Supporting Information

Identification of a novel subtype-selective α_{1B}-adrenoceptor antagonist

Alaa Abdul-Ridha[1], Lazarus A. de Zhang[1], Ashenafi Haileyesus Betrie[1], Mattia Deluigi[2], Tasneem M. Vaid[1,5,6], Alice Whitehead[1], Yifan Zhang[1], Ben Davis[3], Richard Harris[3], Heather Simmonite[3], Roderick E. Hubbard[3,4], Paul R. Gooley[5, 6], Andreas Plückthun[2], Ross A.D. Bathgate[1,5], David K. Chalmers[7], & Daniel J. Scott[1,5]

[1]The Florey Institute, 30 Royal Parade, The University of Melbourne, Parkville, Victoria 3052, Australia

[2] Department of Biochemistry, University of Zurich, CH-8057 Zurich, Switzerland

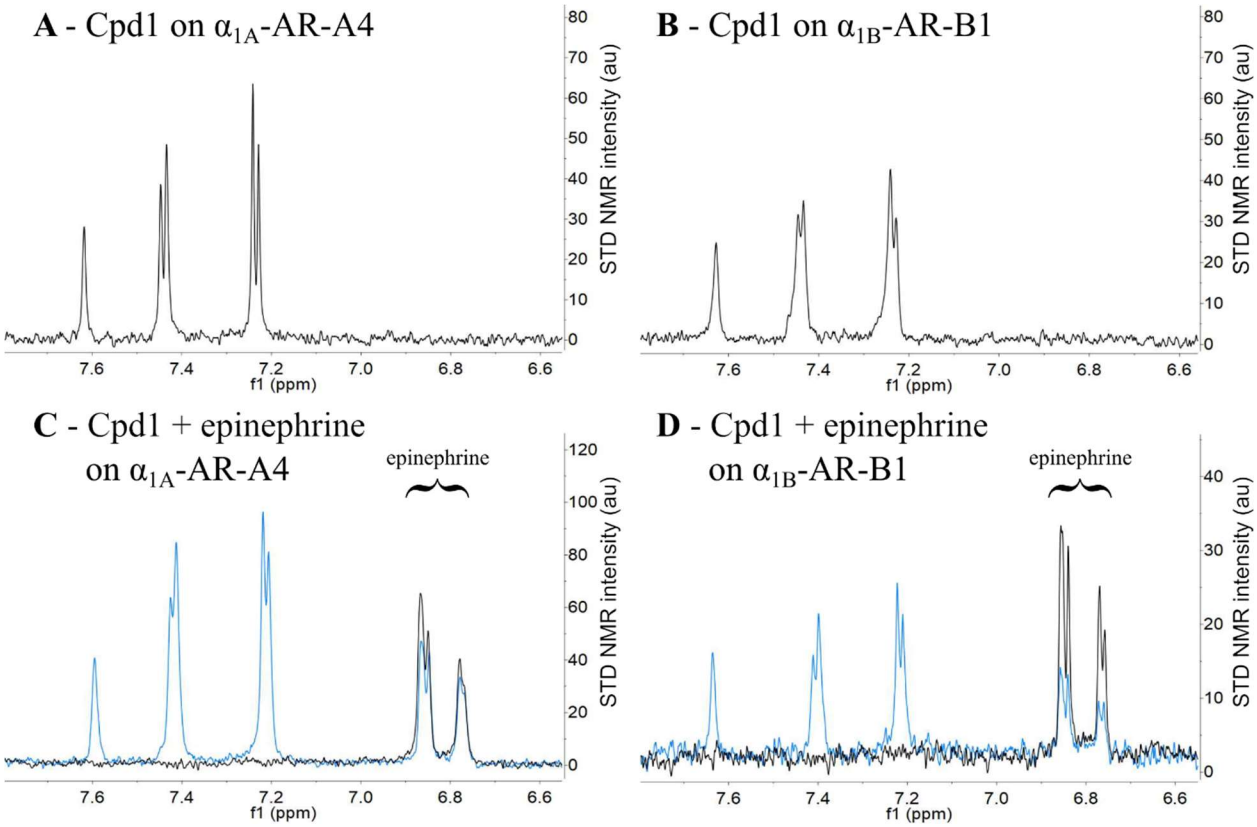
[3] Vernalis (R&D) Ltd, Granta Park, Cambridge, CB21 6GB, UK

[4] Department of Chemistry, University of York, York YO10 5DD, UK

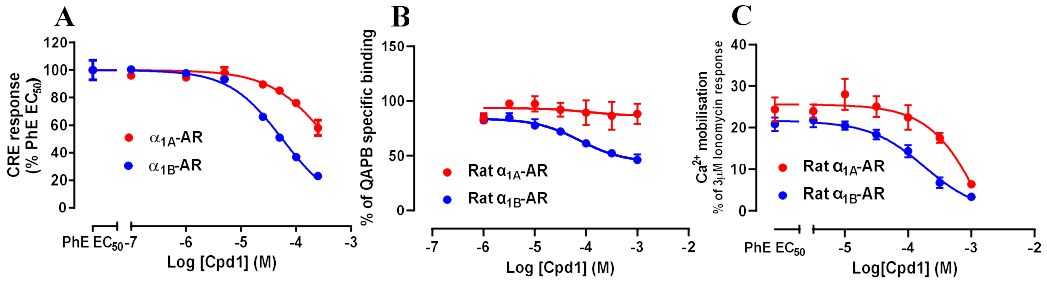
[5] The Department of Biochemistry and Pharmacology, The University of Melbourne, Parkville, Victoria 3010, Australia

[6] The Bio21 Molecular Science and Biotechnology Institute, The University of Melbourne, Parkville, Victoria 3010, Australia

[7] Medicinal Chemistry, Monash Institute of Pharmaceutical Sciences, Monash University, 381 Royal Parade, Parkville, Victoria 3052, Australia

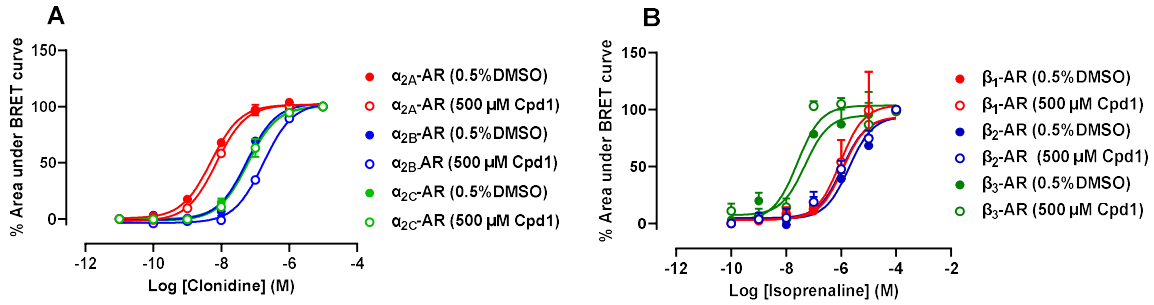


Supporting Figure 1. STD NMR and competition STD NMR of Cpd1 on purified α_{1A}-AR and α_{1B}-AR. STD NMR spectra of Cpd1 (500 µM) binding to purified α_{1A}-AR-A4 (A) and α_{1B}-AR-B1 (B). Epinephrine competition STD NMR was performed on α_{1A}-AR-A4 (C) and α_{1B}-AR-B1 (D) with black spectra epinephrine alone (500 µM) and blue spectra corresponding to epinephrine in the presence of Cpd1. Clear competition of epinephrine binding to α_{1B}-AR-B1 by Cpd1 (100 µM) was observed, which was not the case with α_{1A}-AR-A4, even with 6-fold more Cpd1 added (600 µM).



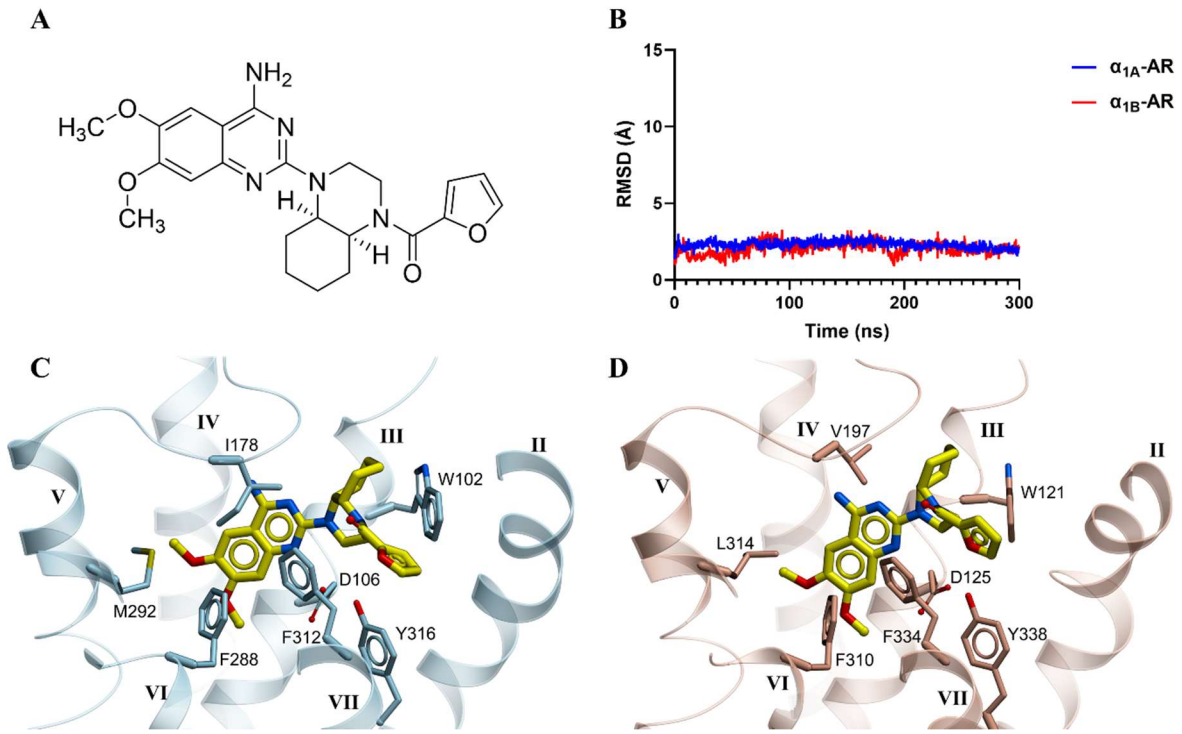
Supporting Figure 2. α_{1B}-AR selectivity of Cpd1 in CRE reporter assay and rat α_1-ARs.

(A) Cpd1 inhibits phenylephrine (PhE)-induced CRE activation in COS-7 cells stably expressing human α_{1B}-AR (blue circles) to a greater extent than at human α_{1A}-AR (red circles) at 37°C. (B) The equilibrium binding of the antagonist QAPB was inhibited by Cpd1 at WT rat α_{1B}-AR (blue circle) but not at WT rat α_{1A}-AR (red circle) in COS-7 cells transiently expressing receptors at 21°C. (C) Cpd1 inhibits PhE-induced Ca^{2+} mobilisation response in rat α_{1B}-AR (blue circles) to a greater extent than in rat α_{1A}-AR (red circles) transiently expressed in COS-7 cells. Cells were pre-incubated with Cpd1 for 30 minutes before addition of an EC_{50} concentration of PhE at 37°C. Points represent the mean ± S.E. of three independent experiments performed in duplicate. Refer to Table 1 for values.



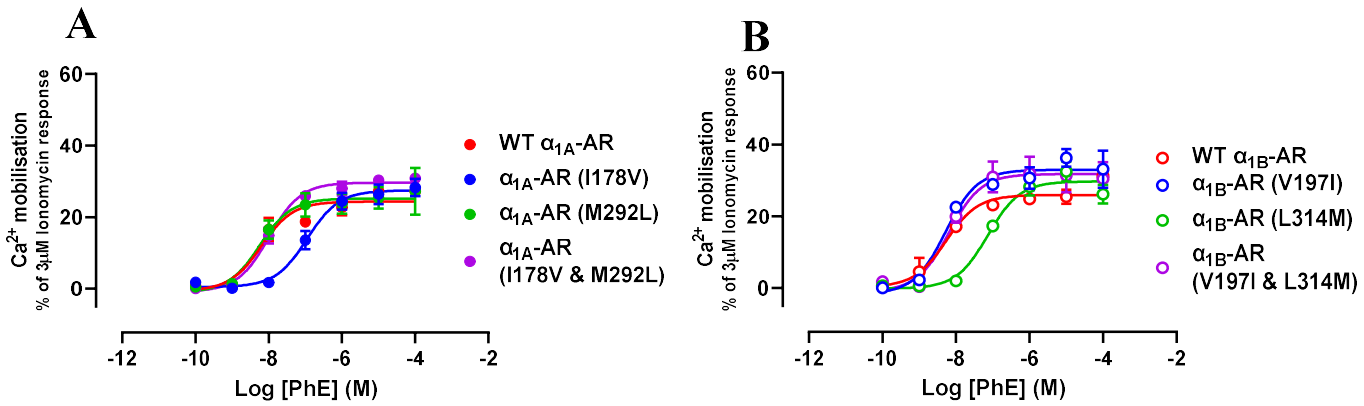
Supporting Figure 3. Screening of Cpd1 at α_2-ARs and β-ARs.

(A) Clonidine induces activation of the $G\alpha_{i3}$ G protein subunit upon binding to α_{2A}-AR (closed red circles), α_{2B}-AR (closed blue circles), and α_{2C}-AR (closed green circles). At 500 µM, Cpd1 weakly competes with clonidine agonist activity at α_{2B}-AR (open blue circles), but not at α_{2A}-AR (open red circles) or α_{2C}-AR (open green circles). (B) Isoprenaline induces activation of the $G\alpha_s$ G protein subunit upon binding to β_1-AR (closed red circles), β_2-AR (closed blue circles), and β_3-AR (closed green circles). At 500 µM, Cpd1 does not compete with isoprenaline at β_1-AR (open red circles), β_2-AR (open blue circles) or β_3-AR (open green circles). COS-7 cells transiently expressing each receptor and BRET sensor pair were pre-incubated with Cpd1 for 30 minutes before addition of either clonidine or isoprenaline at 37°C and measurement of a BRET signal for 1 h. The area under each BRET curve was used to generate a dose-response curve. Points represent the mean ± S.E. of three independent experiments performed in duplicate.



Supporting Figure 4. (+)-Cyclazosin docking and MD simulations studies on α_{1A}-AR and α_{1B}-AR.

(A) The chemical structure of (+)-cyclazosin. (B) RMSD of **(+)**-cyclazosin from MD simulations run on the α_{1A}-AR (blue line) and α_{1B}-AR (red line) WT homology models, revealing that **(+)**-cyclazosin remained stably bound in both receptors during the 400 ns simulation. (C-D) Docking of **(+)**-cyclazosin into the homology models of WT α_{1A}-AR (C) and α_{1B}-AR (D) made using the α_{1B}-AR crystal structure (PDB: 7B6W) as a template.



Supporting Figure 5. Phenylephrine dose-response curves.

(A & B) Phenylephrine (PhE) dose-response curves generated using the intracellular Ca^{2+} mobilisation assay to test the effects of each of the α_{1A}-AR mutants (I178V, M292L, and I178V/M292L) (A) and α_{1B}-AR mutants (V197I, L314M, and V197I/L314M) (B) on agonist potency and efficacy relative to their respective WT receptor. The EC_{50} values derived from these curves were used in subsequent Ca^{2+} mobilisations assay testing Cpd1 (Figure 3 C–D). Assays in (A & B) were conducted using COS-7 cells transiently expressing WT or mutant receptors at 37°C. Points represent the mean ± S.E. of at least three independent experiments performed in duplicate. Refer to Table 1 for values.

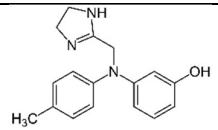
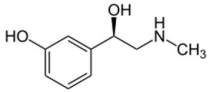
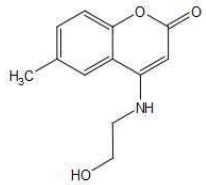
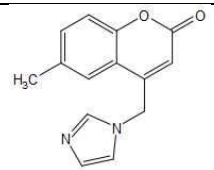
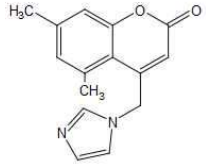
Table 1. Pharmacological characterization for QAPB, Prazosin, Phenylephrine, Cpd1, and Cpd24 at WT and mutant α_1-ARs. Estimated values represent the mean ± S.E. of at least three experiments performed in duplicate.

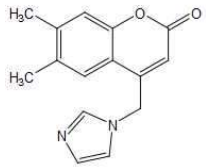
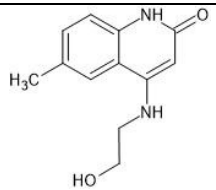
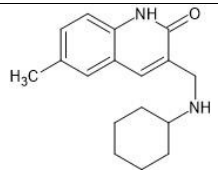
	B_{max}	pK_D[a]	pK_I[b]			pEC_{50}[c]	pIC_{50}[d]		
		QAPB	**Prazosin**	**Cpd1**	**Cpd24**	**Phenylephrine**	**Prazosin**	**Cpd1**	**Cpd24**
α_{1A}-AR WT	235558 ± 45678	8.08 ± 0.08	8.27 ± 0.08	3.38 ± 0.43	6.33 ± 0.06	8.20 ± 0.21	8.54 ± 0.19	3.23 ± 0.41 3.41 ± 0.07 (CRE)	5.24 ± 0.31
α_{1A}-AR I178V	513647 ± 21507*	8.04 ± 0.02	8.32 ± 0.05	ND	6.29 ± 0.05	6.95 ± 0.14*	7.86 ± 0.10*	3.48 ± 0.25	5.34 ± 0.27
α_{1A}-AR M292L	537979 ± 15184*	8.14 ± 0.06	8.75 ± 0.08*	ND	5.64 ± 0.09*	8.24 ± 0.21	8.47 ± 0.14	3.16 ± 0.38	4.96 ± 0.27
α_{1A}-AR I178V & M292L	424599 ± 22546*	8.22 ± 0.08	8.64 ± 0.07*	ND	5.96 ± 0.13	7.99 ± 0.09	8.50 ± 0.16	3.49 ± 0.18	5.79 ± 0.39
α_{1B}-AR WT	97400 ± 15422	8.40 ± 0.09	8.85 ± 0.05	4.76 ± 0.11	6.81 ± 0.14	8.27 ± 0.17	8.92 ± 0.13	4.43 ± 0.11 4.25 ± 0.01 (CRE)	5.54 ± 0.28
α_{1B}-AR V197I	324676 ± 15993*	8.15 ± 0.16	8.95 ± 0.05	3.74 ± 0.36*	5.34 ± 0.14*	8.28 ± 0.16	8.86 ± 0.17	3.52 ± 0.35	4.88 ± 0.39
α_{1B}-AR L314M	273130 ± 16360*	7.75 ± 0.20*	9.01 ± 0.08	3.96 ± 0.24	6.24 ± 0.12	7.13 ± 0.13*	8.77 ± 0.30	3.09 ± 0.44	5.53 ± 0.34
α_{1B}-AR V197I & L314M	315513 ± 23388*	7.90 ± 0.18	9.03 ± 0.06	3.53 ± 0.35*	5.18 ± 0.35*	8.19 ± 0.22	8.77 ± 0.09	2.73 ± 0.74	5.22 ± 0.28
Rat α_{1A}-AR WT	587371 ± 166603	7.80 ± 0.44	ND	ND	ND	7.62 ± 0.37	ND	2.74 ± 0.68	ND
Rat α_{1B}-AR WT	1366189 ± 129077	7.54 ± 0.61	ND	4.20 ± 0.21	ND	8.05 ± 0.30	ND	3.75 ± 0.15	ND
α_{1D}-AR WT	ND	ND	ND	ND	ND	7.76 ± 0.19	ND	ND	ND
Δ 1-79 α_{1D}-AR	ND	9.29 ± 0.63	ND	ND	ND	8.08 ± 0.21	ND	ND	ND

[*] Data are statistically different (P<0.05) from WT values as determined by one way analysis of variance (ANOVA) with Dunnett's post hoc test.

[a] Negative logarithm of the equilibrium dissociation constant for QAPB derived from whole-cell saturation binding assays.
[b] Negative logarithm of the equilibrium constant for each ligand derived from competition binding assays against QAPB (Figures 3 and 4).
[c] Negative logarithm of the EC_{50} of phenylephrine (Supplementary Figure 5).
[d] Negative logarithm of the IC_{50} for each ligand derived from Ca^{2+} mobilisation assays (Figures 3 C–D) or CRE reporter assays as indicated (Supplementary Figure 2A).

Table 2. Inhibition of QAPB binding and effects on Ca^{2+} mobilisation of structural analogues of Cpd1 at α_1-ARs. Estimated values represent the mean ± S.E. of three experiments performed in duplicate.

Compound ID	Structure	% QAPB binding[a] (pK_I)		% Phenylephrine inhibition[b]		% Agonist activity[c]	
		α_{1A}	α_{1B}	α_{1A}	α_{1B}	α_{1A}	α_{1B}
Phentolamine		0.08±2.97*	0.12±0.07*	2.88±0.67**	1.80±0.67**	0.95±0.99	0.51±0.10
Phenylephrine		11.99±6.22*	1.06±0.52*	105.90±4.29	105.54±7.70	79.24±1.90•	68.89±2.37•
Cpd1		84.01±3.57* (3.38 ± 0.43)	37.59±1.57* (4.76 ± 0.07)	99.11±4.46	36.19±6.30**	1.52±0.43	2.68±0.46
Cpd14		67.47±2.68* (2.79 ± 0.30)	42.83±3.85* (4.08 ± 0.11§)	91.77±4.36	74.74±3.03	18.48±3.28•	11.44±6.33
Cpd19		25.23±1.94* (4.16 ± 0.08)	10.58±0.85* (5.18 ± 0.05)	88.04±8.91	11.55±8.19**	1.79±1.12	1.43±1.40

Cpd20 	37.59±4.97* (3.79 ± 0.11)	20.01±0.37* (4.45 ± 0.04)	70.08±8.22**	52.74±12.72**	1.69±0.30	1.47±1.18
Cpd23 	79.79±9.23* (ND)	74.18±6.38* (4.15 ± 0.20§)	94.67±3.91	86.20±12.29	0.44±0.19	0.85±0.86
Cpd24 	1.53±0.53* (6.33 ± 0.06§)	1.63±0.67* (6.81 ± 0.14§)	2.98±0.87**	1.98±1.15**	15.72±4.90•^	19.37±8.65•^

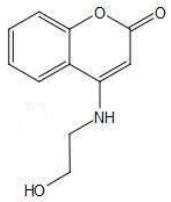
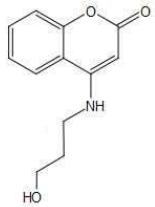
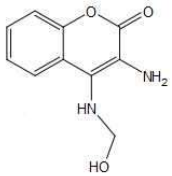
[a] Values are relative to QAPB (6.25 nM) total binding in the absence of other ligands. 1 mM phenylephrine, 1 μM phentolamine, 500 μM all compounds. pK_I values in parentheses.

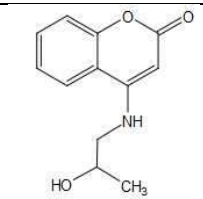
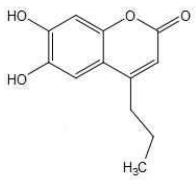
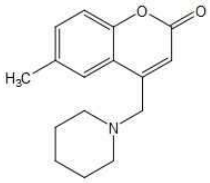
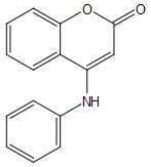
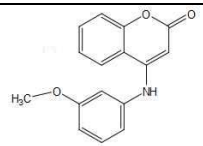
[b] Data normalised to the response elicited by EC_{50} concentration of phenylephrine (10 nM at α_{1A}-AR and α_{1B}-AR). Cells are pre-incubated with either vehicle or 100 μM of phentolamine or test compounds before addition of phenylephrine.

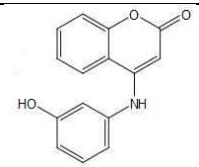
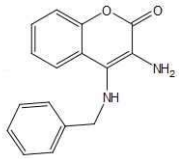
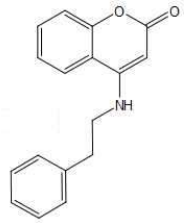
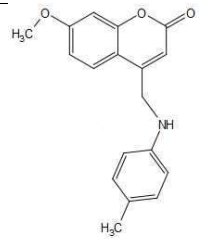
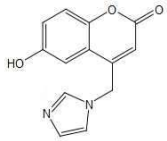
[c] Data is normalised to response elicited by 3 μM ionomycin. Phenylephrine is tested at 1 μM and all compounds are 500 μM.

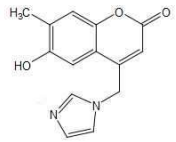
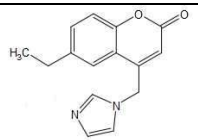
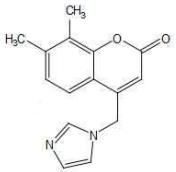
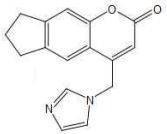
Data are significantly different from: § Cpd1 pK_I value or * total QAPB binding value in the absence of other ligands or ** response elicited by phenylephrine EC_{50} or • vehicle treated cells ($p<0.05$) as determined by one way ANOVA with Dunnett's post hoc test. ND indicates value not determined. ^indicates effects due to higher concentration of DMSO in this sample relative to the rest of the compounds tested (see Table S2).

Table 3. Inhibition of [^{3}H]-prazosin binding and effects on Ca^{2+} mobilisation of structural analogues of Cpd1 at α_1-ARs. Estimated values represent the mean ± S.E. of at least three experiments performed in duplicate.

Cpd ID	Structure	% [^{3}H]-prazosin binding[a]		% Phenylephrine inhibition[b]		% Agonist activity[c]	
		α_{1A}	α_{1B}	α_{1A}	α_{1B}	α_{1A}	α_{1B}
Cpd2		83.29±3.98	85.60±11.76	98.18±4.58	85.54±4.57	8.97±2.04	5.40±1.22
Cpd3		74.80±7.49	78.43±13.09	87.97±7.24	78.08±14.34	1.18±0.42	1.14±0.45
Cpd4		88.97±0.23	89.59±14.89	76.69±9.64	76.67±11.46	3.74±0.35	3.65±0.12

Cpd5 	78.37±8.17	73.90±8.75	89.10±8.76	79.88±10.67	13.16±1.16	9.72±0.88
Cpd6 	78.10±4.48	79.06±19.93	81.78±5.08	68.82±5.94	4.37±2.04	2.80±1.52
Cpd7 	87.42±11.42	84.88±14.80	99.43±3.78	61.11±8.64**	6.32±3.54	6.47±1.56
Cpd8 	86.41±17.92	78.89±12.82	91.34±6.54	72.04±2.45	4.44±2.84	4.37±2.06
Cpd9 	88.76±0.38	98.88±8.63	93.03±6.81	83.19±16.68	20.96±3.86•^	32.99±15.08•^

Cpd10 	88.51±3.49	91.60±17.93	70.19±10.90**	40.47±13.16**	7.39±4.17	9.49±3.76
Cpd11 	88.64±0.46	69.10±3.17	70.50±7.92**	51.29±9.45**	6.24±2.13	9.48±6.63
Cpd12 	54.67±9.34*	77.27±6.56	77.98±9.35	60.01±5.57**	18.14±2.35•^	16.29±2.13^
Cpd13 	93.58±4.38	83.02±11.61	102.42±7.87	89.76±22.00	29.19±2.07•	14.06±2.29
Cpd15 	99.99±6.33	91.02±9.97	88.04±11.32	88.17±7.26	7.31±0.74	0.16±0.31

Cpd16 	89.65±5.91	78.72±9.69	95.53±8.30	99.63±9.59	4.90±1.05	2.65±1.09
Cpd17 	53.08±10.74*	64.15±8.28	104.18±4.63	65.07±3.74**	13.86±6.44^	18.61±8.26^
Cpd18 	68.71±14.28	95.46±11.05	90.53±12.38	73.03±8.75	2.20±0.81	4.03±2.07
Cpd21 	57.31±5.66*	68.22±9.08	89.61±5.47	44.56±8.34**	21.67±8.24•	19.85±2.78•
Cpd22 	79.56±2.93	94.57±8.78	88.14±9.52	87.44±14.13	0.50±0.11	0.31±0.63

[a] Values are relative to total [^{3}H]-prazosin binding in the absence of other ligands. All compounds are 500 μM.

[b] Data normalised to the response elicited by EC_{50} concentration of phenylephrine (10 nM at α_{1A}-AR and α_{1B}-AR). Cells are pre-incubated with either vehicle or 100 μM of phentolamine or test compounds before addition of phenylephrine.

[c] Data is normalised to response elicited by 3 μM ionomycin. Phenylephrine is tested at 1 μM and all compounds are 500 μM.

Data are significantly different from: * total [^{3}H]-prazosin binding in the absence of other ligands or ** response elicited by phenylephrine EC_{50} or • vehicle treated cells ($p<0.05$) as determined by one way ANOVA with Dunnett's post hoc test. ND indicates value not determined.^ indicates these compounds also produced response in untransfected COS-7 cells (see table S2).

Table 4. Ca^{2+} mobilisation signal in response to addition of 500 µM of test compounds or vehicle in untransfected COS-7 cells. Estimated values represent the mean ± S.E. of three experiments performed in duplicate. Signal is relative to vehicle treatment and 3 µM Ionomycin. * Indicates value is significantly different from vehicle treated cells ($p<0.05$) as determined by one way ANOVA with Dunnett's post hoc test. ND indicates value not determined.

Compound	**Ca^{2+} mobilisation signal**
Vehicle	1.62±1.56
Phenylephrine	2.06±0.54
Cpd 1	3.15±1.93
Cpd2	1.95±1.03
Cpd3	0.94±0.35
Cpd4	0.05±0.95
Cpd5	2.09±0.62
Cpd6	0.87±1.22
Cpd7	2.44±1.39
Cpd8	2.46±0.63
Cpd9	10.80±2.07*
Cpd10	2.70±2.21
Cpd11	Nd
Cpd12	11.17±2.41*
Cpd13	2.59±1.02

Cpd14	3.65±3.32
Cpd15	0.44±0.65
Cpd16	6.76±1.70
Cpd17	13.50±2.58*
Cpd18	0.91±1.89
Cpd19	1.09±2.05
Cpd20	0.54±0.64
Cpd21	2.82±1.66
Cpd22	1.66±1.06
Cpd23	1.90±0.76
Cpd24	14.08±0.15*