

SUPPORTING INFORMATION

Identification of V6.51L as a selectivity hotspot in stereoselective A₂ receptor antagonist recognition

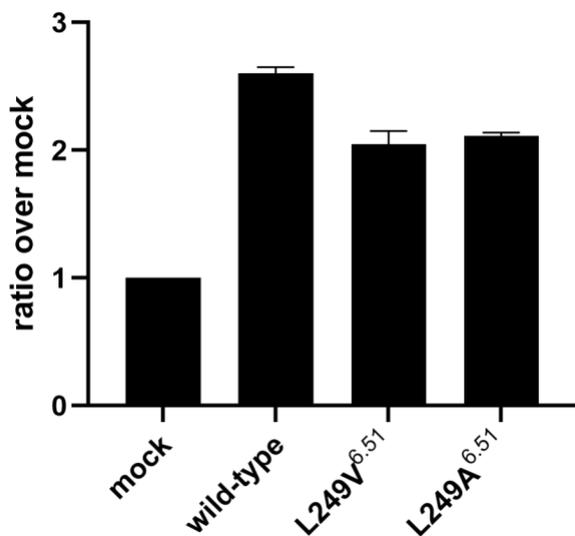
Xuesong Wang^{1*}, Willem Jespers^{1,2*}, Ruben Prieto-Díaz^{3,4*}, Maria Majellaro^{3,4}, Adriaan P.

IJzerman¹, Gerard J. P. van Westen¹, Eddy Sotelo^{3,4#}, Laura H. Heitman^{2,5#} and Hugo Gutiérrez-

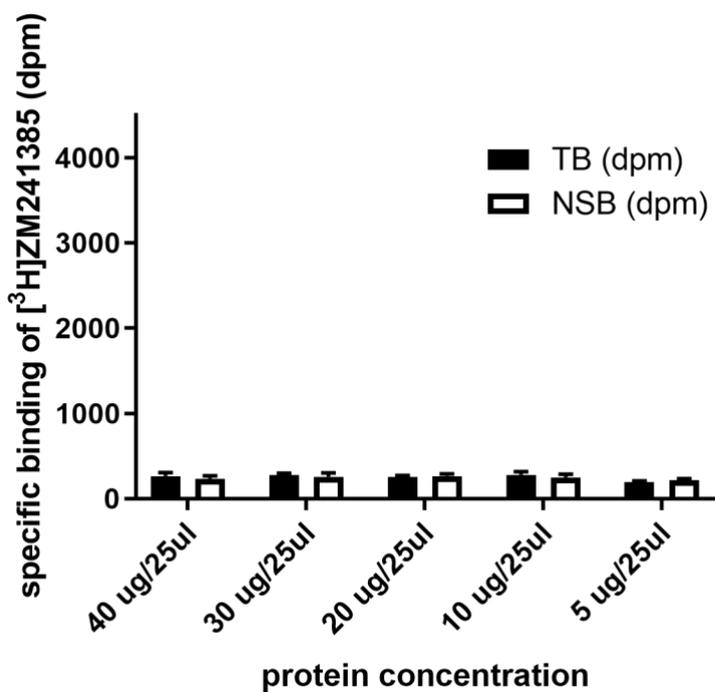
de-Terán^{2#}

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Supplementary Figure S1. Expression level of the transiently transfected WT A_{2A}AR, and L249V^{6.51} and L249A^{6.51} mutant A_{2A}AR at the surface of HEK293 cells. Data are shown as the mean ± SEM of three individual experiments performed in sextuplicate.



Supplementary Figure S2. Window check of HEK293 cell membrane transiently transfected by the L249^{6.51}A mutant A_{2A}AR in the presence of 1.7 nM [³H]ZM241385 in the absence (total binding; TB) and presence (non-specific binding; NSB) of NECA (100 μM). Data is shown as the mean ± SEM of three individual experiments performed in duplicate.

Supplementary Table S1. X-ray diffractometry experimental details of crystallographic (*R*)-ISAM-140 and (*S*)-ISAM-140.

Crystal data		(<i>R</i>)-ISAM140		(<i>S</i>)-ISAM140	
CCDC		1966312		1966450	
Chemical	formula	C ₁₉ H ₁₉ N ₃ O ₃		C ₁₉ H ₁₉ N ₃ O ₃	
M _r		337.37		337.37	
Crystal system		Monoclinic		Monoclinic	
Space group		C2		C2	
Temperature	(K)	100		100	
<i>a</i>	(Å)	16,4552	(9)	16.4553	(4)
<i>B</i>	(Å)	8.0613	(4)	8.0605	(2)
<i>c</i>	(Å)	13.5259	(7)	13.5260	(3)
α	(°)	90		90	
β	(°)	112.684	(3)	112.678	(1)
γ	(°)	90		90	
<i>V</i>	(Å ³)	1655.42	(16)	1655.35	(7)
<i>Z</i>		4		4	
Radiation	type	Cu-K α		Cu-K α	
μ	(mm ⁻¹)	0.76		0.76	
Crystal size	(mm)	0.12 × 0.11 × 0.10		0.11 × 0.01 × 0.03	
Tmin, Tmax		0.852, 0.929		-	
(sin θ/λ)	max (Å ⁻¹)	0.633		0.625	
Measured/Independent/ observed [I>2 σ (I)]	reflection	20568/3488/3266		17353/3370/3346	
Rint		0.068		0.076	
$R[F^2 > 2\sigma(F^2)], wR(F2), S$		0.043, 0.102, 1.10		0.025, 0.069, 1.01	
$\Delta\rho_{\max}/\Delta\rho_{\min}$	(eÅ ⁻³)	0.19, -0.25		0.16, -0.20	
Absolute structure (Flack)		-0.1(2)		-0.02 (4)	

Supplementary Table S2. Experimental and FEP calculated energies for the L6.51V mutation, with the value for each FEP leg in the thermodynamic cycle included. The $\Delta\Delta G$ values are plotted on Fig 5 on the main text. All $(\Delta)\Delta G$ values and expressed in Kcal·mol⁻¹. Standard error of the mean (sem) calculated from 10 replica simulations (FEP) or from the experimental data (see main text). $\Delta\Delta G_{\text{exp}} = -RT\ln(K_i^{\text{wt}}/K_i^{\text{mut}})$

	$\Delta\Delta G_{\text{exp}}$	$\Delta G_{\text{calc}} = \Delta G_{\text{apo}} - \Delta G_{\text{holo}}$		
		$\Delta\Delta G_{\text{calc}}$	ΔG_{holo}	ΔG_{apo}
ISAM-140(R)	-0.70±0.06	-0.39±0.36	-4.52±0.24	-4.91±0.27
ISAM-140(S)	-0.56±0.05	-0.50±0.35	-4.41±0.22	-4.91±0.28
ZM241385	0.57±0.11	0.20±0.42	-5.11±0.31	-4.91±0.29

Spectroscopic and analytical data for racemates and enantiomers isolated through chiral HPLC.

(±) Isopropyl 4-(furan-2-yl)-2-methyl-1,4-dihydrobenzo[4,5]imidazo[1,2-*a*]pyrimidine-3-carboxylate [(±) ISAM-140].¹ ¹H NMR (300 MHz, DMSO-*d*₆), δ (ppm): 10.78 (brs, 1H), 7.67–7.23 (m, 3H), 7.19–6.84 (m, 2H), 6.52 (s, 1H), 6.44 (d, *J* = 3.3 Hz, 1H), 6.37–6.23 (m, 1H), 4.86 (h, *J* = 6.3 Hz, 1H), 2.44 (s, 3H), 1.21 (d, *J* = 6.2 Hz, 3H), 1.05 (d, *J* = 6.1 Hz, 3H). ¹³C NMR (75 MHz, DMSO-*d*₆), δ (ppm): 165.0, 153.3, 148.0, 146.0, 143.0, 142.6, 132.0, 122.3, 120.7, 117.2, 110.8, 110.2, 108.2, 94.9, 67.0, 49.7, 22.3, 22.0, 19.1. HRMS (ESI) *m/z*: calcd for C₁₉H₂₀N₃O₃ [M + H]⁺: 338.1488; found: 338.7927.

Isopropyl (R)-4-(furan-2-yl)-2-methyl-1,4-dihydrobenzo[4,5]imidazo[1,2-a]pyrimidine-3-carboxylate [(R)-ISAM-140]. ¹H NMR (300 MHz, DMSO-*d*₆), δ (ppm): 10.76 (s, 1H), 7.41 (d, *J* = 11.0 Hz, 2H), 7.34 (d, *J* = 7.7 Hz, 1H), 7.04 (dt, *J* = 18.2, 7.0 Hz, 2H), 6.52 (s, 1H), 6.44 (d, *J* = 3.3 Hz, 1H), 6.35 – 6.27 (m, 1H), 4.87 (p, *J* = 6.3 Hz, 1H), 2.44 (s, 3H), 1.21 (d, *J* = 6.3 Hz, 3H), 1.05 (d, *J* = 6.4 Hz, 3H). ¹³C NMR (75 MHz, DMSO-*d*₆), δ (ppm): 165.0, 153.3, 148.0, 146.0, 143.1, 142.6, 132.0, 122.3, 121.0, 117.2, 110.8, 110.2, 108.7, 95.0, 67.0, 49.7, 22.3, 22.1, 19.1. HRMS (APCI) *m/z* calcd for C₁₉H₁₉N₃O₃ [M+H]⁺: 338.1499; found: 338.1501.

Isopropyl (R)-4-(furan-2-yl)-2-methyl-1,4-dihydrobenzo[4,5]imidazo[1,2-a]pyrimidine-3-carboxylate [(S)-ISAM-140]. ¹H NMR (300 MHz, DMSO-*d*₆), δ (ppm): 10.78 (s, 1H), 7.42 (d, *J* = 10.9 Hz, 2H), 7.35 (d, *J* = 7.8 Hz, 1H), 7.04 (dt, *J* = 18.2, 7.0 Hz, 2H), 6.54 (s, 1H), 6.44 (d, *J* = 3.3 Hz, 1H), 6.40 – 6.29 (m, 1H), 4.87 (p, *J* = 6.3 Hz, 1H), 2.44 (s, 3H), 1.21 (d, *J* = 6.3 Hz, 3H), 1.05 (d, *J* = 6.4 Hz, 3H). ¹³C NMR (75 MHz, DMSO-*d*₆), δ (ppm): 165.0, 153.3, 148.0, 146.0, 143.0, 142.6, 132.0, 122.3, 120.7, 117.5, 110.8, 110.3, 108.4, 94.9, 67.0, 49.7, 22.3, 22.2, 19.0. HRMS (APCI) *m/z* calcd for C₁₉H₁₉N₃O₃ [M+H]⁺: 338.1499; found: 338.1501.

Enzyme-linked Immunosorbent assay (ELISA). The experiment was performed as described previously.² Briefly, 24 hours after transfection, cells were split into a 96-well poly-D- lysine-coated plates at a density of 10⁶ cells per well. After an additional 24 h, the cells were fixed with 4% formaldehyde and blocked with 2% bovine serum albumin (BSA) (Sigma-Aldrich Chemie N.V., Zwijndrecht, The Netherlands) in Tris-buffered saline (TBS). Then, the cells

were incubated with monoclonal M1-anti-FLAG antibody (1:2250) (Sigma-Aldrich Chemie N.V. Zwijndrecht, The Netherlands) in Tris-buffered saline (TBS)/1 mM CaCl₂ for 2 hours at room temperature (RT). Next, the antibody was removed and the cells were washed with TBS/1 mM CaCl₂ before adding the secondary antibody, monoclonal anti-Mouse-HRP (1:5000) (Jackson ImmunoResearch Europe Ltd., Cambridgeshire, UK) and incubating for 1 hour at RT. After removing the secondary antibody and washing the cells with TBS/1 mM CaCl₂, 3, 3',5,5'-tetramethyl-benzidine (TMB) was added and incubated for 5 minutes in the dark. The reaction was stopped with 1 M H₃PO₄, and absorbance was read at 450 nm using a Wallac EnVision 2104 Multilabel reader (PerkinElmer).

References

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