Comparison of orexin 1 and orexin 2 ligand binding modes using X-ray crystallography and computational analysis

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Table 1: Sources of compounds used in X-ray crystallographic studies.

Compound	Source
Suvorexant	Synthesized according to reported procedures.[1]
Lemborexant	Purchased: MedChem Express, Catalogue Number HY-16725; ChemShuttle, Catalogue Number 157318. CAS # 1369764-02-2.
Filorexant	Purchased: eNovation Chemicals LLC, Catalogue Number D480467. CAS # 1088991-73-4.
GSK1059865	Purchased: Shanghai Haoyuan Chemexpress Co., Ltd., Catalogue Number HY-101534. CAS # 1191044-58-2.
EMPA	Synthesized according to reported procedures.[2]
Nemorexant	Purchased: Shanghai Haoyuan Chemexpress Co., Ltd., Catalogue Number HY-109095. CAS # 1505484-82-1.
HTL6641	Synthesized according to reported procedures.[3]

Compound **14**	Synthesized by analogy to reported procedures.[3,4]
ACT-462206	Purchased: Tocris Bioscience, Catalogue Number 5319. CAS # 1361321-96-1.
Compound **16**	Synthesized according to reported procedures.[5]
SB-334867	Purchased: Tocris Bioscience, Catalogue Number 1960. CAS # 792173-99-0.
SB-408124	Purchased: Tocris Bioscience, Catalogue Number 1963. CAS # 288150-92-5.

Cloning, Baculovirus generation, large-scale infection of Sf21 cells and membrane preparation. Human wild-type OX_1 or OX_2 receptors were cloned into the Bac-to-Bac Baculovirus Expression System (ThermoFisher Scientific, UK). P0 baculovirus was generated by transfecting Sf9 cells with bacmid DNA using Cellfectin^{TM} II transfection reagent (ThermoFisher Scientific, UK). Following P0 generation P1 virus was then generated ready for large scale infection and membrane preparation. Sf21 cells were grown in expression medium ESF921 (Expression Systems, USA) supplemented with 10% heat-inactivated FBS and 1% Pen/Strep and were infected at a cell density of $2.5x10^6$ cells/mL and a MOI of 1.0 for both Human OX_1 and OX_2 receptors. Expression was carried out at over 48 h in a shaking incubator set at 27 °C. The cell culture

was centrifuged at 2,500 rcf for 10 min at 4 °C. The pellets were resuspended in cold PBS supplemented with cOmplete™ EDTA-free protease inhibitor cocktail tablets (Sigma-Aldrich, UK), 1 mM PMSF and 1 mM EDTA. The resuspended cell paste was centrifuged was then centrifuged at 3,273 rcf for 12 min at 4 °C. The supernatant was discarded and the pellet frozen at -80 °C. The cell pellet from a 4 L culture was resuspended in buffer containing 50 mM Hepes pH 7.5, 150 mM NaCl, 8 cOmplete™ EDTA-free protease inhibitor cocktail tablets and 1 mM PMSF. The suspension was left stirring at rt for 1 h and then homogenised for 90 s at 9,500 rpm using a VDI 25 (VWR, USA) homogeniser. The cells were lysed using a M-110L microfluidizer® processor (Microfluidics, USA). After lysis, the mixture was homogenised for 90 s at 9,500 rpm, and then centrifuged at 335 rcf for 10 min. The supernatant was further ultra-centrifuged at 335 rcf for 10 min, the supernatant was discarded and the pellet was resuspended in 50 mL (25 mL for each 2 L culture) of buffer containing 50 mM Hepes pH 7.5, 150 mM NaCl, 3 cOmplete™ EDTA-free protease inhibitor cocktail tablets and 1 mM PMSF. The suspension was homogenised for 90 s at 9,500 rpm and the resulting membranes were stored at -80 °C.

Radioligand Binding Assay. Cell membranes from Sf21 Insect cells infected with P1 virus expressing either the wild-type human OX_1 or OX_2 receptor were incubated with [^{3}H]-Compound 23† in Krebs assay buffer (8.5 mM HEPES, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 118 mM NaCl, 4.7 mM KCl, 4 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 11 mM glucose, pH 7.4) in a total assay volume of 0.25 mL with a final DMSO concentration of 1%. After 90 min incubation at rt the reaction was terminated by rapid filtration through GF/B 96-well glass fibre plates with 5 x 0.25 mL washes H_2O using a Tomtec cell harvester. Bound radioactivity was determined through liquid scintillation using Lablogic SafeScint and detected on a microbeta liquid scintillation counter. Non-specific binding was determined as that remaining in the presence of a 10 μM saturating concentration of suvorexant. Saturation studies were carried out by incubating membranes (6.4 μg (OX_1) or 1.4 μg (OX_2) protein per well) with a range of concentrations of [^{3}H]-Compound 23 (0.6-30 nM). Radioligand concentrations were determined using SafeScint and a Beckman LS 6000 liquid scintillation counter. Competition binding was performed incubating membranes (6.4 μg (OX_1) or 1.4 μg (OX_2) protein per well) with K_D concentrations of [^{3}H]-Compound 23 and a range of concentrations of the test compound.

† 4-(2,6-Difluoro-4-methoxybenzyl)-2-(5,6-dimethoxypyridin-3-yl)-2*H*-1,2,4-benzothiadiazin-3(4*H*)-one 1,1-dioxide (Compound 23 in reference 3). [^{3}H]-Compound 23 was prepared by RC Tritec, Teufen, Switzerland by reaction of cold Compound 23 with Crabtree's catalyst in DCM at rt for 14 h in the presence of T_2 gas.

Table 2: X-ray data collection and refinement statistics.

	OX_1 suvorexant	**OX_1^{A127T} EMPA**	**OX_1 GSK1059865**	**OX_1 lemborexant**	**OX_1 filorexant**	**OX_1 daridorexant**	**OX_1 ACT-462206**
Data collection							
Number of crystals	4	2	2	1	2	2	3
Space group	I2	I2	$P2_1$	$P2_1$	$P2_1$	$P2_1$	$P2_1$
Cell dimensions							
a, *b*, *c* (Å)	57.90, 158.47 183.62	57.91, 158.89, 182.35	59.69, 146.19, 71.51	59.73, 145.71, 71.70	59.89, 146.20, 72.33	61.22, 146.42, 73.60	61.72, 146.67, 73.75
α, β, γ (°)	90, 95.31, 90	90, 95.77, 90	90, 112.43, 90	90, 112.25, 90	90, 111.12, 90	90, 109.55, 90	90, 108.29, 90
Resolution (Å)	44.39-2.29(2.54-2.29)[a]	48.57-2.13(2.35-2.13)[a]	44.04-2.16(2.29-2.16)[a]	33.18-2.22(2.49-2.22)[a]	34.03-2.34(2.61-2.34)[a]	69.36-3.03(3.39-3.03)[a]	34.63-3.01(3.42-3.01)[a]
R_{pim}	0.141(1.110)[a]	0.099(1.180)[a]	0.075(1.911)[a]	0.054(0.721)[a]	0.048(0.682)[a]	0.070(0.928)[a]	0.131(0.851)[a]
$I/\sigma(I)$	4.8(1.7)[a]	9.3(1.5)[a]	8.8(1.6)[a]	12.1(1.7)[a]	13.5(1.6)[a]	8.9(1.3)[a]	10.9(1.3)[a]
$CC_{1/2}$[b]	0.985(0.397)[a]	0.893(0.000)[a]	0.990(0.338)[a]	0.999(0.442)[a]	0.997(0.486)[a]	0.985(0.416)[a]	0.993(0.439)[a]
Completeness (%)							
spherical	60.7(8.2)[a]	63.4(11.9)[a]	74.6(18.2)[a]	64.0(11.2)[a]	60.0(10.1)[a]	58.6(9.3)[a]	55.0(8.1)[a]
ellipsoidal	92.9(52.4)[a]	93.3(65.5)[a]	87.2(38.9)[a]	91.4(60.7)[a]	89.4(59.0)[a]	90.0(61.5)[a]	89.3(60.1)[a]
Redundancy	5.4(5.7)[a]	3.3(3.6)[a]	3.3(3.9)[a]	3.8(3.8)[a]	5.2(5.3)[a]	5.8(5.4)[a]	7.9(7.8)[a]
Refinement							
Resolution (Å)	44.39-2.29	30.62-2.13	30.47-2.16	24.02-2.22	34.03-2.34	25.86-3.03	34.63-3.01

No. reflections	45066	57581	44881	35526	29373	13842	13576
R_{work} / R_{free}	0.185/0.210	0.189/0.208	0.194/0.218	0.207/0.239	0.211/0.244	0.205/0.230	0.226/0.264
No. atoms							
Protein	4828	4881	4902	4826	4851	4982	4771
Ligand	110	116	100	100	112	110	51
Other	873	1009	706	340	338	353	213
B factors							
Protein	42.22	43.53	55.95	69.61	76.81	121.00	85.72
Ligand	33.44	38.79	44.91	46.45	60.04	85.70	106.32
Other	68.80	72.76	89.37	88.70	91.17	140.50	103.54
R.m.s. deviations							
Bond lengths (Å)	0.010	0.010	0.010	0.010	0.002	0.010	0.005
Bond angles (°)	1.03	1.00	0.96	1.00	0.57	1.00	1.05

	OX_1 Compound 16	**OX_1 Compound 14**	**OX_1 SB-334867**	**OX_1 SB-408124**	**OX_2 suvorexant**	**OX_2 EMPA**	**OX_2 HTL6641**
Data collection							
Number of crystals	1	1	1	3	1	5	8
Space group	$P2_1$	$P2_1$	$P2_1$	$P2_1$	P1	$C222_1$	$C222_1$
Cell dimensions							
a, *b*, *c* (Å)	59.34, 145.89, 71.34	59.83, 147.07, 72.26	59.77, 148.24, 71.67	60.36, 147.07, 72.54	55.32, 76.25, 82.74	91.10, 172.91, 77.81	90.94, 173.95, 78.33
α, β, γ (°)	90, 112.33, 90	90, 111.56, 90	90, 112.17, 90	90, 111.09, 90	89.99, 85.25, 89.97	90, 90, 90	90, 90, 90
Resolution (Å)	33.75-2.30(2.42-2.30)[a]	33.60-2.55(2.69-2.55)[a]	33.18-2.66(2.94-2.66)[a]	44.71-2.65(2.89-2.65)[a]	47.69-2.76(2.97-2.76)[a]	45.55-2.74(2.98-2.74)[a]	46.49-2.61(2.79-2.61)[a]
R_{pim}	0.039(0.736)[a]	0.064(0.903)[a]	0.092(0.761)[a]	0.074(1.224)[a]	0.165(0.512)[a]	0.181(1.168)[a]	0.164(0.943)[a]
$I/\sigma(I)$	13.4(1.2)[a]	11.2(1.1)[a]	10.6(1.2)[a]	8.7(1.8)[a]	2.5(1.4)[a]	6.7(1.5)[a]	5.9(1.4)[a]
$CC_{1/2}$	0.999(0.460)[a]	0.998(0.368)[a]	0.997(0.336)[a]	0.998(0.562)[a]	0.984(0.500)[a]	0.977(0.346)[a]	0.996(0.426)[a]
Completeness (%)							
spherical	77.1(26.3)[a]	79.1(26.6)[a]	58.4(9.4)[a]	58.3(9.6)[a]	68.7(17.2)[a]	68.8(13.0)[a]	60.6(8.2)[a]
ellipsoidal	93.4(69.5)[a]	92.1(58.6)[a]	89.5(58.6)[a]	90.8(69.9)[a]	86.3(48.0)[a]	89.1(44.3)[a]	91.6(79.3)[a]
Redundancy	3.5(3.4)[a]	8.4(7.8)[a]	5.3(4.7)[a]	9.6(9.7)[a]	1.6(1.5)[a]	8.0(4.4)[a]	8.3(14.5)[a]
Refinement							
Resolution (Å)	33.75-2.30	33.60-2.55	33.18-2.66	44.71-2.65	47.69-2.76	45.55-2.74	41.47-2.61
No. reflections	38396	29922	19273	19853	23783	11345	11694
R_{work} / R_{free}	0.209/0.236	0.215/0.253	0.220/0.255	0.236/0.265	0.207/0.254	0.218/0.275	0.221/0.255

No. atoms							
Protein	4762	4744	4878	4833	7979	4048	4100
Ligand	102	92	148	132	55	58	48
Other	353	366	311	322	555	80	136
B factors							
Protein	81.12	76.23	68.75	84.63	41.69	42.17	53.20
Ligand	122.39	69.99	90.80	91.25	48.64	43.66	52.38
Other	108.06	98.15	89.60	109.18	52.06	29.67	57.88
R.m.s. deviations							
Bond lengths (Å)	0.003	0.004	0.003	0.002	0.019	0.005	0.002
Bond angles (°)	0.67	0.66	0.64	0.58	1.20	0.68	0.400

[a] Values in parentheses indicate highest resolution shell. [b] See Reference 6.

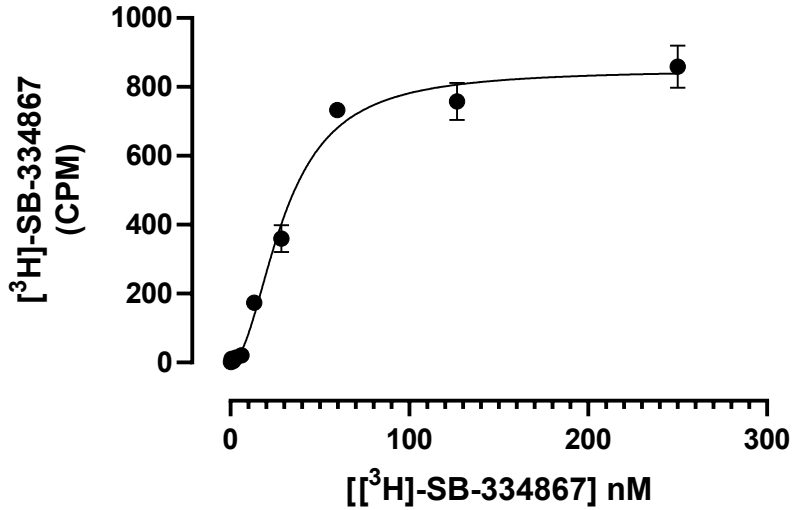

Figure 1: Saturation binding of [^{3}H]-SB-334867 to HEK293T cell membranes expressing WT OX_1. The data was analyzed by 'Saturation model with Hillslope' in GraphPad Prism and showed best fit with an unconstrained Hillslope (K_d 30.1, Hillslope 2.02). The data is representative of 3 independent experiments. Consistent with the crystal structure, both WT OX_1 and OX_1 StaR showed binding with a Hillslope of ~2, indicating that the ligand binds with positive cooperativity.

Table 3: Summary of Crystallography Constructs

	Boundaries				ICL3		
	N-terminus	C-terminus	StaR mutations	PTM site mutations	deletion	fusion	fusion insertion points
OX_1	28	380	E46A, I85L, V95A, R162L, L198A, Y211A, L304V, C339A	N194A, C375W, C376W	254-285	n/a	n/a
OX_2	1	388	E54A, Y91L, D100A, V142A, R170L, L206A, Y219A, M233A, A242L, L310V, L318A, T347A	N14D, N22D, N30D, N202D, C381W, C382W, C383W	255-293	*Pyrococcus abyssi* glycogen synthase (218 -413)	255-293

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