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Supplementary information

Positive allosteric mechanisms of adenosine A_1 receptor-mediated analgesia

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Supplementary Table 1. Stability characteristics of VCP171 and MIPS521.

Parameter	VCP171	MIPS521
Plasma Stability[a]		
% Compound remaining (rat plasma)	93	100
% Compound remaining (human plasma)	95	95
Stability in Liver Microsomes		
$t_{1/2}$ rat (min)	< 2[a]	11
$t_{1/2}$ human (min)	8	17
CL_{int} rat (µL/min/mg protein)	>866	159
CL_{int} human (µL/min/mg protein)	220	105

[a] Apparent degradation half-lives could not be calculated due to minimal compound loss over the incubation period.

[b] No measurable concentration of the parent compound was detected past the first time point (i.e. 2 min) in the rat metabolism incubation, hence, the clearance parameters could not be determined. Degradation half-life was considered to be < 2 min.

Supplementary Table 2. Binding affinity estimates for MIPS521 from [^{3}H]-DPCPX binding.

[a], negative logarithm of the equilibrium dissociation constant of the PAM for the unoccupied receptor.

Receptor	pK_b [a]	Log α[b]
A1R wild type	5.74 ± 0.14	1.41 ± 0.08
L242$^{6.43}$A L245$^{6.46}$A	5.50 ± 0.11	0.11 ± 0.50 *
S246$^{6.47}$A	5.12 ± 0.11 *	0.53 ± 0.17 *
F275$^{7.40}$V	5.14 ± 0.12 *	1.24 ± 0.11
L276$^{7.41}$A	5.20 ± 0.05 *	1.71 ± 0.10
G279$^{7.44}$A	4.88 ± 0.07 *	0.33 ± 0.09 *
G279$^{7.44}$T	4.99 ± 0.06 *	0.02 ± 0.27 *
N280$^{7.45}$A	4.73 ± 0.06 *	1.13 ± 0.06
M283$^{7.48}$V	5.21 ± 0.11 *	1.34 ± 0.25

[b], Logarithm of allosteric modulator equilibrium dissociation constant.

*, pK_B or Log α significantly different ($p < 0.05$) from wild type as determined by one-way ANOVA with Dunnett's post hoc test.

Supplementary Table 3. [^{3}H]DPCPX and NECA equilibrium binding parameter estimates

Receptor	pK_d [a]	Bmax[b]	pK_i[c]
A1R wild type	8. 38 ± 0.35	2255 ± 388	5.70 ± 0.13
L242$^{6.43}$A L245$^{6.46}$A	8.58 ± 0.54	1033 ± 229	6.89 ± 0.14 *
S246$^{6.47}$A	8.02 ± 0.27	2293 ± 352	6.18 ± 0.12 *
F275$^{7.40}$V	8.57 ± 0.35	2820 ± 386	5.43 ± 0.11
L276$^{7.41}$A	8.64 ± 0.34	1630 ± 227	5.44 ± 0.07
G279$^{7.44}$A	8.59 ± 0.30	1963 ± 228	5.03 ± 0.15 *
G279$^{7.44}$T	8.44 ± 0.45	2063 ± 376	5.93 ± 0.10
N280$^{7.45}$A	8.33 ± 0.57	2839 ± 817	6.30 ± 0.06 *
M283$^{7.48}$V	8.53 ± 0.46	1566 ± 376	6.07 ± 0.05

Data represent the mean ± S.E.M of three or more independent experiments performed in duplicate.

[a], negative logarithm of the equilibrium dissociation constant of [^{3}H]DPCPX

[b], Receptor density expressed as total radioligand counts (CPM).

[c], negative logarithm of the equilibrium dissociation constant of NECA

*, pK_B or Log α significantly different ($p < 0.05$) from wild type as determined by one-way ANOVA with Dunnett's post hoc test.

Supplementary Table 4. Summary of GaMD simulations performed on the ADO-bound A1R, A1R-MIPS521, A1R-G_{i2} and A1R-Gi2-MIPS521 complexes. Since all systems contained ADO, it was dropped off from the system names for simplicity.

System	A1R	A1R-MIPS521	A1R-G_{i2}	A1R-G_{i2}-MIPS521
Dimension ($Å^3$)	87x87x102	87x87x102	106x124x148	106x124x148
N_{atoms}	60,386	60,425	177,316	176,790
Simulation Length (ns)	1000 x 3	1000 x 3	500 x 3	500 x 3
Boost Potential (kcal/mol)	16.85 ± 5.06	16.31 ± 4.99	18.95 ± 6.36	20.31 ± 6.39

Supplementary Table 5. Effects of MIPS521 and Gi2 on the kinetics of [3H]DPCPX dissociation estimates

	Control	Control + 30 µM MIPS521	G_{i2}	G_{i2} + 30 µM MIPS521
k_{off} (min^{-1})	0.414 ± 0.016	0.411 ± 0.065	0.128 ± 0.013[a]	0.051 ± 0.006[a,b]
$t_{1/2}$ (min)	1.7 ± 0.1	1.8 ± 0.3	5.7 ± 0.6	14.0 ± 1.6

[^{3}H]DPCPX dissociation was initiated by the addition of 10 µM SLV320. Data represent the mean ± S.E.M of three or more independent experiments performed in duplicate. $t_{1/2}$ is the half-life of dissociation. Statistical testing was done by one-way ANOVA with Tukey's multiple comparison test on the logarithm of the K_{off} values as the values are log normally distributed.

[a] Significantly different from control (p value < 0.05, by one-way ANOVA with a Tukey's multiple comparison test)

[b], Significantly different from G_{i2} (p value < 0.05, by one-way ANOVA with a Tukey's multiple comparison test)

Supplementary Table 6. Primer sequences used to generate mutations

Mutation	**Forward primer 5'-3'**
L242A + L245A	GCAGCCAGCTGGCGGCAAAGGCGAAGAGGATGAG
S246A	GTGCAAAGGCAGCCAG
F275V	GCCGTGCGTGAGGACGATGGCAATGTAG
L276A	CGAGTTGCCGTGCGTGGCGAAGATGGCAATGTAG
G279A	CATGGCCGAGTTGGCGTGCGTGAG
G279T	GTTCATGGCCGAGTTGGTGTGCGTGAGGAAGATG
N280A	GTACTCCATGCTGATGACCGCCTCGAACTCGCACTTGATC
M283V	GACAATGGGGTTCACGGCCGAGTTGCCG