilka, B.K.
403 Structure of a nanobody-stabilized active state of the $\beta(2)$ adrenoceptor. *Nature* **469**,
404 175-180 (2011).
- 405 (5) Ring, A.M., Manglik, A., Kruse, A.C., Enos, M.D., Weis, W.I., Garcia, K.C., &
406 Kobilka, B.K. Adrenaline-activated structure of 2-adrenoceptor stabilized by an engi-
407 neered nanobody. *Nature* **502**, 575-579 (2013).
- 408 (6) Manglik, A., Kobilka, B.K., & Steyaert, J. Nanobodies to Study G Protein-Coupled
409 Receptor Structure and Function. *Annu. Rev. Pharmacol. Toxicol.* **57**, 19-37 (2017).
- 410 (7) Weis, W.I., & Kobilka, B.K. The Molecular Basis of G Protein-Coupled Receptor
411 Activation. *Annu. Rev. Biochem.* **87**, 897-919 (2018).
- 412 (8) Erlandson, S.C., McMahon, C., & Kruse, A.C. Structural Basis for G Protein-Coupled
413 Receptor Signaling. *Annu. Rev. Biophys.* **47**, 1-18 (2018).
- 414 (9) Hilger, D., Masureel, M., & Kobilka, B.K. Structure and dynamics of GPCR signaling
415 complexes. *Nat. Struct. Mol. Biol.* **25**, 4-12 (2018).
- 416 (10) Thal, D.M., Vuckovic, Z., Draper-Joyce, C.J., Liang, Y.L., Glukhova, A., Christopou-
417 los, A., & Sexton, P.M. Recent advances in the determination of G protein-coupled
418 receptor structures. *Curr. Opin. Struct. Biol.* **51**, 28-34 (2018).
- 419 (11) Casiraghi, M., Damian, M., Lescop, E., Point, E., Moncoq, K., Morellet, N., Levy,
420 D., Marie, J., Guittet, E., Banères, J.L., & Catoire L.J. Functional Modulation of a
421 G Protein-Coupled Receptor Conformational Landscape in a Lipid Bilayer. *J. Am.*
422 *Chem. Soc.* **138**, 11170-11175 (2016).

- 423 (12) Casiraghi, M., Banères J.L., & Catoire L.J. NMR Spectroscopy for the Characteri-
424 zation of GPCR Energy Landscapes. *In: Topics in Medicinal Chemistry*. Springer,
425 Berlin, Heidelberg, pp 1-26 (2017).
- 426 (13) Casiraghi, M., Damian, M., Lescop, E., Banères J.L., & Catoire L.J. Illuminating
427 the Energy Landscape of GPCRs: The Key Contribution of Solution-State NMR
428 Associated with Escherichia coli as an Expression Host. *Biochemistry* **57**, 2297-2307
429 (2018).
- 430 (14) Casiraghi, M., Point, E., Pozza, A., Moncoq, K., Banères, J.L., & Catoire, L.J. NMR
431 analysis of GPCR conformational landscapes and dynamics. *Mol. Cell. Endocrinol.*
432 **484**, 69-77 (2019).
- 433 (15) Shimada, I., Ueda, T., Kofuku, Y., Eddy, M.T., & Wüthrich, K. GPCR drug discovery:
434 integrating solution NMR data with crystal and cryo-EM structures. *Nat. Rev. Drug.*
435 *Discov.* **18**, 59-82 (2019).
- 436 (16) Bostock, M.J., Solt, A.S., & Nietlispach, D. The role of NMR spectroscopy in mapping
437 the conformational landscape of GPCRs. *Curr. Opin. Struct. Biol.* **57**, 145-156 (2019).
- 438 (17) Inooka, H., Ohtaki, T., Kitahara, O., Ikegami, T., Endo, S., Kitada, C., Ogi, K.,
439 Onda, H., Fujino, M., & Shirakawa, M. Conformation of a peptide ligand bound to
440 its G-protein coupled receptor. *Nat. Struct. Biol.* **8**, 161-165 (2001).
- 441 (18) Catoire, L.J., Damian, M., Giusti, F., Martin, A., van Heijenoort, C., Popot, J.L.,
442 Guittet, E., & Banères, J.L. Structure of a GPCR ligand in its receptor-bound state:
443 leukotriene B4 adopts a highly constrained conformation when associated to human
444 BLT2. *J. Am. Chem. Soc.* **132**, 9049-9057 (2010).
- 445 (19) Catoire, L.J., Damian, M., Baaden, M., Guittet, E., & Banères J.L. Electrostatically-
446 driven fast association and perdeuteration allow detection of transferred cross-

- 447 relaxation for G protein-coupled receptor ligands with equilibrium dissociation con-
448 stants in the high-to-low nanomolar range. *J. Biomol. NMR.* **50**, 191-195 (2011).
- 449 (20) O'Connor, C., White, K.L., Doncescu, N., Didenko, T., Roth, B.L., Czaplicki, G.,
450 Stevens, R.C., Wüthrich, K., & Milon, A. NMR structure and dynamics of the agonist
451 dynorphin peptide bound to the human kappa opioid receptor. *Proc. Natl. Acad. Sci.*
452 *U. S. A.* **112**, 11852-11857 (2015).
- 453 (21) Yong, K.J., Vaid, T.M., Shilling, P.J., Wu, F.J., Williams, L.M., Deluigi, M.,
454 Plückthun, A., Bathgate, R.A.D., Gooley, P.R., & Scott, D.J. Determinants of Ligand
455 Subtype-Selectivity at 1A-Adrenoceptor Revealed Using Saturation Transfer Differ-
456 ence (STD) NMR. *ACS Chem. Biol.* **13**, 1090-1102 (2018).
- 457 (22) Brancaccio, D., Diana, D., Di Maro, S., Di Leva, F.S., Tomassi, S., Fattorusso,
458 R., Russo, L., Scala, S., Trotta, A.M., Portella, L., Novellino, E., Marinelli, L.,
459 & Carotenuto A. Ligand-Based NMR Study of C-X-C Chemokine Receptor Type
460 4 (CXCR4)-Ligand Interactions on Living Cancer Cells. *J. Med. Chem.* **61**, 2910-2923
461 (2018).
- 462 (23) Chen, S., Lu, M., Liu, D., Yang, L., Yi, C., Ma, L., Zhang, H., Liu, Q., Frimurer,
463 T.M., Wang, M.W., Schwartz, T.W., Stevens, R.C., Wu, B., Wüthrich, K., & Zhao
464 Q. Human substance P receptor binding mode of the antagonist drug aprepitant by
465 NMR and crystallography. *Nat. Commun.* **10**, 638 (2019).
- 466 (24) Bender, B.J., Vortmeier, G., Ernicke, S., Bosse, M., Kaiser, A., Els-Heindl, S., Krug,
467 U., Beck-Sickinger, A., Meiler, J., & Huster, D. Structural Model of Ghrelin Bound
468 to its G Protein-Coupled Receptor. *Structure* **27**, 537-544 (2019).
- 469 (25) Ferré, G., Louet, M., Saurel, O., Delort, B., Czaplicki, G., MKadmi, C., Damian, M.,
470 Renault, P., Cantel, S., Gavara, L., Demange, P., Marie, J., Fehrentz, J.A., Floquet,
471 N., Milon, A., & Banères, J.L. Structure and dynamics of GPCR-bound ghrelin reveal

- 472 the critical role of the octanoyl chain. *Proc. Natl. Acad. Sci. U. S. A.* **116**, 17525-17530
473 (2019).
- 474 (26) Yokomizo, T., Izumi, T., Chang, K., Takuwa, Y., & Shimizu, T. A G-protein-coupled
475 receptor for leukotriene B4 that mediates chemotaxis. *Nature* **387**, 620-624 (1997).
- 476 (27) Kamohara, M., Takasaki, J., Matsumoto, M., Saito, T., Ohishi, T., Ishii, H., & Fu-
477 ruichi, K. Molecular cloning and characterization of another leukotriene B4 receptor.
478 *J. Biol. Chem.* **275**, 27000-27004 (2000).
- 479 (28) Tryselius, Y., Nilsson, N.E., Kotarsky, K., Olde, B., & Owman, C. Cloning and char-
480 acterization of cDNA encoding a novel human leukotriene B(4) receptor. *Biochem.*
481 *Biophys. Res. Commun.* **274**, 377-382 (2000).
- 482 (29) Wang, S., Gustafson, E., Pang, L., Qiao, X., Behan, J., Maguire, M., Bayne, M.,
483 & Laz, T. A novel hepatointestinal leukotriene B4 receptor. Cloning and functional
484 characterization. *J. Biol. Chem.* **275**, 40686-40694 (2000).
- 485 (30) Yokomizo, T., Kato, K., Terawaki, K., Izumi, T., & Shimizu, T. A second leukotriene
486 B(4) receptor, BLT2. A new therapeutic target in inflammation and immunological
487 disorders. *J. Exp. Med.* **192**, 421-432 (2000).
- 488 (31) Izumi, T., Yokomizo, T., Obinata, H., Ogasawara, H., & Shimizu, T. Leukotriene
489 Receptors: Classification, Gene Expression, and Signal Transduction. *J. Biochem.*
490 **132**, 1-6 (2002).
- 491 (32) Tager, A.M., & Luster, A.D. BLT1 and BLT2: the leukotriene B4 receptors.
492 *Prostaglandins Leukot. Essent. Fatty Acids* **69**, 123-134 (2003).
- 493 (33) Yokomizo, T. Leukotriene B4 receptors: Novel roles in immunological regulations *Adv.*
494 *Enzyme Regul.* **51**, 59-64 (2011).

- 495 (34) Yokomizo, T. Two distinct leukotriene B4 receptors, BLT1 and BLT2. *J. Biochem.*
496 **157**, 65-71 (2015).
- 497 (35) Borgeat, P., & Samuelsson, B. Transformation of arachidonic acid by rabbit polymor-
498 phonuclear leukocytes. Formation of a novel dihydroxyeicosatetraenoic acid. *J. Biol.*
499 *Chem.* **254**, 2643-2646 (1979).
- 500 (36) Yokomizo, T., Kato, K., Hagiya, H., Izumi, T., & Shimizu, T. Hydroxyeicosanoids
501 bind to and activate the low affinity leukotriene B4 receptor, BLT2. *J. Biol. Chem.*
502 **276**, 12454-12459 (2001).
- 503 (37) Hamberg, M., Svensson, J., & Samuelsson, B. Prostaglandin endoperoxides. A new
504 concept concerning the mode of action and release of prostaglandins. *Proc. Natl. Acad.*
505 *Sci. U. S. A.* **71**, 3824-3828 (1974).
- 506 (38) Okuno, T., Iizuka, Y., Okazaki, H., Yokomizo, T., Taguchi, R., & Shimizu, T. 12(S)-
507 Hydroxyheptadeca-5Z, 8E, 10E-trienoic acid is a natural ligand for leukotriene B4
508 receptor 2. *J. Exp. Med.* **205**, 759-766 (2008).
- 509 (39) Hecker, M., Haurand, M., Ullrich, V., Diczfalusy, U., & Hammarström, S. Products,
510 kinetics, and substrate specificity of homogeneous thromboxane synthase from human
511 platelets: development of a novel enzyme assay. *Arch. Biochem. Biophys.* **254**, 124-135
512 (1987).
- 513 (40) Goetzl, E.J., & Gorman, R.R. Chemotactic and chemokinetic stimulation of hu-
514 man eosinophil and neutrophil polymorphonuclear leukocytes by 12-L-hydroxy-5,8,10-
515 heptadecatrienoic acid (HHT). *J. Immunol.* **120**, 526-531 (1978).
- 516 (41) Iizuka, Y., Okuno, T., Saeki, K., Uozaki, H., Okada, S., Misaka, T., Sato, T., Toh,
517 H., Fukayama, M., Takeda, N., Kita, Y., Shimizu, T., Nakamura, M., & Yokomizo, T.
518 Protective role of the leukotriene B4 receptor BLT2 in murine inflammatory colitis.
519 *FASEB J.* **24**, 4678-4690 (2010).

- 520 (42) Matsunaga, Y., Fukuyama, S., Okuno, T., Sasaki, F., Matsunobu, T., Asai, Y., Mat-
521 sumoto, K., Saeki, K., Oike, M., Sadamura, Y., Machida, K., Nakanishi, Y., Kubo,
522 M., Yokomizo, T., & Inoue, H. Leukotriene B4 receptor BLT2 negatively regulates
523 allergic airway eosinophilia. *FASEB J.* **27**, 3306-3314 (2013).
- 524 (43) Liu, M., Saeki, K., Matsunobu, T., Okuno, T., Koga, T., Sugimoto, Y., Yokoyama,
525 C., Nakamizo, S., Kabashima, K., Narumiya, S., Shimizu, T., & Yokomizo T. 12-
526 Hydroxyheptadecatrienoic acid promotes epidermal wound healing by accelerating
527 keratinocyte migration via the BLT2 receptor. *J. Exp. Med.* **211**, 1063-1078 (2014).
- 528 (44) Tong, W.G., Ding, X.Z., Hennig, R., Witt, R.C., Standop, J., Pour, P.M., & Adrian,
529 T.E. Leukotriene B4 receptor antagonist LY293111 inhibits proliferation and induces
530 apoptosis in human pancreatic cancer cells. *Clin. Cancer Res.* **8**, 3232-3242 (2002).
- 531 (45) Hennig, R., Osman, T., Esposito, I., Giese, N., Rao, S.M., Ding, X.Z., Tong, W.G.,
532 Büchler, M.W., Yokomizo, T., Friess, H., & Adrian, T.E. BLT2 is expressed in PanINs,
533 IPMNs, pancreatic cancer and stimulates tumour cell proliferation. *Br. J. Cancer* **99**,
534 1064-1073 (2008).
- 535 (46) Lee, J.W., & Kim, J.H. Activation of the leukotriene B4 receptor 2-reactive oxy-
536 gen species (BLT2-ROS) cascade following detachment confers anoikis resistance in
537 prostate cancer cells. *J. Biol. Chem.* **288**, 30054-30063 (2013).
- 538 (47) Houthuijzen, J.M., Daenen L.G., Roodhart J.M., Oosterom I., van Jaarsveld M.T.,
539 Govaert K.M., Smith, M., Sadatmand, S.J., Rosing, H., Kruse, F., Helms, B.J., van
540 Rooijen, N., Beijnen, J.H., Haribabu, B., van de Lest, C.H., & Voest, E.E. Lysophos-
541 pholipids secreted by splenic macrophages induce chemotherapy resistance via inter-
542 ference with the DNA damage response. *Nat. Commun.* **5**, 5275 (2014).
- 543 (48) van der Velden, D.L., Cirkel G.A., Houthuijzen J.M., van Werkhoven E., Roodhart
544 J.M.L., Daenen L.G.M., Kaing, S., Gerrits, J., Verhoeven-Duif, N.M., Grootsholten,

- 545 C., Boot, H., Sessa, C., Bloemendal, H.J., De Vos, F.Y., & Voest, E.E. Phase I study
546 of combined indomethacin and platinum-based chemotherapy to reduce platinum-
547 induced fatty acids. *Cancer Chemother. Pharmacol.* **81**, 911-921 (2018).
- 548 (49) Dahmane, T., Damian, M., Mary, S., Popot, J.-L., & Banères, J.-L. Amphipol-assisted
549 in vitro folding of G protein-coupled receptors. *Biochemistry* **48**, 6516-6521 (2009).
- 550 (50) Giusti, F., Rieger, J., Catoire, L.J., Qian, S., Calabrese, A.N., Watkinson, T.G.,
551 Casiraghi, M., Radford, S.E., Ashcroft, A.E., & Popot, J.L. Synthesis, characterization
552 and applications of a perdeuterated amphipol. *J. Membr. Biol.* **247**, 909-924 (2014).
- 553 (51) Arcemisbèhère, L., Sen, T., Boudier, L., Balestre, M.N., Gaibelet, G., Detouillon,
554 E., Orcel, H., Mendre, C., Rahmeh, R., Granier, S., Vivès, C., Fieschi, F., Damian,
555 M., Durroux, T., Banères, J.L., & Mouillac, B. Leukotriene BLT2 receptor monomers
556 activate G(i2) GTP-binding protein more efficiently than dimers. *J. Biol. Chem.* **285**,
557 6337-6347(2010) .
- 558 (52) Hwang, T.L., & Shaka, A.J. Water Suppression That Works. Excitation Sculpting
559 Using Arbitrary Wave-Forms and Pulsed-Field Gradients *J. Magn. Reson.* **A112**,
560 275-279 (1995).
- 561 (53) A. W. Schüttelkopf, A.W., & van Aalten, D.M.F. PRODRG: a tool for high-
562 throughput crystallography of protein-ligand complexes. *Acta Crystallogr.* **D60**, 1355-
563 1363 (2004).
- 564 (54) Kleywegt, G. J., & Jones, T.A. Model-building and refinement practice. *Methods En-*
565 *zymol.* **277**, 208-230 (1997).
- 566 (55) Rieping, W., Habeck, M., Bardiaux, B., Bernard, A., Malliavin, T.E., & Nilges, M.
567 ARIA2: automated NOE assignment and data integration in NMR structure calcula-
568 tion. *Bioinformatics* **23**, 381-382 (2007).

- 569 (56) Brünger, A.T., Adams, P.D., Clore, G.M., DeLano, W.L., Gros, P., Grosse-Kunstleve,
570 R.W., Jiang, J.S., Kuszewski, J., Nilges, M., Pannu, N.S., Read, R.J., Rice, L.M.,
571 Simonson, T., & Warren, G.L. Crystallography & NMR system: A new software
572 suite for macromolecular structure determination. *Acta Crystallogr., Sect. D: Biol.*
573 *Crystallogr.* **54**, 905-921 (1998).
- 574 (57) Bloembergen, N. On the interaction of nuclear spins in a crystalline lattice. *Physica*
575 **15**, 386-426 (1949).
- 576 (58) Linge, J. P., Habeck, M., Rieping, W., & Nilges, M. Correction of spin diffusion during
577 iterative automated NOE assignment. *J. Magn. Reson.* **167**, 334-342 (2004).
- 578 (59) Sali, A., & Blundell, T.L. Comparative protein modelling by satisfaction of spatial
579 restraints. *J. Mol. Biol.* **234**, 779-815 (1993).
- 580 (60) Fiser, A., Do, R.K., & Sali, A. Modeling of loops in protein structures. *Prot. Sci.* **9**,
581 1753-1773 (2000).
- 582 (61) Marti-Renom, M.A., Stuart, A., Fiser, A., Sánchez, R., Melo, F., & Sali, A. Compar-
583 ative protein structure modeling of genes and genomes. *Annu. Rev. Biophys. Biomol.*
584 *Struct.* **29**, 291-325 (2000).
- 585 (62) Hori, T, Okuno, T, Hirata, K, Yamashita, K, Kawano, Y, Yamamoto, M, Hato, M,
586 Nakamura, M, Shimizu, T, Yokomizo, T, Miyano, M, & Yokoyama, S. Na⁺-mimicking
587 ligands stabilize the inactive state of leukotriene B4 receptor BLT1. *Nat Chem Biol.*
588 **14**, 262-269 (2018).
- 589 (63) Zoonens, M., Catoire, L.J., Giusti, F., & Popot, J.L. NMR study of a membrane
590 protein in detergent-free aqueous solution. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 8893-
591 8898 (2005).

- 592 (64) Banères, J.-L., Popot, J.-L., & Mouillac, B. New advances in production and functional
593 folding of G-protein-coupled receptors. *Trends Biotechnol.* **29**, 314-322 (2011).
- 594 (65) Baneres, J.-L., Martin, A., Hullot, P., Girard, J.-P., Rossi, J.-C., & Parello, J.
595 Structure-based analysis of GPCR function: conformational adaptation of both ag-
596 onist and receptor upon leukotriene B4 binding to recombinant BLT1. *J. Mol. Biol.*
597 **329**, 801-814 (2003).
- 598 (66) Popot, J.-L., Althoff, T., Bagnard, D., Banres, J.-L., Bazzacco, P., Billon-Denis, E.,
599 Catoire, L.J., Champeil, P., Charvolin, D., Cocco, M.J., Crémel, G., Dahmane, T., de
600 la Maza, L.M., Ebel, C., Gabel, F., Giusti, F., Gohon, Y., Goormaghtigh, E., Guittet,
601 E., Kleinschmidt, J.H., Kühlbrandt, W., Le Bon, C., Martinez, K.L., Picard, M.,
602 Pucci, B., Sachs, J.N., Tribet, C., van Heijenoort, C., Wien, F., Zito, F., & Zoonens,
603 M. Amphipols from A to Z. *Annu. Rev. Biophys.* **40**, 379-408 (2011).
- 604 (67) Kumar, A., Ernst, R.R., & Wüthrich, K. A two-dimensional nuclear Overhauser en-
605 hancement (2D NOE) experiment for the elucidation of complete proton-proton cross-
606 relaxation networks in biological macromolecules. *Biochem. Biophys. Res. Commun.*
607 **95**, 1-6 (1980).
- 608 (68) Wassenaar, T.A., van Dijk, M., Loureiro-Ferreira, N., van der Schot, G., de Vries,
609 S.J., Schmitz, C., van der Zwan, J., Boelens, R., Giachetti, A., Ferella, L., Rosato,
610 A., Bertini, I., Herrmann, T., Jonker, H.R.A., Bagaria, A., Jaravine, V., Gntert, P.,
611 Schwalbe, H., Vranken, W.F., Doreleijers, J.F., Vriend, G., Vuister, G.W., Franke, D.,
612 Kikhney, A., Svergun, D.I., Fogh, R.H., Ionides, J., Laue, E.D., Spronk, C., Jurksa, S.,
613 Verlato, M., Badoer, S., Dal Pra, S., Mazzucato, M., Frizziero, E., & Bonvin, A.M.J.J.
614 WeNMR: Structural Biology on the Grid. *J. Grid. Comp.* **10**, 743-767 (2012).
- 615 (69) van Zundert, G.C.P., Rodrigues, J.P.G.L.M., Trellet, M., Schmitz, C., Kastiris, P.L.,
616 Karaca, E., Melquiond, A.S.J., van Dijk, M., de Vries, S.J., & Bonvin, A.M.J.J.

- 617 The HADDOCK2.2 webserver: User-friendly integrative modeling of biomolecular
618 complexes. *J. Mol. Biol.* **428**, 720-725 (2016).
- 619 (70) Ballesteros, J. A., & Weinstein, H. Integrated methods for the construction of three-
620 dimensional models and computational probing of structure-function relations in G
621 protein-coupled receptors. *Methods Neurosci.* **25**, 366-428 (1995).
- 622 (71) Basu, S., Jala, V.R., Mathis, S., Rajagopal, S.T., Del Prete, A., Maturu, P., Trent,
623 J.O., & Haribabu, B. Critical role for polar residues in coupling leukotriene B4 binding
624 to signal transduction in BLT1. *J. Biol. Chem.* **282**, 10005-10017 (2007).
- 625 (72) Sabirsh, A., Wetterholm, A., Bristulf, J., Leffler, H., Haeggström, J.Z., & Owman, C.
626 Fluorescent leukotriene B4: potential applications. *J. Lipid Res.* **46**, 1339-1346 (2005).
- 627 (73) Wheatley, M., Wootten, D., Conner, M.T., Simms, J., Kendrick, R., Logan, R.T.,
628 Poyner, D.R., & Barwell, J. Lifting the lid on GPCRs: the role of extracellular loops.
629 *Br. J. Pharmacol.* **165**, 1688-1703 (2012).
- 630 (74) Unal, H., & Karnik, S.S. Domain coupling in GPCRs: the engine for induced confor-
631 mational changes. *Trends Pharmacol. Sci.* **33**, 79-88 (2012).
- 632 (75) Peeters, M.C., van Westen, G.J.P., Li, Q., & IJzerman, A.P. Importance of the ex-
633 tracellular loops in G protein-coupled receptors for ligand recognition and receptor
634 activation. *Trends Pharmacol. Sci.* **32**, 35-42 (2011).
- 635 (76) Nguyen, A.T., Baltos, J.A., Thomas, T., Nguyen, T.D., Muñoz, L.L., Gregory, K.J.,
636 White, P.J., Sexton, P.M., Christopoulos, A., & May, L.T. Extracellular Loop 2 of the
637 Adenosine A1 Receptor Has a Key Role in Orthosteric Ligand Affinity and Agonist
638 Efficacy. *Mol. Pharmacol.* **90**, 703-714 (2016).
- 639 (77) Peeters, M.C., Wisse, L.E., Dinaj, A., Vroling, B., Vriend, G., & Ijzerman, A.P.

- 640 The role of the second and third extracellular loops of the adenosine A1 receptor in
641 activation and allosteric modulation. *Biochem. Pharmacol.* **84**, 76-87 (2012).
- 642 (78) Pantel, J., Legendre, M., Cabrol, S., Hilal, L., Hajaji, Y., Morisset, S., Nivot, S.,
643 Vie-Luton, M.P., Grouselle, D., de Kerdanet, M., Kadiri, A., Epelbaum, J., Le Bouc,
644 Y., & Amselem S. Loss of constitutive activity of the growth hormone secretagogue
645 receptor in familial short stature. *J. Clin. Invest.* **116**, 760-768 (2006).
- 646 (79) Gabdouliline, R.R., & Wade, R.C. Biomolecular diffusional association. *Curr. Opin.*
647 *Struct. Biol.* **12**, 204-213 (2002).
- 648 (80) Morimoto, K., Suno, R., Hotta, Y., Yamashita, K., Hirata, K., Yamamoto, M., Naru-
649 miya, S., Iwata, S., & Kobayashi, T. Crystal structure of the endogenous agonist-
650 bound prostanoid receptor EP3. *Nat. Chem. Biol.* **15**, 8-10 (2019).
- 651 (81) Coin, I., Katritch, V., Sun, T., Xiang, Z., Siu, F.Y., Beyermann, M., Stevens, R.C.,
652 and Wang, L. (2013) Genetically encoded chemical probes in cells reveal the binding
653 path of urocortin-I to CRF class B GPCR. *Cell* 155, 1258-69.
- 654 (82) Koole, C., Reynolds, C.A., Mobarec, J.C., Hick, C., Sexton, P.M., and Sakmar, T.P.
655 (2017) Genetically encoded photocross-linkers determine the biological binding site
656 of exendin-4 peptide in the N-terminal domain of the intact human glucagon-like
657 peptide-1 receptor (GLP-1R). *J. Biol. Chem.* 292, 7131-7144.
- 658 (83) Schmidt, P., Bender, B.J., Kaiser, A., Gulati, K., Scheidt, H.A., Hamm, H.E., Meiler,
659 J., Beck-Sickinger, A.G., and Huster, D. (2018). Improved in Vitro Folding of the Y(2)
660 G Protein-Coupled Receptor into Bicelles. *Front. Mol. Biosci.* 4, 100.
- 661 (84) West, G.M., Chien, E.Y., Katritch, V., Gatchalian, J., Chalmers, M.J., Stevens, R.C.,
662 and Griffin, P.R. (2011) Ligand-dependent perturbation of the conformational ensem-
663 ble for the GPCR 2 adrenergic receptor revealed by HDX. *Structure* 19, 1424-32.