Similarity- and substructure-based development of β_2-adrenergic receptor ligands based on unusual scaffolds

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SUPPLEMENTARY PROCEDURES

Substructure searches

Eight substructure queries (**S1**-**S8**), which are depicted in Chart S2, were manually derived from the six original hits (Chart S1). These queries were encoded as SMARTS and run against the complete ZINC database (*1*), which comprised 8.5 million molecules at that time.

Similarity searches

At the same time, FCFP4 fingerprints, as implemented in Pipeline Pilot, of the six query molecules **Q1**-**Q6** were used to screen the same database of 8.5 million entries for molecules with a Tanimoto similarity ≥ 0.45.

Docking

All molecules originating from the two searches were, after removal of duplicates, docked into the orthosteric pocket of the inverse-agonist bound X-ray structure of the β_2AR (PDB 2RH1), as previously described. (*2*) Briefly, molecules were placed by DOCK, using guiding points inside the pocket that had been derived from carazolol, the inverse agonist bound to the β_2AR in this X-ray structure.

Cell culture

CHO-K1 cells stably expressing either the human β_1AR or the human β_2AR and CRE-SPAP reporter gene were used (CHO-β_1, CHO-β_2 (*3*)) and grown in Dulbecco's modified Eagle's medium nutrient mix F12 (DMEM/F12) containing 10% foetal calf serum and 2 mM L-glutamine in a 37°C humidified 5% CO_2:95% air atmosphere.

[^{3}H](-)CGP 12177 whole cell binding

Media was removed from confluent cells in white-sided 96-well plated and immediately replaced by 100 µL of the ligand under investigation (diluted in serum-free media (sfm), DMEM/F12 containing 2 mM L-glutamine only) followed immediately by 100 µL [^{3}H](-)CGP 12177 (in sfm) to give a final [^{3}H](-)CGP 12177 concentration of 0.44-1.12 nM. The plates were the incubated for 2 h at 37°C before being washed twice with 200 µL 4°C phosphate buffered saline. Microscint 20 (100 µL) was added to each well, a white base added to the plate, the plated left for a minimum of 8 h in the dark then counted on a TopCount.

K_D values were determined from the IC_{50} values using the Cheng-Prusoff equation (see ref. (*3*) for details). For all ligands that completely inhibited specific binding, a pK_D value is given. For ligands where significant specific binding was inhibited, but the maximum concentration of a ligand was not quite sufficient to completely inhibit specific binding, an apparent pK_D value is given (based on the assumption that a higher concentration of the competing ligand would inhibit all specific binding). For ligands with less than 50% inhibition of binding, despite maximum concentration of ligand (maximum possible concentration of ligand ranged from 20-100 µM), no K_D value is stated. Propranolol (10 µM) was used to determine non-specific binding and the K_D values for [^{3}H](-)CGP 12177 were 0.42 nM for the human β_1AR and 0.17 nM for the human β_2AR. (*3*)

CRE-SPAP production

Confluent cells (96-well plates) were serum starved with sfm for 24 h before experimentation. The media was then removed and replaced with 100 µL sfm or sfm containing final concentration of antagonist. Agonist (10 µL, diluted in sfm) was then added and the plates incubated for 5 h at 37°C.

CRE-SPAP production was then measured as previously described. (*4*)The intrinsic efficacy of all ligands was assessed from 7-point concentration response curves. Isoprenaline (10 µM) was used as the positive control in all plates. Maximum responses and pEC_{50} values were obtained from sigmoidal dose response curves (see ref. (*4*) for full details). The affinity of antagonists was determined from a rightward shift of the agonist response using the Gaddam equation, and for the partial agonist **3**, using the method of Stephenson (see ref. (*4*) or full details).

SUPPLEMENTARY RESULTS

[^{3}H](-)CGP 12177 whole cell binding and CRE-SPAP production validation

[^{3}H](-)CGP 12177 whole cell binding demonstrated that the known β_1-selective antagonist CGP 20712A, as expected, had high affinity for the human β_1AR (pK_D 8.96±0.13, n=4) whilst the known β_2-antagonist ICI 118551 had high affinity for the human β_2AR (pK_D 9.61±0.05, n=5, Table 1).

Cimaterol stimulated a full agonist response at both receptors. At the β_1AR, this response was 3.3±0.5-fold over basal, 105±2% that of the isoprenaline maximum (n=12) and at the β_2AR, the response was 4.4±0.1-fold over basal and 95±1% that of isoprenaline (n=9) (Table ~~S3~~). As expected, CGP 20712A inhibited the CHO-β_1 cimaterol response with high affinity, and ICI 118551 inhibited the CHO-β_2 cimaterol response with high affinity to yield similar selectivities to those obtained from the binding assay (Table ~~2~~).

Compound 3 acts through the primary catecholamine conformation of β_1AR

Compound **3** was clearly a partial agonist at both the β_1 and β_2-AR (Table S4, Figure ~~S2~~). At the β_2AR, this partial agonist response was inhibited by ICI 118551 to give a K_D value for ICI 118551 very similar to that obtained in the presence of cimaterol (Table S4), confirming that this partial agonist response is indeed occurring through interaction with the β_2AR.

The β_1AR, however, exists in at least two active agonist conformations~~:~~ (*4-8*), a high affinity catecholamine conformation (through which cimaterol and catecholamines stimulate agonist responses, and for which CGP 20712A and CGP 12177 have high affinities), and a secondary conformation through which higher concentrations of CGP 12177 stimulate agonist responses (although these responses are relatively resistant to antagonism). The conformation through which **3** was stimulating β_1 partial agonist response was therefore assessed.

The affinity measured by both the binding assay (pK_D 9.01) and the functional assay (pK_D 9.19) were very similar. The concentrations of [^{3}H](-)CGP 12177 used in the binding assay would only measure binding to the catecholamine conformation. Compound **3** also inhibited the cimaterol response (known to act through the catecholamine conformation, (~~*4-7*~~) with high affinity, again suggesting high affinity catecholamine conformation interaction. The partial agonist response (pEC_{50} 8.80) is also very similar to the pK_D values, again suggesting interactions with the catecholamine conformation. This therefore suggests that both the binding of **3** and the agonist response obtained in the functional assay are occurring through the same high affinity conformation of the β1AR.

The partial agonist response of **3** in CHO-β1 cells was inhibited by CGP 20712A with high affinity, suggesting that the response is indeed β_1AR-mediated. However, the K_D value for CGP 20712A obtained was part-way between that of cimaterol (high affinity catecholamine conformation) and that of CGP 12177 (secondary conformation, Table S4). Thus, although the similarity of the K_D and EC_{50} values suggests single-site, high affinity conformation interactions, further evidence for which site of actions the response was occurring through was sought. When increasing concentrations of **3** were added to fixed concentrations of cimaterol (Figure S2), the cimaterol response was inhibited in a manner suggestive of competition at a single conformation (compare with Figure 1 of ref (*5*); Figure 4 of ref (*6*); Figure 8 of ref (*7*) and Figure 6 of ref (*4*)). Overall therefore, **3** is also a high affinity partial agonist of the human β_1AR, with the agonist response occurring through the primary catecholamine conformation of the receptor.

Dose response curves of the other ligands

The dose response curve for several compounds showed no stimulation of either receptor (e.g. Figure S3, compound **1**). For some ligands, e.g. **16** and **17**, there was also no inhibition of [^{3}H](-)CGP 12177 binding and no shift of the cimaterol-induced concentration response curve. These ligands were therefore found to not be interacting with either the β_1 or β_2AR at concentrations up to the maximum studies (100 µM for many). Other compounds, e.g. **1**, although no stimulation occurred in response to the ligands alone, they did inhibit binding and cause a shift of the cimaterol-induced dose response. These compounds are therefore neutral antagonists.

For some compounds, e.g. **10** and **11**, the highest concentrations possible (20 µM for **10**, 100 µM for **11**) caused a marked fall to below basal (e.g. Figure S3). This pattern of CRE-SPAP production is consistent with toxicity (i.e. cell death, or major assay interference). In these instances, the concentration of compound used to antagonize cimaterol was reduced, until such a time as the reduction in basal was minimal or non-existent (i.e. for **10**, reduced to 2 µM as this no longer caused a reduction

in basal). The functional assay is far more sensitive to issues such as toxicity because the cells need to be living in order to generate responses, whereas in the binding assay, binding to the receptors can be measured even if the cells are dead. In some cases, this reduction in compound concentration still allowed a shift to be observed and thus a K_D value to be measured.

Receptor-mediated inverse agonism as an explanation for the marked fall in CRE-SPAP production is very unlikely as i) this gene transcription assay is relatively poor at detecting inverse agonism, including compound ICI 118551, which is known to be an inverse agonist in these cells; (*9*) ii) identical results were seen in both β_1- and β_2-cells despite the fact that the ligands e.g. **10** had different affinities for the two receptors (and therefore receptor mediated effects should have been observed at different ~~doses~~); iii) the logIC_{50} of the apparent fall in CRE-SPAP production (e.g. for **10**, 10μM at the β_2AR) is not the same as the K_D value obtained from the binding studies (1μM), again suggesting the fall is a non-receptor mediated issue and iv) if the fall below basal was due to inverse agonism, there should still be a cimaterol concentration response in the presence of 20μM **10**, that was further right shifted, than that at 2 μM (Figure S1). As can be seen in Figure S4, there is absolutely no cimaterol response in the presence of 20 μM **10** and the whole response is below basal. This strongly suggests a non-receptor mediated cause for the fall.

Novel compounds

As well as a small fall in maximum cimaterol response, **10** causes a rightward shift in the cimaterol concentration response at the β_2AR but not at the β_1AR. This suggests that **10** is indeed interacting with the β_2AR in this functional assay and it is showing some β_2AR selectivity, with an apparent K_D value that is similar to that obtained from the binding study. The lack of a rightward shift of the cimaterol-concentration response in the presence of 2 μM **10** at β_1AR are entirely as expected, given the K_D value obtained from the binding studies (3 μM). Thus, despite the apparent toxicity issues at high concentrations in the functional assay, **10** appears to be a β_2-selective ligand with an affinity of 300-1000 nM in both the binding and functional assay.

SUPPLEMENTARY FIGURES

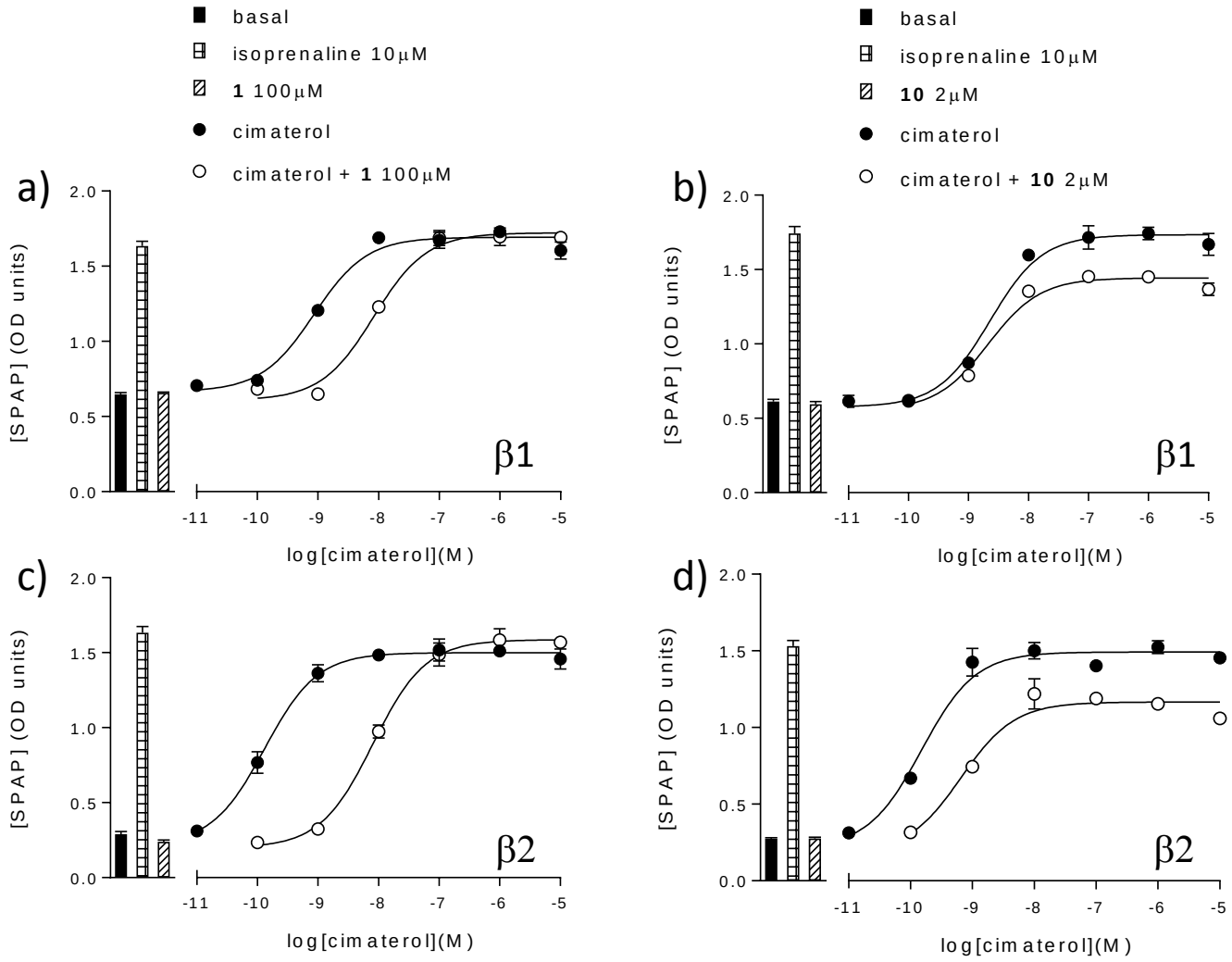


Figure S1: CRE-SPAP production in a) and b) CHO-β1 cells and c) and d) CHO-β_2 cells in response to cimaterol in the absence and presence of a) and c) 100 μM **1,** and b) and d) ~~2mM~~ **10**. Bars represent basal CRE-SPAP production and that in response to 10 μM isoprenaline and 100 μM **1** or ~~2mM~~ **10** alone. Data points are mean ± sem of triplicate values and these individual experiments are representative of a) and c) 6 separate experiments and b) and d) 3 separate experiments.

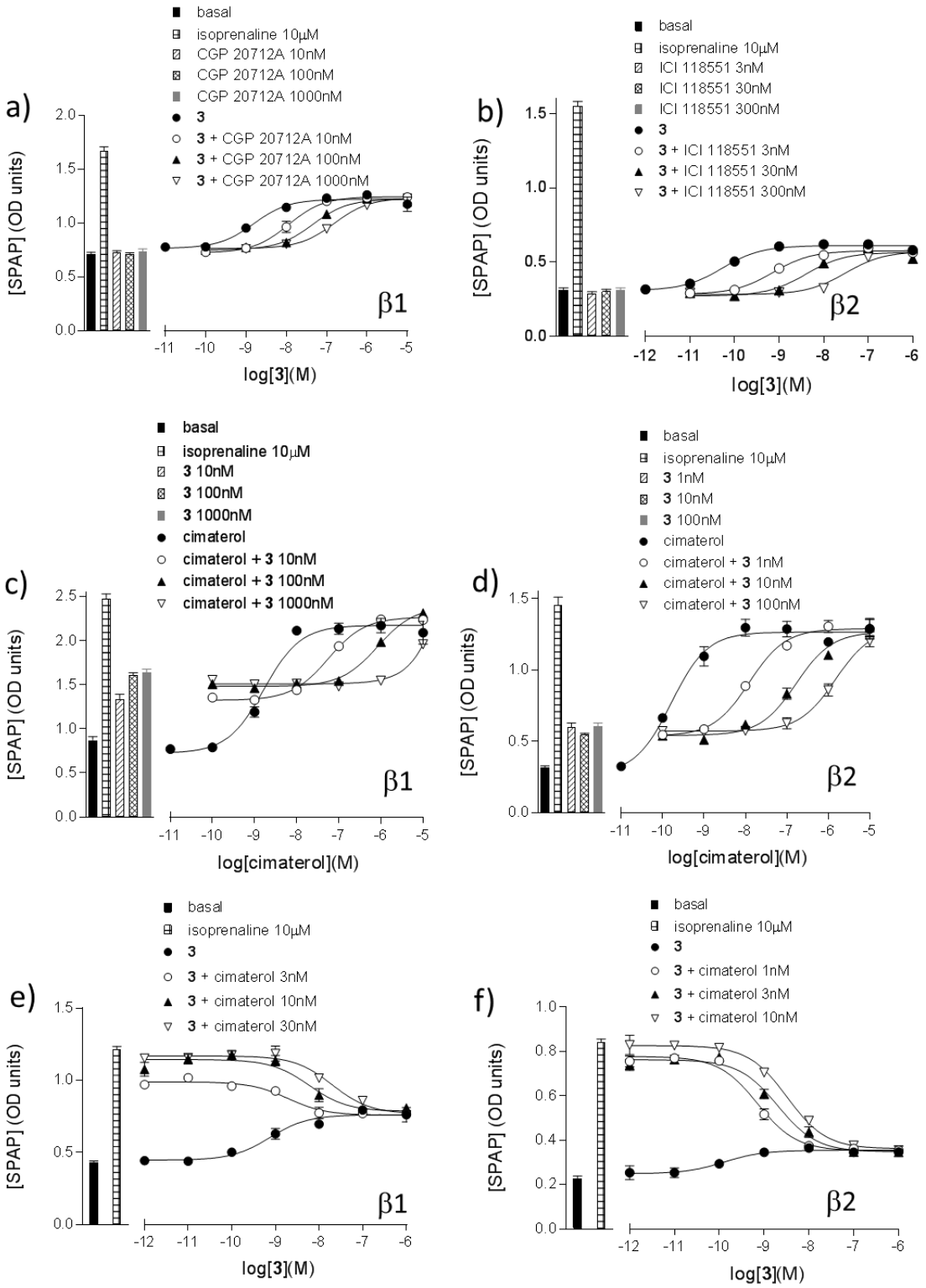


Figure S2: CRE-SPAP production in CHO-β_1 cells (a, c and e) and CHO-β_2 cells (b, d and f). a and b) show response to **3** inhibited by CGP20712A in the β_1 cells and inhibited by ICI 118551 in the β_2 cells thus confirming the responses are mediated via the respective receptors. c and d) show inhibition of the cimaterol response by increasing concentration of **3** in a manner consistent with that of a partial agonist; e and f) show inhibition of the cimaterol response by **3** in a manner consistent with competition at a single site. Bars represent basal CRE-SPAP production and that in response to 10 μM isoprenaline or various concentrations of CGP 20712A, ICI 118551 or **3** alone. Data points are mean ± sem of triplicate values and these individual experiments are representative of five or more separate experiments in each case.

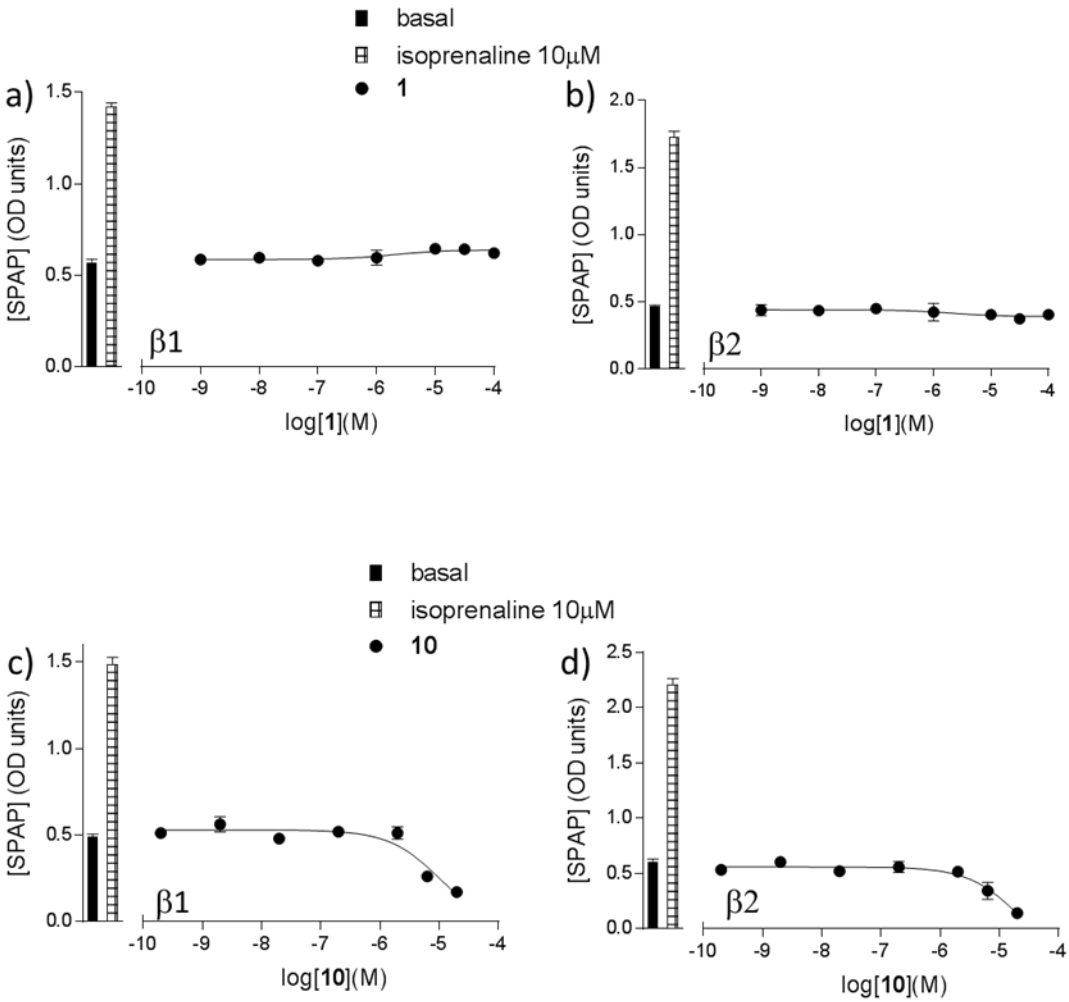


Figure S3: CRE-SPAP production in a) and c) in CHO-β_1 cells and b) and d) in CHO-β_2 cells in response to **1** (a and b) and **10** (c and d). Bars represent basal response and that to10 μM isoprenaline. Data points are mean ± sem of triplicate determinations and these individual experiments are representative of 4 separate experiments in each case.

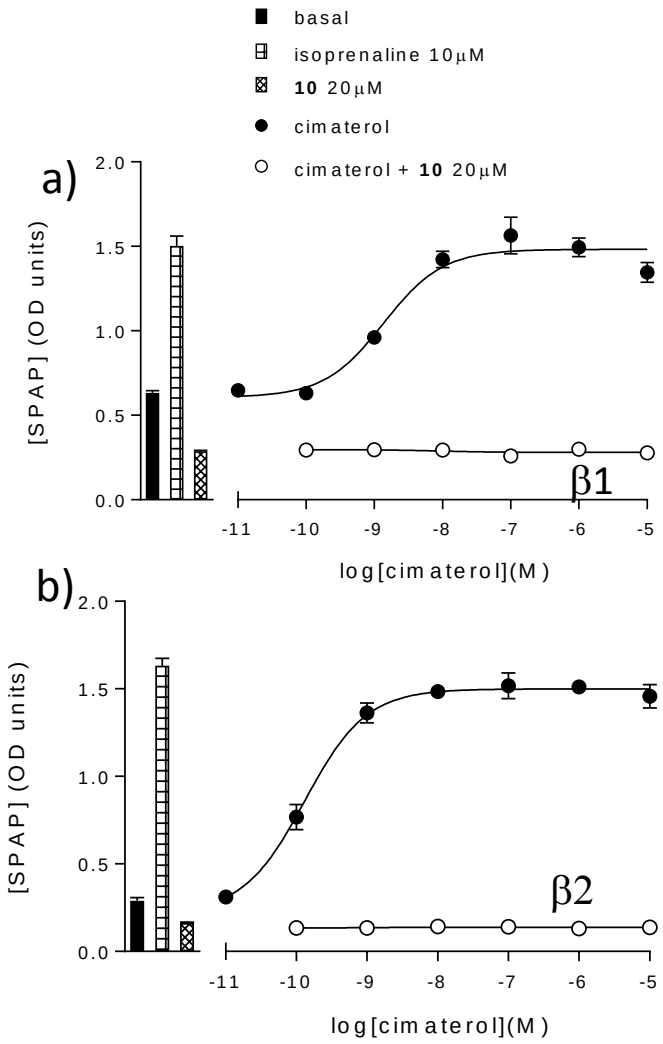


Figure S4: CRE-SPAP production in a) in CHO-β_1 cells and b) in CHO-β_2 cells in response to cimaterol in the presence and absence of 20μM **10**. Bars represent basal response and that in response to 10μM isoprenaline or 20μM **10** alone. Data points are mean ± sem of triplicate determinations and are representative of three separate experiments in each case.

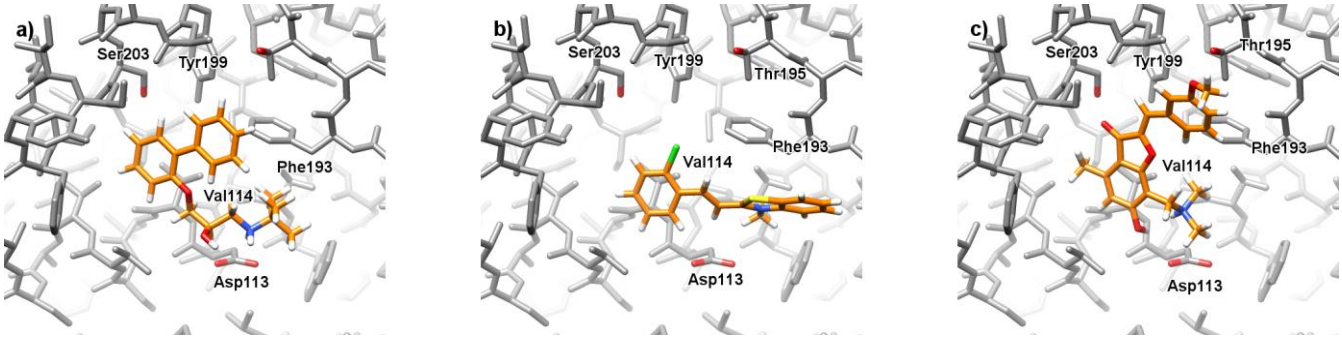


Figure S5: Docking poses for selected compounds. The β_2AR is shown in gray stick representation. Residues discussed in the text are labeled and shown with colored heteroatoms. Selected residues in TM6 and TM7 (including Phe289$^{6.51}$ and Phe290$^{6.52}$) are hidden for clarity. Ligands are shown in orange stick representation. Perspective as in ref. ~~(5)~~ for comparability. (a) **3**, (b) **11**, (c) **7**.

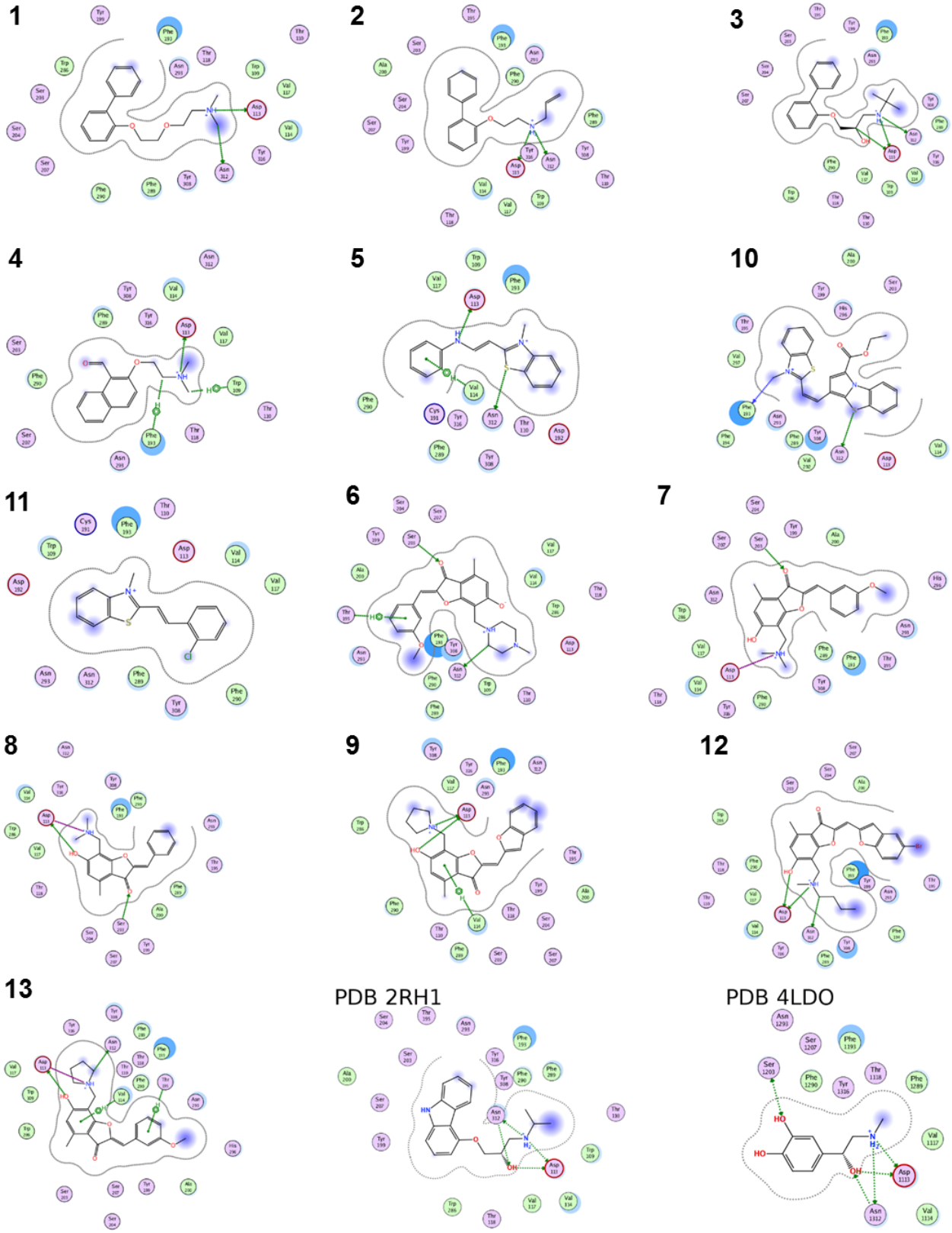


Figure S6: 2D binding mode depictions for all compounds for which binding has been correctly predicted (1,2,3,5,6,10,11,12,13,14,15,16,17). For comparison, the binding modes for Carazolol (PDB 2RH1) and adrenaline (PDB 4LDO) are shown. For new compounds, the depictions have been calculated based on binding mode predicted by docking. Depictions created using the Molecule Operating Environment (MOE). (*10*)

SUPPLEMENTARY CHARTS

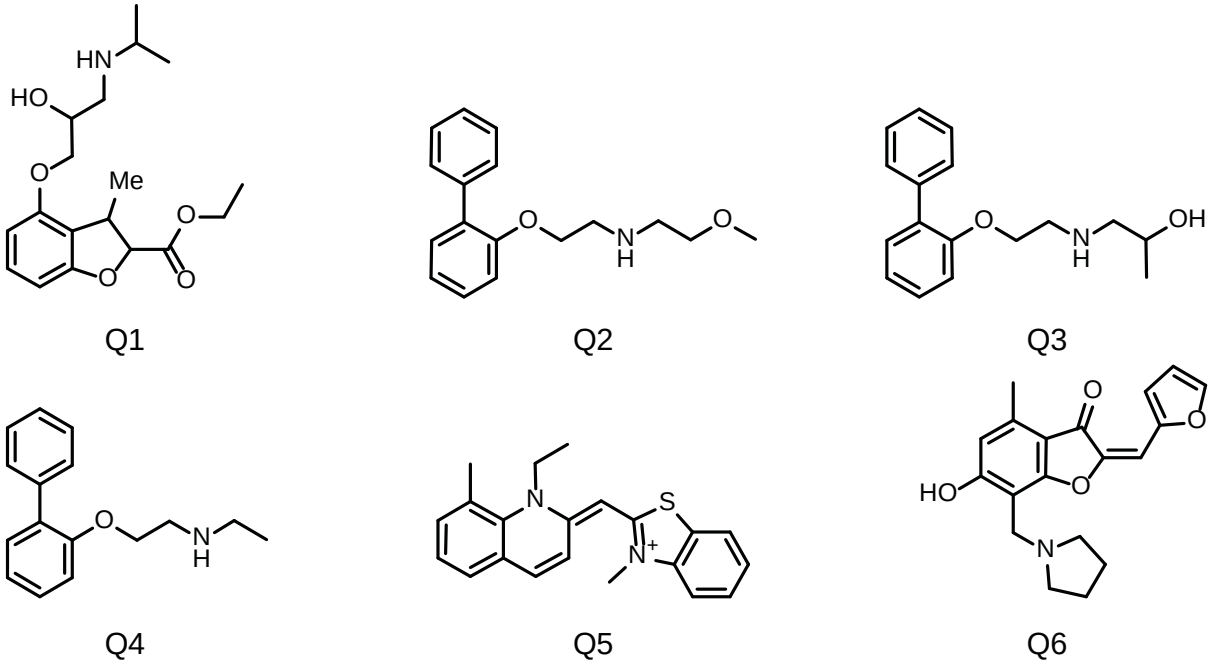


Chart S1: The six query molecules from ref. ~~(5)~~ used for similarity search and the derivation of eight substructures.

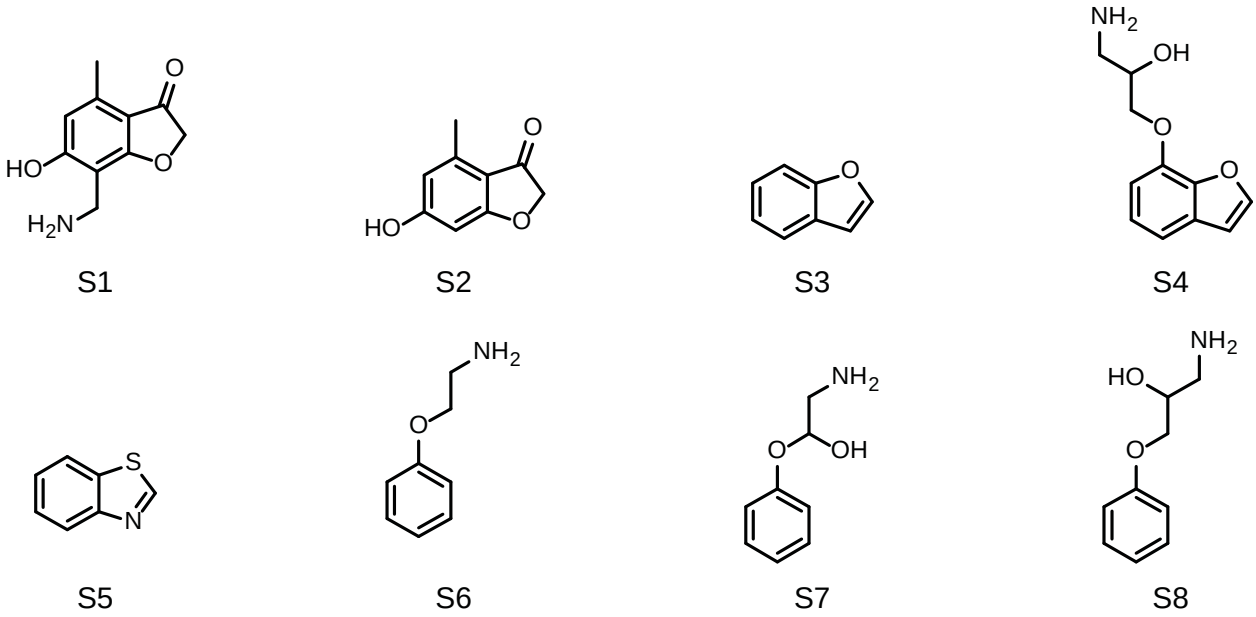


Chart S2: The eight substructures, based on the ligands of ref. ~~(5)~~, used for screening in this study.

SUPPLEMENTARY TABLES

Table S1: Number of molecules resulting from the similarity search with TC ≥ 0.45 for each query molecule of ref. (*2*). The sum reflects the number of molecules after removing duplicates.

Query	Q1	Q2	Q3	Q4	Q5	Q6	Σ
N_{Hits}	1538	2381	946	1310	1053	284	6363

Table S2: Affinity (K_D values) and β_2-selectivity for compounds as measured by a parallel shift inhibition of cimaterol concentration responses in the CRE-SPAP assay in CHO-β_1 and CHO-β_2 cells. Values are mean ± sem of n separate experiments.

ID	β_2AR pK_D		n	β_1AR pK_D		n	β_2/β_1[a]
1[c]	5.63	±0.06	6	4.81	±0.07	6	6.6
2[c]	5.73	±0.06	4	4.85	±0.05	5	7.6
3[b,d]	10.74	±0.03	4	9.19	±0.04	10	35.5
4	4.98	±0.05	6	4.62	±0.08	4	2.3
5	n.c.		8	n.c.		4	
6	5.30	±0.07	4	5.01	±0.06	6	1.9
7	5.30	±0.06	4	5.02	±0.07	6	1.9
8	5.06	±0.04	4	5.17	±0.12	7	0.78
9	n.c.		6	n.c.		6	
10	6.62	±0.12[e]	3	n.c.		3	
11	n.c.		8	n.c.		6	
12	n.c.		8	n.c.		6	
13	5.58	±0.1	6	4.82	±0.08[e]	5	5.8
14	n.c.		4	n.c.		4	
15	4.13	±0.06	6	n.c.		4	
16	n.c.		8	n.c.		4	
17	n.c.		6	n.c.		4	
ICI 118551	9.81	±0.15	5	7.16	±0.07	6	447
CGP 20712A	6.21	±0.04	6	9.21	±0.06	15	0.001

[a] Selectivity: $\beta_2/\beta_1 = K_D(\beta_2)/K_D(\beta_1)$

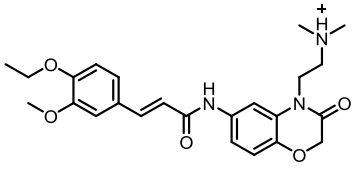
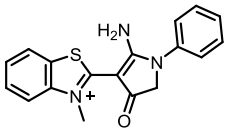
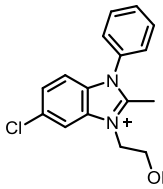
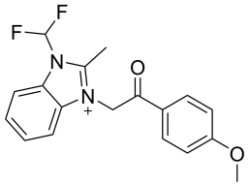
[b] the partial agonist method of Stephenson 1956 was used to calculate the K_D value for **3**.

[c] US 20090163545

[d] Antiarrythmic pharmaceutical (Bipranol/Berlafenone), Arzneimittel-Forschung **1992**, *42*, 289-291

[e] estimated K_D. Here a shift and a small reduction of the maximum response obtained when incubated with cimaterol rather than an absolute parallel shift was obtained e.g. Figure 2d. The shift was calculated from a parallel response of the lower part of the curve (as per the Gaddam equation) but noted here as an estimated K_D given the slight fall in maximum.

Table S3: Affinity (K_D values) ~~and β_2-selectivity~~ for compounds as measured by [^{3}H](-)CGP 12177 whole cell binding to CHO-β_1 and CHO-β_2 cells. Each compound was measured in n separate experiments.

ID	Structure	β_2AR pK_D	n	β_1AR pK_D	n	~~β_2/β_1[a]~~
14		n.c.	5	n.c.	5	
15		n.c.	3	n.c.	3	
16		n.c.	5	n.c.	5	
17		n.c.	6	n.c.	6	

[a] ~~Selectivity: $\beta_2/\beta_1 = K_D(\beta_2)/K_D(\beta_1)$~~

[n.c.] For ligands with less than 50% inhibition of specific binding, the IC_{50} value could not be determined and thus a K_D value could not be calculated (n.c.)

Table S4: EC_{50} values and % isoprenaline maximum values for cimaterol, 3 and CGP 12177 as agonists and log K_D values for CGP 20712A and ICI 118551 as antagonists of these agonist response in the CHO-β_1 and CHO-β_2 cells respectively, as determined from CRE-SPAP production. Values are mean ± sem of n separate determinations.

Agonist	pEC_{50}		% isoprenaline maximum	n	pK_D		n
β_1AR					CGP 20712A		
cimaterol	8.81	±0.02	104.7 ± 1.9	12	9.21	±0.06	15
3	8.80	±0.06	45.0 ± 2.0	11	8.35	±0.08	21
CGP 12177	8.39	±0.03	86.8 ± 2.8	7	7.47	±0.04	14
β_2AR					ICI 118551		
cimaterol	9.71	±0.02	94.9 ± 1.4	9	9.81	±0.15	5
3	9.94	±0.1	22.0 ± 2.1	8	9.56	±0.06	11

Table S5: SMILES codes, vendor information and ZINC ID for described compounds

ID	SMILES string	Vendor	Vendor ID	ZINC ID
1[b]	CN(C)CCOCCOc1ccccc1-c1ccccc1	Ambinter[a]	Amb8591782	2825338
2[b]	C=CCNCCOc1ccccc1-c1ccccc1	Ambinter[a]	Amb10982638	3001189
3[c]	CC(C)(C)NCC(O)COc1ccccc1-c1ccccc1	Innovapharm[b]	STT-00320296	4353
4	CN(C)CCOc1ccc2ccccc2c1C=O	Otava[c]	7020663309	11992987
5	C[n+]1c(C=CNc2ccccc2)sc2ccccc21	Enamine[d]	T0504-1129	
6	COc1cccc(C=C2Oc3c(c(C)cc(O)c3CN3CCN(C)CC3)C2=O)c1	IBS[f]	STK854129	20573542
7	COc1cccc(C=C2Oc3c(c(C)cc(O)c3CN(C)C)C2=O)c1	IBS[f]	Amb807687	6764660
8	Cc1cc(O)c(CN(C)C)c2c1C(=O)C(=Cc1ccccc1)O2	IBS[f]	Amb800928	6670218
9	Cc1cc([O-])c(C[NH+]2CCCC2)c2c1C(=O)C(=Cc1cc3ccccc3o1)O2	Ambinter[a]	Amb2448714	9575977
10	CCOC(=O)c1cc(C=Cc2sc3ccccc3[n+]2C)c2sc3ccccc3n12	Otava[c]	107910005	15222345
11	C[n+]1c(C=Cc2ccccc2Cl)sc2ccccc21	Otava[c]	107910003	4158946
12	CCCC[NH+](C)Cc1c2c(c(C)cc1[O-])C(=O)C(=Cc1cc3cc(Br)ccc3o1)O2	Ambinter[a]	Amb2453954	9531929
13	COc1cccc(C=C2Oc3c(c(C)cc(O)c3CN3CCCC3)C2=O)c1	Ambinter[a]	Amb804798	6759304
14	CCOc1ccc(C=CC(=O)Nc2ccc3c(c2)N(CCN(C)C)C(=O)CO3)cc1OC	Otava[c]	1082925	12082453
15	C[n+]1c2ccccc2sc1C1=C(N)N(c2ccccc2)CC1=O	Ambinter[a]	Amb471924	8394352
16	Cc1n(-c2ccccc2)c2ccc(Cl)cc2[n+]1CCO	Ambinter[a]	Amb8495562	3127921
17	COc1ccccc1C(=O)C[n+]1c(C)n(C(F)F)c2ccccc21	Timtec[e]	ST51248084	5571431

[a] Ambinter c/o Greenpharma, 3, allée du titane 45100 Orléans, FRANCE

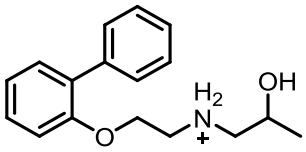
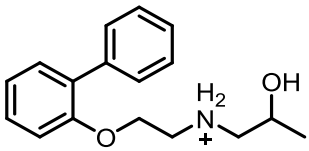
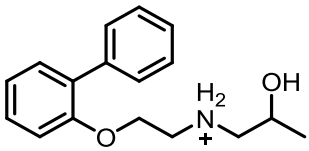
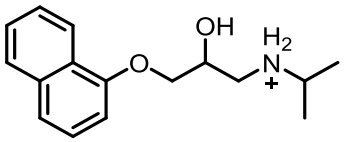
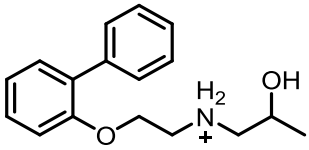
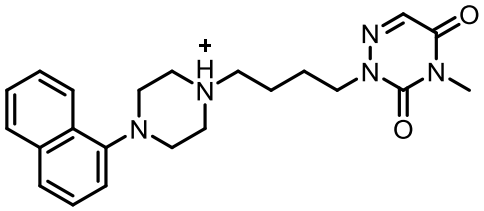
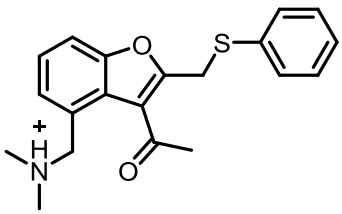
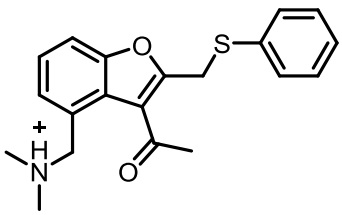
[b] Innovapharm Ltd., 42 Krasnotkatskaya Street, app. 111, Kiev – 02660, UKRAINE

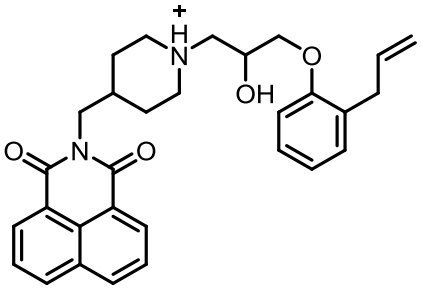
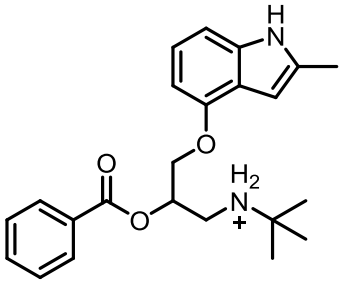
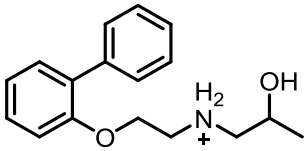
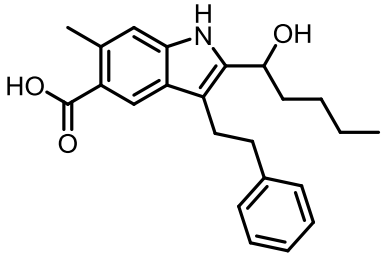
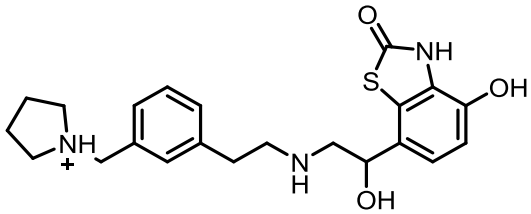
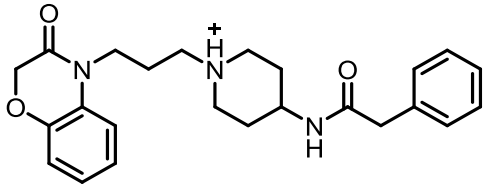
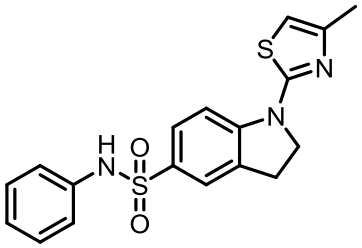
[c] OTAVA Ltd., 400 Applewood Crescent, Unit 100, Vaughan, Ontario, L4K 0C3, CANADA

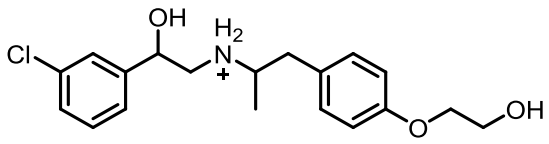
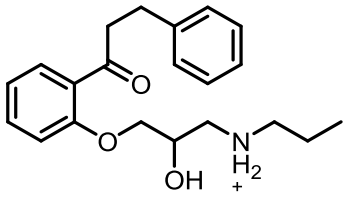
[d] SIA Enamine, Vestienas iela 2 B, V-1035 Riga, LATVIA

[e] TimTec LLC, 301-A Harmony Business Park, Newark, DE 19711, USA
[f] InterBioScreen Ltd., Institutsky Prospect, 7a, 142432 Chernogolovka, RUSSIA

Table S6: Most similar molecules (ChEBML ID and structure) for each compound by Tanimoto ECFP4 similarity at the time of the investigation

ID	ChEMBL ID	Structure	Tanimoto ECFP4
1[a]	CHEMBL1626224		0.5870
2[a]	CHEMBL1626224		0.7270
3[a]	CHEMBL1626224		0.6220
4	CHEMBL275742		0.3820
5	CHEMBL1626224		0.3260
6	CHEMBL2068762		0.3150
7	CHEMBL1622248		0.2990
8	CHEMBL1622248		0.3960

9	CHEMBL1083366		0.2180
10	CHEMBL357995		0.2540
11	CHEMBL1626224		0.2700
12	CHEMBL403296		0.2310
13	CHEMBL1945294		0.3200
14	CHEMBL1242923		0.2710
15	CHEMBL433454		0.2900

16	CHEMBL41113		0.2890
17	CHEMBL631		0.3500

[a] Compounds are annotated in the latest ChEMBL version (ChEMBL 22)

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