Supplementary Table 1. The affinities of Nb80 and Nb71 for HDL-reconstituted β_2AR with different agonists measured by Octet Red.

	Agonist	K_{on} ($M^{-1}s^{-1}$)	K_{off} (s^{-1})	K_d (nM)
Nb80	Epinephrine	$7.2x10^5 \pm 0.1x10^5$	$1.6x10^{-3} \pm 0.1x10^{-3}$	2.2 ± 0.1 (n.s.)
	BI-167107	$4.0x10^5 \pm 0.1x10^5$	$1.3x10^{-3} \pm 0.1x10^{-3}$	3.2 ± 0.3 (n.s.)
	Salmeterol	$1.8x10^5 \pm 0.1x10^5$	$1.6x10^{-3} \pm 0.1x10^{-3}$	8.8 ± 0.7 (***)
Nb71	Epinephrine	$2.4x10^5 \pm 0.2x10^5$	$2.5x10^{-3} \pm 0.1x10^{-3}$	10 ± 1 (n.s.)
	BI-167107	$3.3x10^5 \pm 0.1x10^5$	$2.6x10^{-3} \pm 0.3x10^{-3}$	8.0 ± 0.9 (n.s.)
	Salmeterol	$2.4x10^5 \pm 0.5x10^5$	$2.1x10^{-3} \pm 0.1x10^{-3}$	8.8 ± 0.5 (n.s.)

The on- and off-rates (K_{on} and K_{off}) and the K_d values are listed as mean ± SEM from three independent experiments. Analysis of the binding affinities using Ordinary One-way ANOVA (Tukey) test at a multiplicity adjusted P value = 0.05 indicate that the difference in Nb80 affinities between epinephrine and BI-167107 and the differences in Nb71 affinities between epinephrine, BI-167107 and salmeterol bound b2ar are non-significant (n.s, P values > 0.3), while the difference in Nb80 affinities between epinephrine and salmeterol and BI-167107 and salmeterol are (***), at respective P values of 0.0001 and 0.0003.

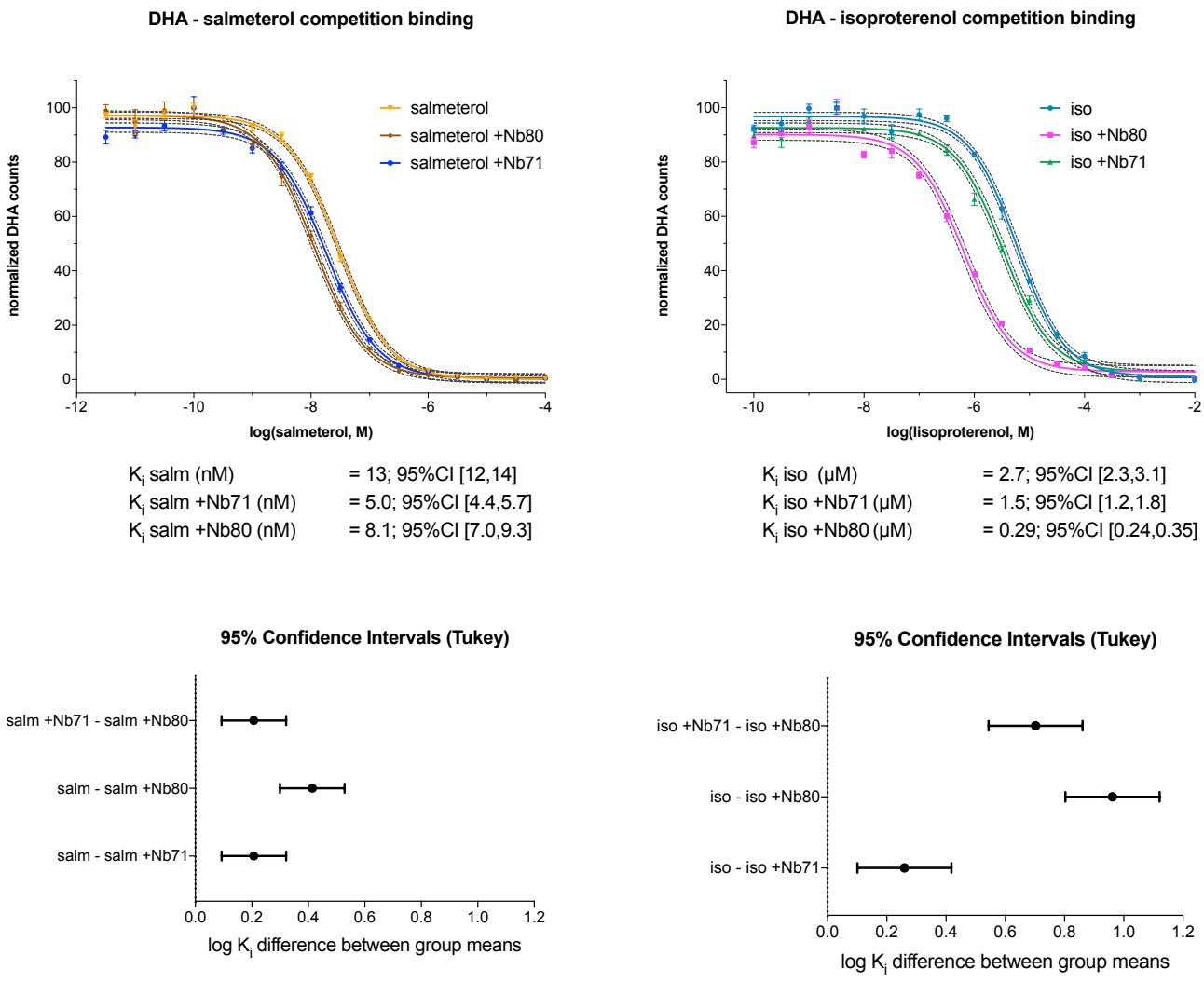
Supplementary Table 2: Data collection and refinement statistics (molecular replacement)

	β_2AR-Nb71-Salmeterol* (PDB code: 6CSY)
Data collection	
Space group	*C2*
Cell dimensions	
a, *b*, *c* (Å)	178.68, 52.59, 125.92
α,β,γ (°)	90, 123.86, 90
Resolution (Å)	28.52 – 2.96 (3.07 – 2.96)[a]
R_{merge} (%)[b]	16.7 (52.5)
$\langle I/\sigma I \rangle$	7.5 (1.5)
Completeness (%)	92.6 (75.1)
Redundancy	3.3 (2.0)
Refinement	
Resolution (Å)	28.52 – 2.96 (3.09 – 2.96)
No. reflections	18742 (1012)
R_{work} / R_{free} [c]	20.9 (29.8) / 24.6 (33.2)
Number of atoms	
Protein	4560
Ligand/ion	111
Water	20
Average B factors (Å^2)	
β_2AR	47.2
T4L	61.5
Nb71	50.6
Salmeterol	36.4
Water	36.3
R.m.s. deviations	
Bond lengths (Å)	0.007
Bond angles (°)	0.8

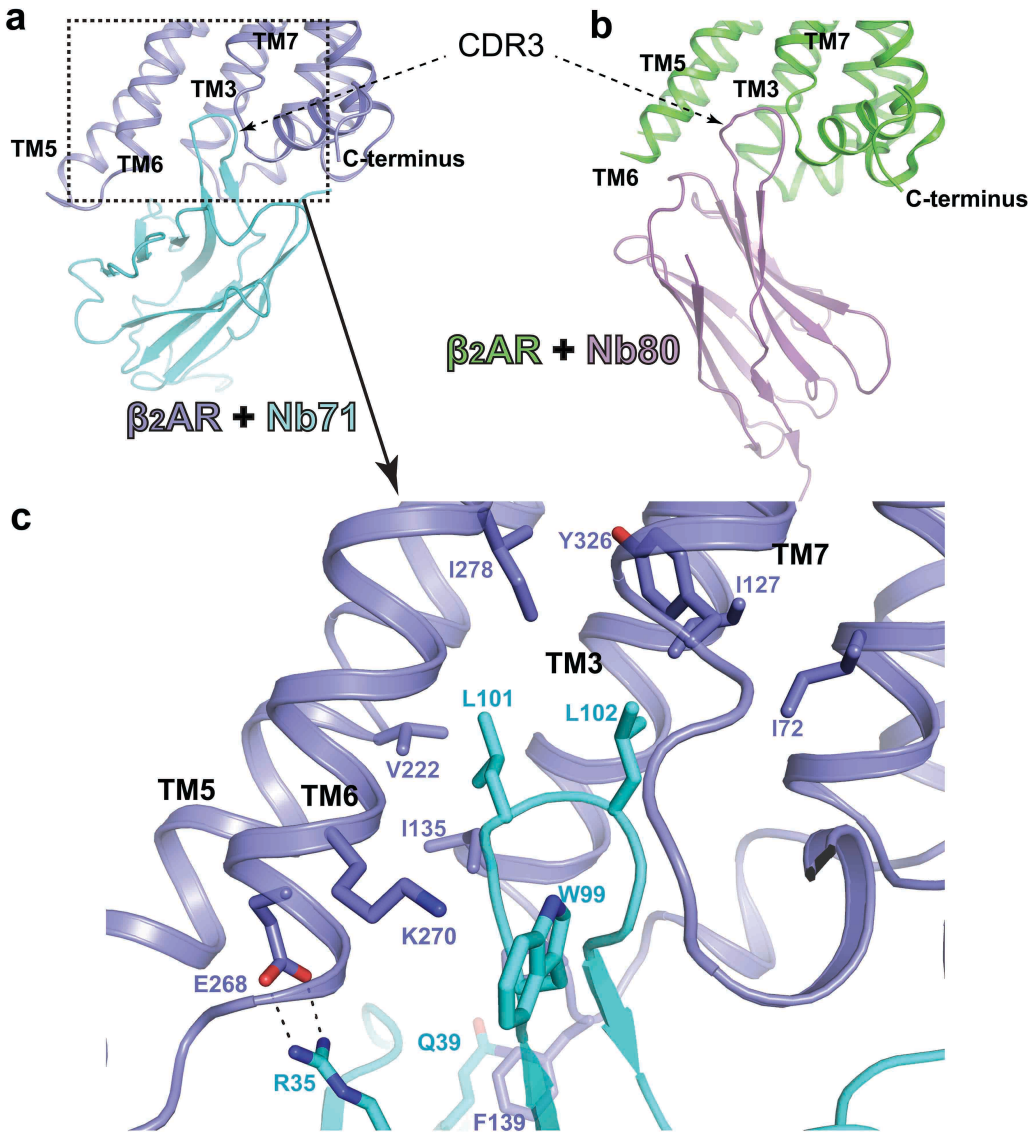
*X-ray diffraction data from 24 LCP crystals was merged to get the final complete data set.
[a]Values in parentheses are for highest-resolution shell.
[b] $R_{merge} = \sum|I_i - I_m|/\sum I_i$, where I_i is the intensity of the measured reflection and I_m is the mean intensity of all symmetry related reflections.
[c] $R_{cryst} = \Sigma||F_{obs}| - |F_{calc}||/\Sigma|F_{obs}|$, where F_{obs} and F_{calc} are observed and calculated structure factors.
$R_{free} = \Sigma_T||F_{obs}| - |F_{calc}||/\Sigma_T|F_{obs}|$, where T is a test data set of about 5% of the total reflections randomly chosen and set aside prior to refinement.

Supplementary Table 3. E_{max} and pEC50 values for BRET-based βarrestin-2 recruitment and Gs activation assays in cells from Figure 4. NR = not reported

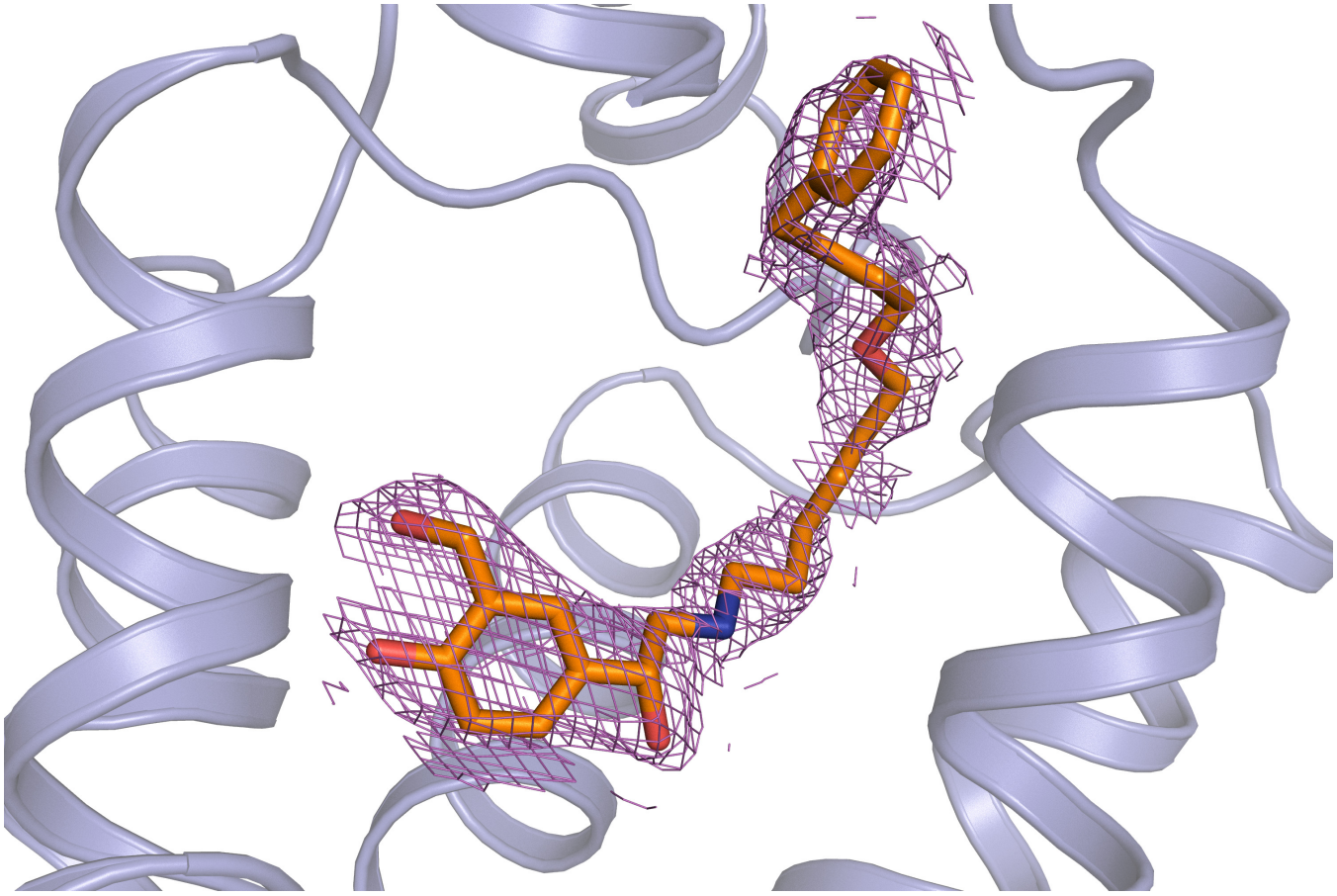
	βarr-2 recruitment (ISO)		βarr-2 recruitment (SALM)		Gs activation (ISO)		Gs activation (SALM)	
	Emax	**pEC50**	**Emax**	**pEC50**	**Emax**	**pEC50**	**Emax**	**pEC50**
WT	100 ± 1	-7.1 ± 0.1	NR	NR	99 ± 5	-6.8 ± 0.1	84 ± 7	-8.4 ± 0.3
N293Q	13 ± 1	-5.4 ± 0.1	NR	NR	80 ± 9	-5.6 ± 0.2	82 ± 6	-8.9 ± 0.3
N293D	11 ± 2	-5.4 ± 0.2	NR	NR	75 ± 7	-6.0 ± 0.2	85 ± 7	-8.4 ± 0.3
N293A	61 ± 1	-6.0 ± 0.1	17 ± 1	-8.3 ± 0.1	86 ± 6	-6.2 ± 0.2	91 ± 6	-8.7 ± 0.3
S204T	4 ± 2	-5.5 ± 0.8	NR	NR	76 ± 10	-5.5 ± 0.3	44 ± 7	-8.1 ± 0.6
S204A	40 ± 4	-5.2 ± 0.1	NR	NR	93 ± 8	-5.8 ± 0.2	71 ± 7	-8.3 ± 0.4



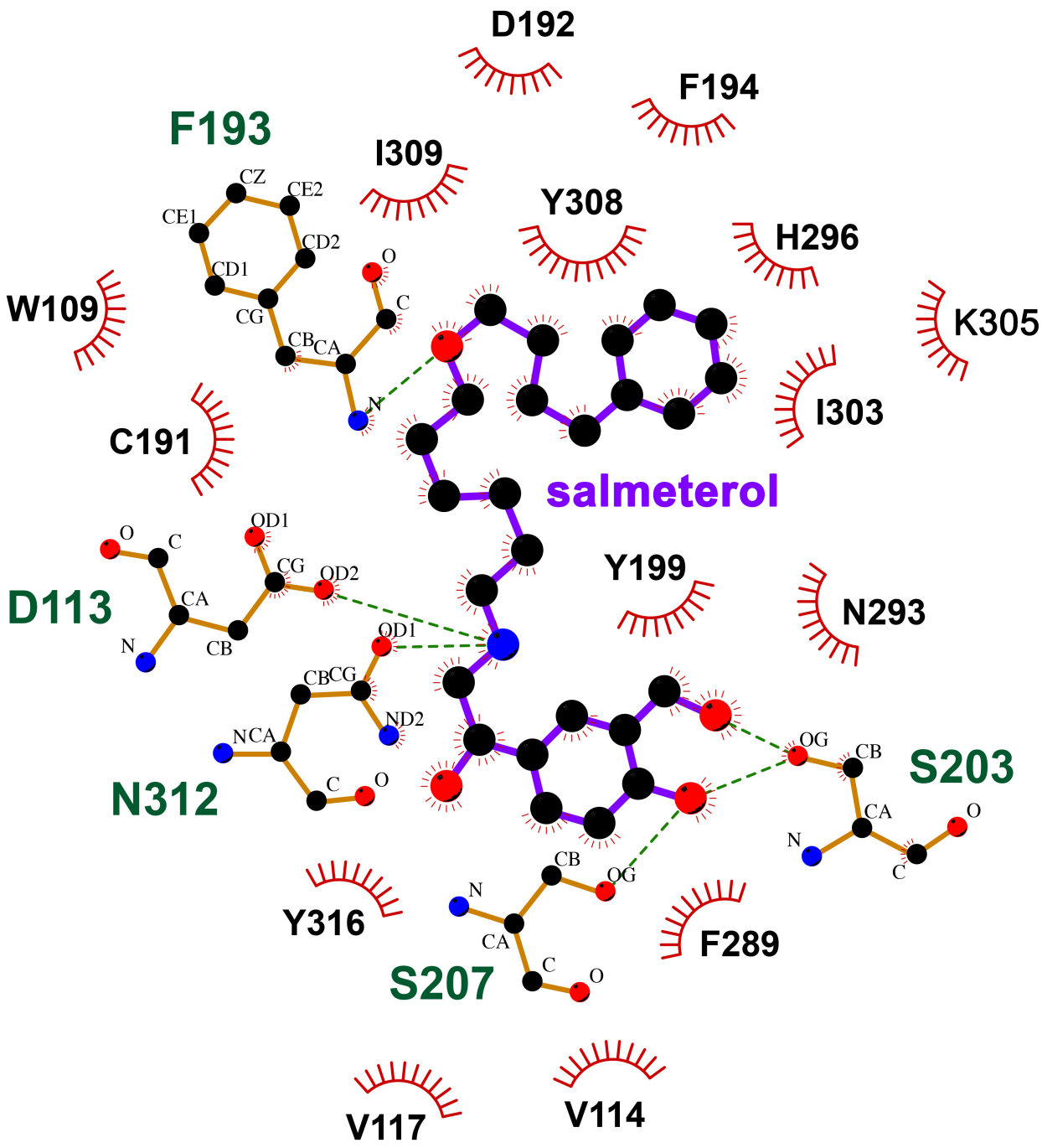
Supplementary Figure 1. Salmeterol and isoproterenol competition binding on purified β_2AR in detergent, in the presence of Nb71 and Nb80. Purified β_2AR (1nM) in detergent buffer was incubated for 1h at room temperature in the presence of 5nM [^{3}H]dihydroalprenolol and, when indicated, 2μM Nb71 or Nb80, in the presence of increasing concentrations of salmeterol (left panel) or isoproterenol (right panel). Data points for the competition binding curves are presented as mean values +/- SEM from three independent measurements. The data were analyzed in Prism (GraphPad) using "Nonlinear Regression – One site – Fit Ki" fitting, with the fitted curve shown in color and 95% confidence intervals plotted as dashed black lines for each fit (top plots). K_i values are reported as means with 95% confidence intervals in square brackets. To assess whether the difference between the calculated logKi values was statistically significant, a one-way ANOVA Tukey test was performed to compare the fitted mean logK_i values. As shown in the bottom plots, all differences are statistically significant at P values of 0.05 (adjusted for multiple comparisons), although the 95% confidence intervals clearly indicate that the logKi difference +/- Nb is larger and more significant for Nb80 than for Nb71.



Supplementary Figure 2. Different modes of Nb71 and Nb80 binding to the β_2AR. (a) β_2AR with Nb71. **(b)** β_2AR with Nb80. **(c)** Interactions between β_2AR and Nb71. Two residues L101 and L102 in the Nb71 CDR3 insert into a hydrophobic cavity at the cytoplasmic region of β_2AR to form extensive hydrophobic interactions with residues I278, Y326, I127, V222, I135 and I72. Additional salt bridge interaction between E268 in β_2AR and R35 in Nb71 and cation-π interaction between K270 in β_2AR and W99 in Nb71 also contribute to the binding of Nb71.

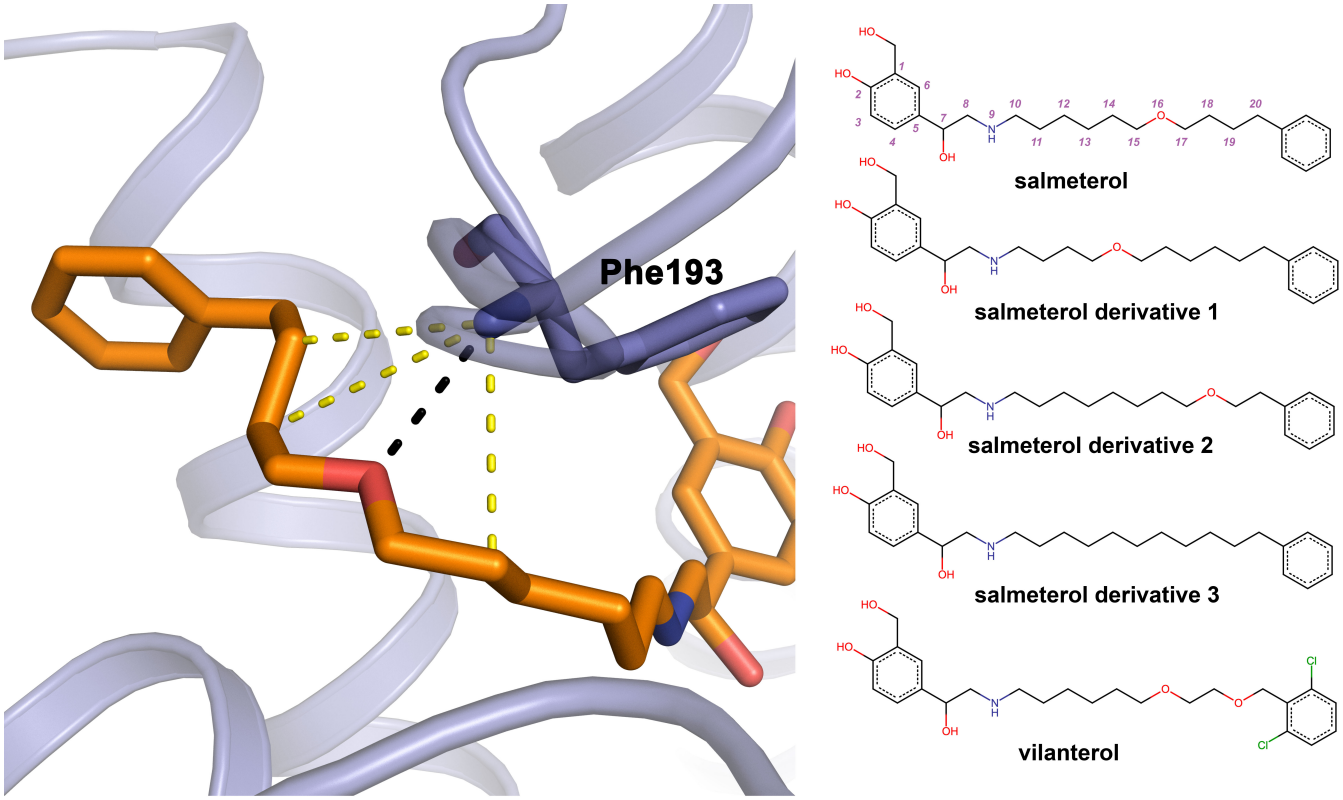


Supplementary Figure 3. Composite omit electron density map of salmeterol. β_2AR is colored blue. Salmeterol is colored orange. The $2F_o$-F_c composite omit electron density map of salmeterol is shown as purple mesh contoured at 1.2σ.



Supplementary Figure 4. Interactions between salmeterol and β_2AR. The 2-D representation figure was generated by LigPlot+[1]. The bonds in the salmeterol molecule are colored purple, while the bonds in the β_2AR residues are colored orange. Residues forming hydrogen-bonding interactions with the ligand and the hydrogen bonds are colored green. Residues and atoms involved in the hydrophobic interactions are colored black and marked with red half-circles.

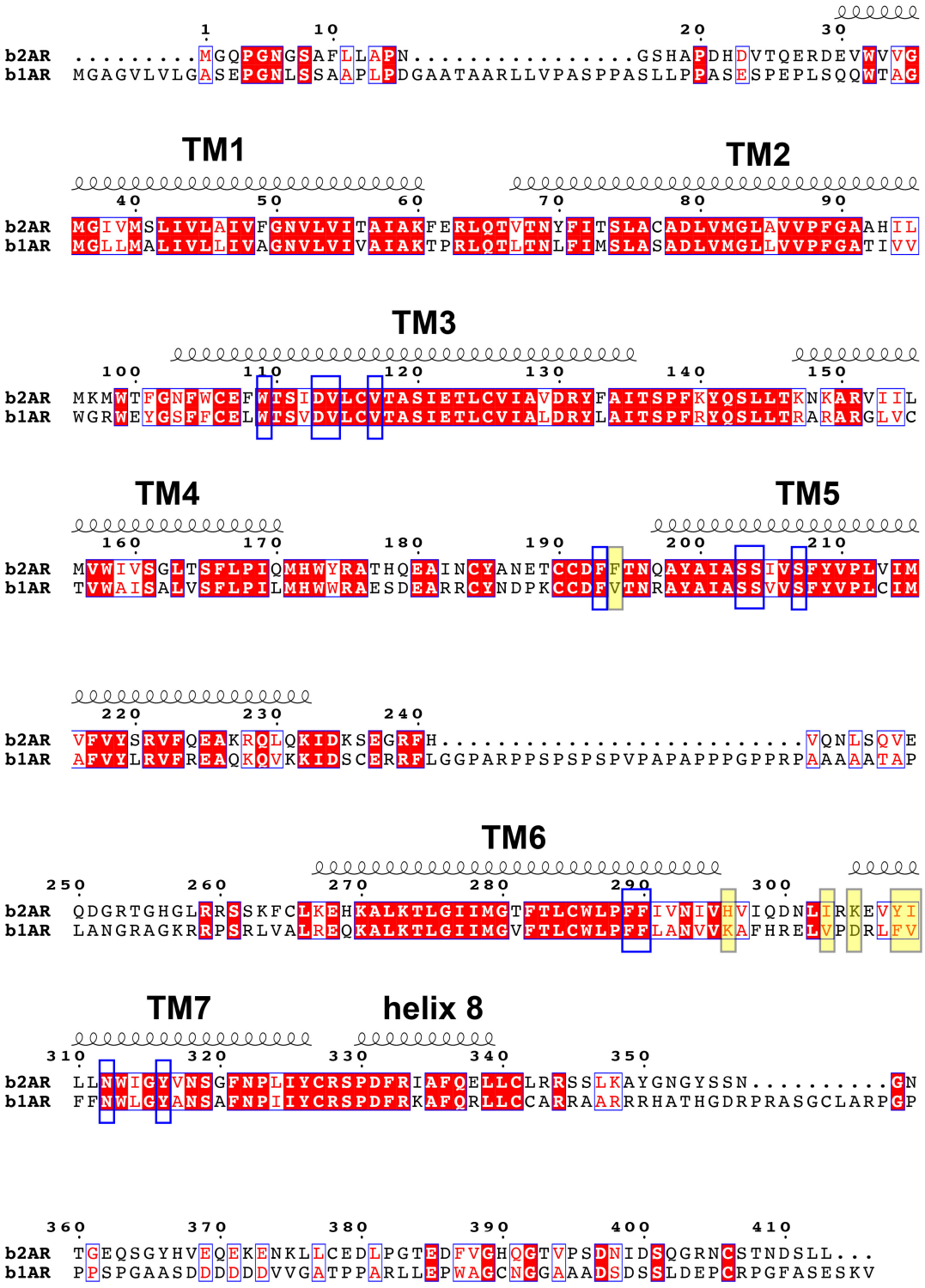
1. Laskowski, R. A. & Swindells, M. B. LigPlot+: multiple ligand-protein interaction diagrams for drug discovery. *J Chem Inf Model* **51**, 2778-2786, (2011).



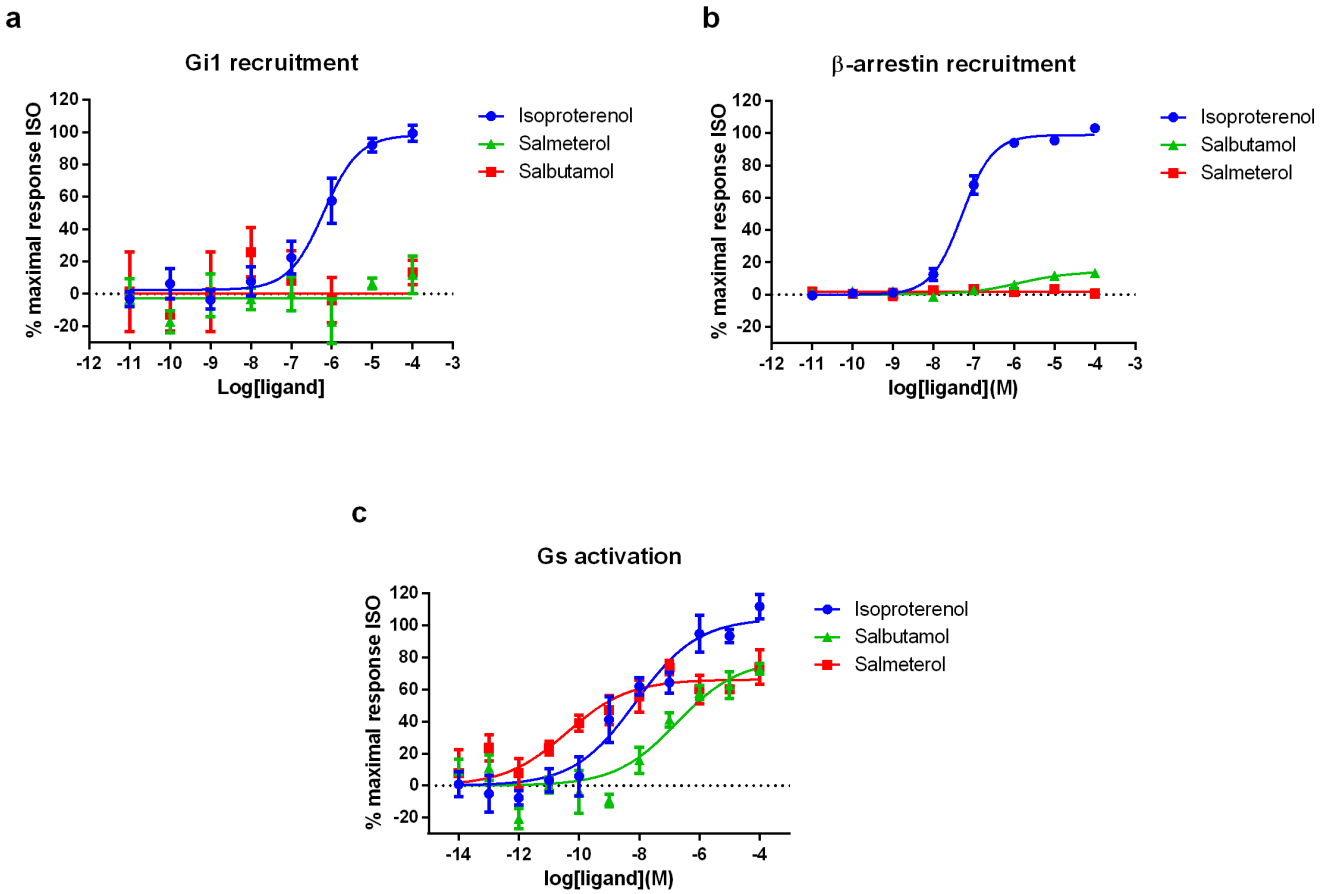
Supplementary Figure 5. Important role of the ether oxygen in salmeterol. The ether oxygen at position 16 in salmeterol forms a hydrogen bond with the main chain amine of residue Phe193 (black dashed line). Changing the position of the ether oxygen to positions 14 (salmeterol derivative 1) or position 18 (salmeterol derivative 2) greatly reduces the ligand affinities, and removing this oxygen (salmeterol derivative 3) reduces the affinity even further[2]. This is most likely due to the loss of the hydrogen bond, since the distance between the main chain amine of Phe193 and the atom at position 14 or 18 in salmeterol (represented by yellow dashed lines) is longer than 3.5Å. On the other hand, another long-lasting β_2AR agonist vilanterol, which shares a high structural similarity with salmeterol but has one more ether oxygen atom at position 19 that may form an additional hydrogen bond, exhibits higher affinity and longer *in vivo* duration of action compared to salmeterol[3].

2 Isogaya, M. *et al.* Identification of a key amino acid of the beta2-adrenergic receptor for high affinity binding of salmeterol. *Mol Pharmacol* **54**, 616-622 (1998).

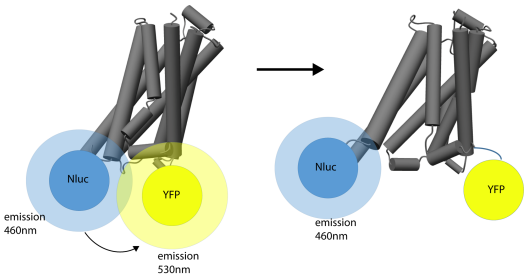
3 Slack, R. J. *et al.* In vitro pharmacological characterization of vilanterol, a novel long-acting beta2-adrenoceptor agonist with 24-hour duration of action. *J Pharmacol Exp Ther* **344**, 218-230 (2013).



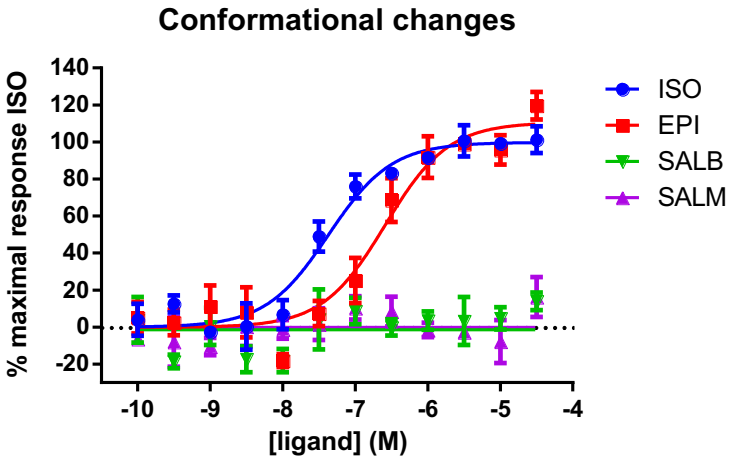
Supplementary Figure 6. Sequence alignment of human β_2AR (b2AR) and β_1AR (b1AR). The seven transmembrane helices in the human β_2AR-salmeterol structure are shown above the sequences. The residues of β_2AR that interact with the head group of salmeterol in the orthosteric-binding pocket are indicated by dark blue boxes, which are highly conserved in β_2AR and β_1AR. The yellow boxes highlight residues of β_2AR in the exosite that interact with the tail of salmeterol, which are far less conserved.



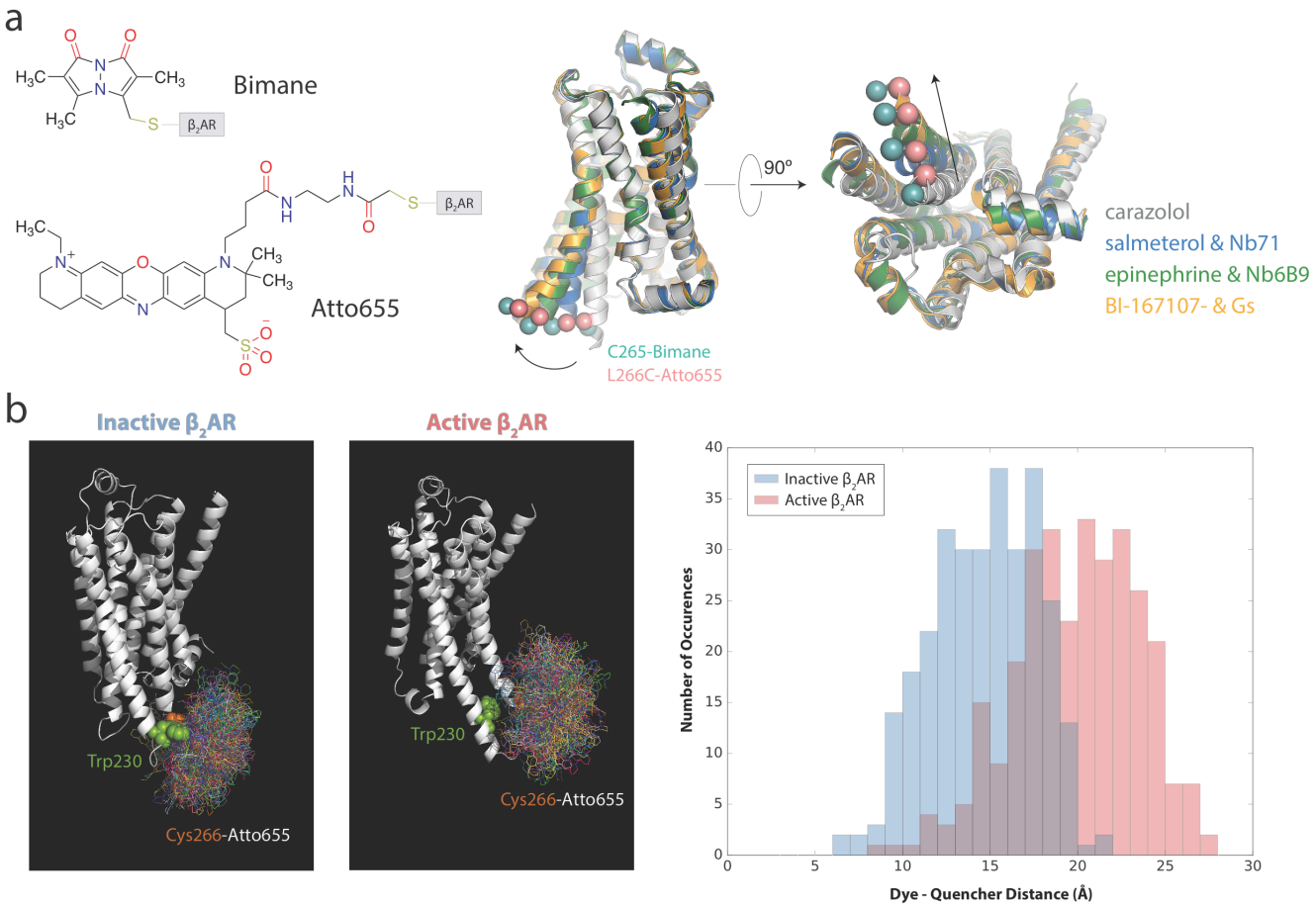
Supplementary Figure 7. (a) Dose-response curves of β_2AR full agonist isoproterenol (ISO) and partial agonsits salmeterol (SALM) and salbutamol (SALB) on cell-based BRET measurements between Gαi-91-RLuc and nic/myc- β_2AR-GFP10. Isoproterenol and salmeterol increased the interaction between Gαi-91-RLuc and nic/myc- β2-AR-GFP10 in a concentration-dependent manner (pEC50 for isoproterenol = -6.2 ± 0.2) Salbutamol and Salmeterol had no effect in this assay. Data are the mean ± SEM of 3 independent experiments performed in duplicates. **(b)** Dose-response curves of isoproterenol, salmeterol and salbutamol on cell-based BRET measurements between β-arrestin 2-RLucII and rGFP-CAAX. Isoproterenol and Salmeterol increased the recruitment of β-arrestin 2-RLucII to the rGFP-CAAX in a dose-dependent manner (pEC50 for isoproterenol= -7.3 ± 0.04; pEC50 for salbutamol= -5.9 ± 0.5, n=4). Data are the mean ± SEM of four experiments with repeats in duplicates. **(c)** Dose-response curves of isoproterenol, salbutamol and salmeterol on cell-based BRET measurements between Gαs-117-RLuc and Gγ1-GFP10. 5min stimulation with isoproterenol, salbutamol or salmeterol decreased the BRET signal between Gαs-117-RLucII and Gγ1-GFP10 in a concentration dependent manner (pEC50 for isoproterenol = -8.1 ± 0.3, pEC50 for salbutamol= -6.7 ± 0.5 and for salmeterol = -10 ± 0.4). Data are the mean ± SEM of 3 independent experiments performed in duplicates. Error bars shorter than the height of the symbol are not shown.



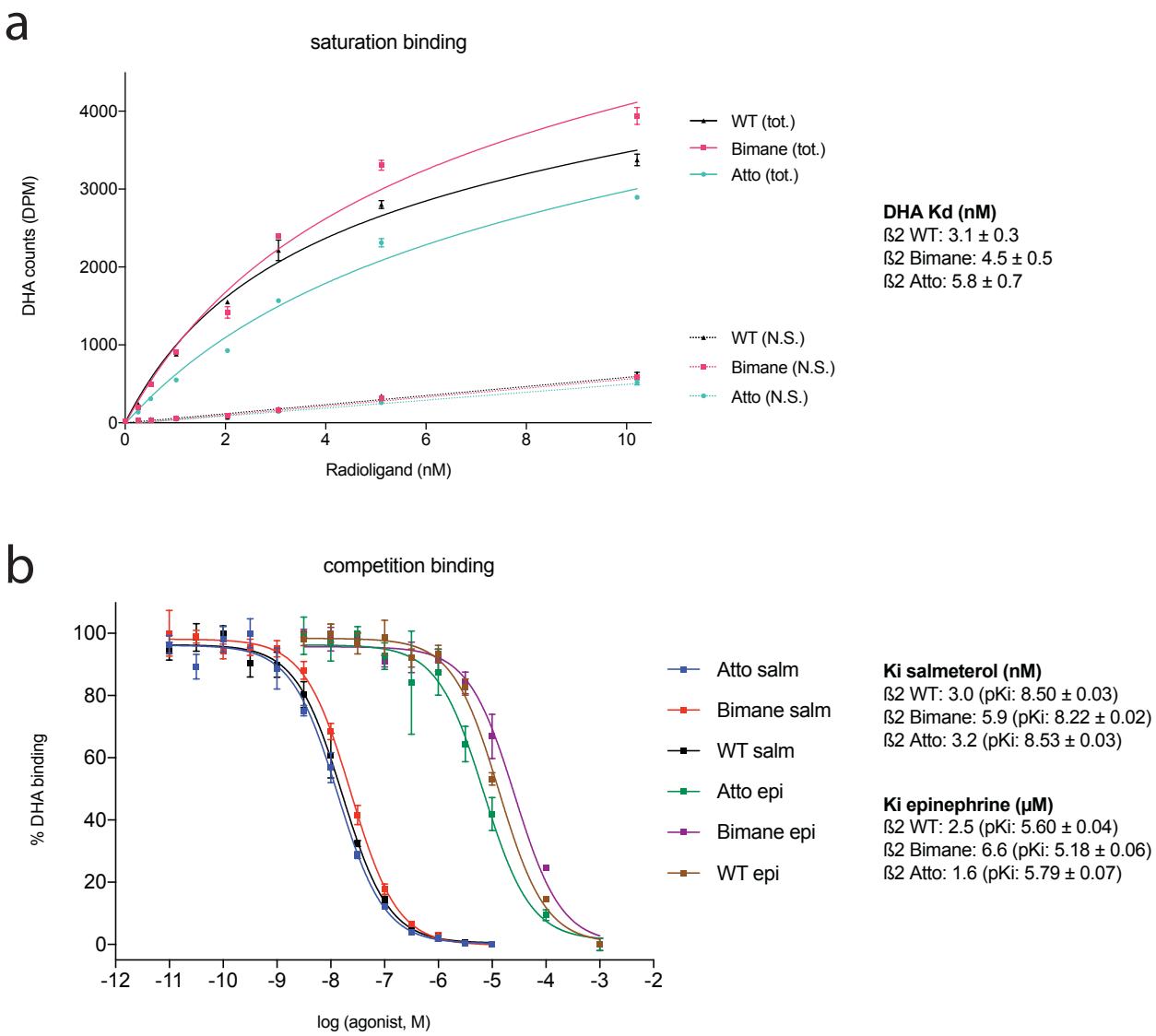
	eMAX	pEC50
ISO	100 ± 4	-7.4 ± 0.1
EPI	110 ± 6	-6.6 ± 0.1
SALB	NR	NR
SALM	NR	NR



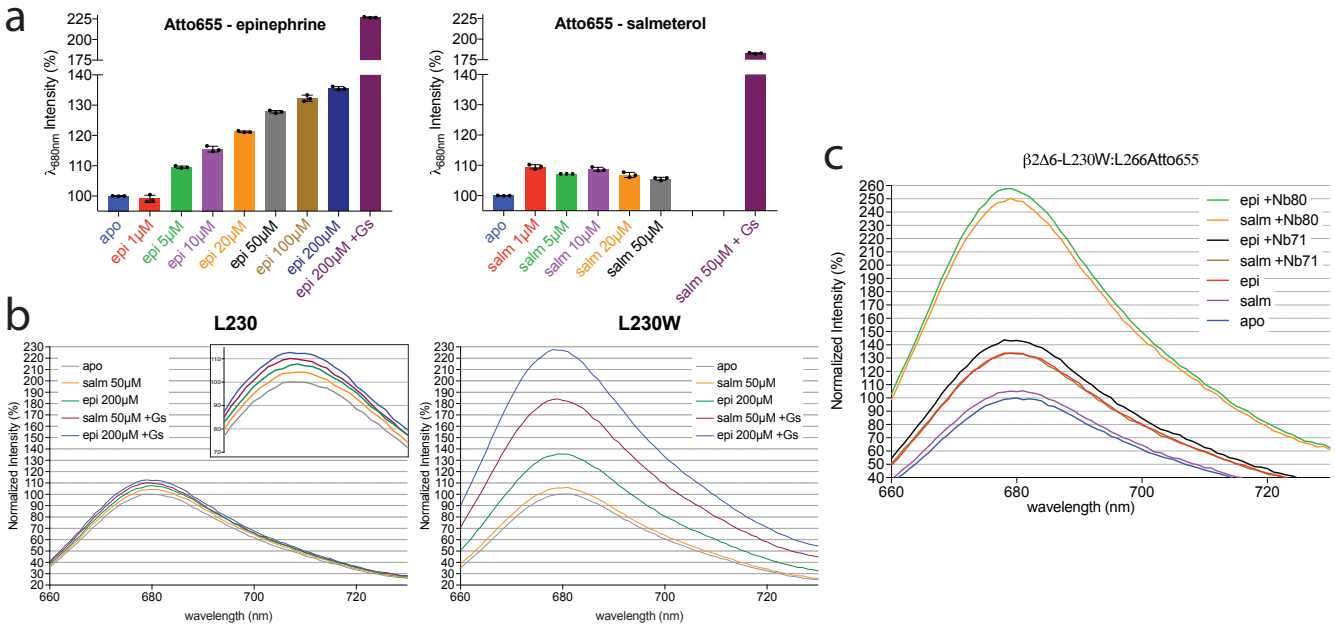
Supplementary Figure 8. The β_2AR conformational sensor in live cells shows distinct ligand responses. Full and unbiased agonists induce strong conformational changes on the β_2AR in live cells, whereas partial and biased agonists do not induce detectable conformation changes, suggesting that the 2 types of ligand stabilize different conformations of the receptor. Data represent the mean ± SEM of 3 independent experiments. NR = not reported



Supplementary Figure 9. Fluorescent reporters of β_2AR activation. (a) Chemical structures of the β_2AR-conjugated fluorophores used in spectroscopic studies of receptor activation (left panel), and overlay of carazolol-bound (grey), salmeterol-bound (blue), epinephrine-bound (green) and BI-167107-bound (yellow) β_2AR crystal structures (right panel). The side view and cytoplasmic view show the outward TM6 motion upon activation, as indicated by black arrows. The labeling sites for bimane and Atto655 are shown as spheres, colored green and pink, respectively. **(b)** Simulating the ensembles of Atto655 bound to Cys 266 in the inactive (antagonist-bound structure, PDB ID 2RH1) and active (agonist- and Gs-bound structure, PDB ID 3SN6) β2AR structures (left panel), yields an average Atto655 -Trp 230 (Dye-Quencher) distance change of about 5 Å upon receptor activation (right panel), compatible with the reported threshold quenching distance.



Supplementary Figure 10. Radioligand binding on purified wild-type, bimane-labeled and Atto655-labeled β_2AR in detergent. The engineered β_2AR constructs labeled with bimane (β_2 Bimane) or Atto655 (β_2 Atto) show similar ligand binding properties as the wild type β_2AR (β_2 WT). **(a)** [^{3}H]-dihydroalprenolol (DHA) saturation binding assays, total binding (tot.), non-specific binding (N.S.). Purified β_2AR (1nM) in detergent buffer was incubated for 1h at room temperature in the presence of increasing amounts of DHA. **(b)** Salmeterol and epinephrine competition binding assays. Purified β_2AR in detergent buffer (1nM) was incubated for 1h at room temperature in the presence of 5nM DHA and in the presence of increasing concentrations of salmeterol or epinephrine. Data points are presented as mean values ± SD from 3 independent measurements. The data was analyzed in Prism (GraphPad).



Supplementary Figure 11. a) Dose-response of the fluorescence of Atto655-labeled ß$_2$AR in detergent, in the presence of epinephrine and salmeterol. The intensity changes at 680nm for the emission spectra presented in **Fig. 5d** are calculated relative to the unliganded response (apo, 100%). The Atto-labeled β$_2$AR shows a dose-dependent increase in Atto655 fluorescence intensity in the presence of epinephrine, indicating a conformational change upon binding sufficient to partly unquench the Atto fluorescence. Little change is observed in the presence of increasing amounts of salmeterol, suggesting that the receptor conformational changes associated with salmeterol binding are not sufficient to substantially alter Atto quenching. Bar graphs represent the mean ± S.D. (error bars) of triplicate measurements (data points shown as black dots). **b)** Emission spectra of Atto655-labeled ß$_2$ARΔ6-L266C with and without the L230W mutation in detergent. The minor fluorescence intensity changes observed on Atto-labeled β$_2$AR with the native Leu230 compared to β$_2$AR with the L230W mutation indicate that the engineered tryptophan is responsible for the observed quenching/unquenching of Atto655 at residues L266C. The spectra are normalized to the respective unliganded data (apo, grey curve). **c)** Atto-β2AR response in the presence of Nb71 and Nb80. Fluorescence emission spectra of Atto655-labeled β2ARΔ6 L230W:L266C in detergent, in the presence of saturating concentrations of epinephrine or salmeterol, with and without Nb71 or Nb80. For both ligands the Atto response in the presence of Nb80 is much larger than in the presence of Nb71. Nb71 in the presence of epinephrine or salmeterol gives a response similar to epinephrine alone. These data indicate that when both nanobody and ligand are present, the intracellular receptor conformation is mainly imposed by the former and not the latter.