Materials and methods

Homology modeling and molecular docking screening. A homology model of TAAR1 was generated based on a crystal structure of the β_2 adrenergic receptor (PDB accession code: 2RH1[1]) and a sequence alignment generated with PROMALS3D[2] using MODELLER9v8.[3] Additional side chain restraints were put on binding site residues to improve ligand recognition, as previously described.[4] A total of 1,000 models were generated and ranked by their DOPE scores.[5] The 200 models with the best structure quality scores were further evaluated using molecular docking screens using the program DOCK3.6.[6–8] A set of 63 known TAAR1 ligands (agonists) from the ChEMBL09 database,[9] together with 160,000 fragment-like molecules from the ZINC database,[10] were docked to each model. The flexible-ligand sampling algorithm in DOCK3.6 superimposes atoms of the docked molecule onto binding site matching spheres, which represent favourable positions for ligand atoms. Forty-five matching spheres based on the co-crystallized ligand of the template structure and docking poses of known ligands were used. The degree of ligand sampling is determined by the bin size, bin size overlap and distance tolerance, which were set to 0.4 Å, 0.3 Å, and 1.5 Å, respectively, for both the matching spheres and the docked molecules. The receptor-ligand binding energy was calculated as the sum of the electrostatic and van der Waals interaction energies, corrected for ligand desolvation. These three energy terms were estimated from pre-calculated grids. For the top scoring conformation of each molecule, 50 steps of rigid-body energy minimization were also carried out. The enrichment of known TAAR1 ligands by each homology model was quantified using the adjusted LogAUC metric.[8] The receptor model with the strongest enrichment of known ligands was further refined manually by modifying the side chain rotamers of binding site residues to match those observed in adrenergic receptor crystal structures.[1,11] In addition, the Protein Local Optimization Program (PLOP) was used to optimize the second extracellular loop two with the rest of the receptor held rigid.[12,13] The ZINC fragment- and lead-like libraries of commercially available compounds (0.357 and 2.7 million unique molecules, respectively)[10] were screened against the orthosteric site of the resulting TAAR1 model. All docked compounds were prepared for docking using the ZINC database

protocol.[10] Prior to compound selection, molecules with high internal energy motifs were removed automatically, as described previously.[14]

2D Molecular Similarity Calculations. Human TAAR1 ligands were extracted from ChEMBL19 database.[9] This compound set was used to assess the novelty of the discovered ligands by calculating the maximum Tanimoto coefficient (T_c) to all TAAR1 ligands using 2D ECFP4 fingerprints as implemented in the ScreenMD software from ChemAxon.[15]

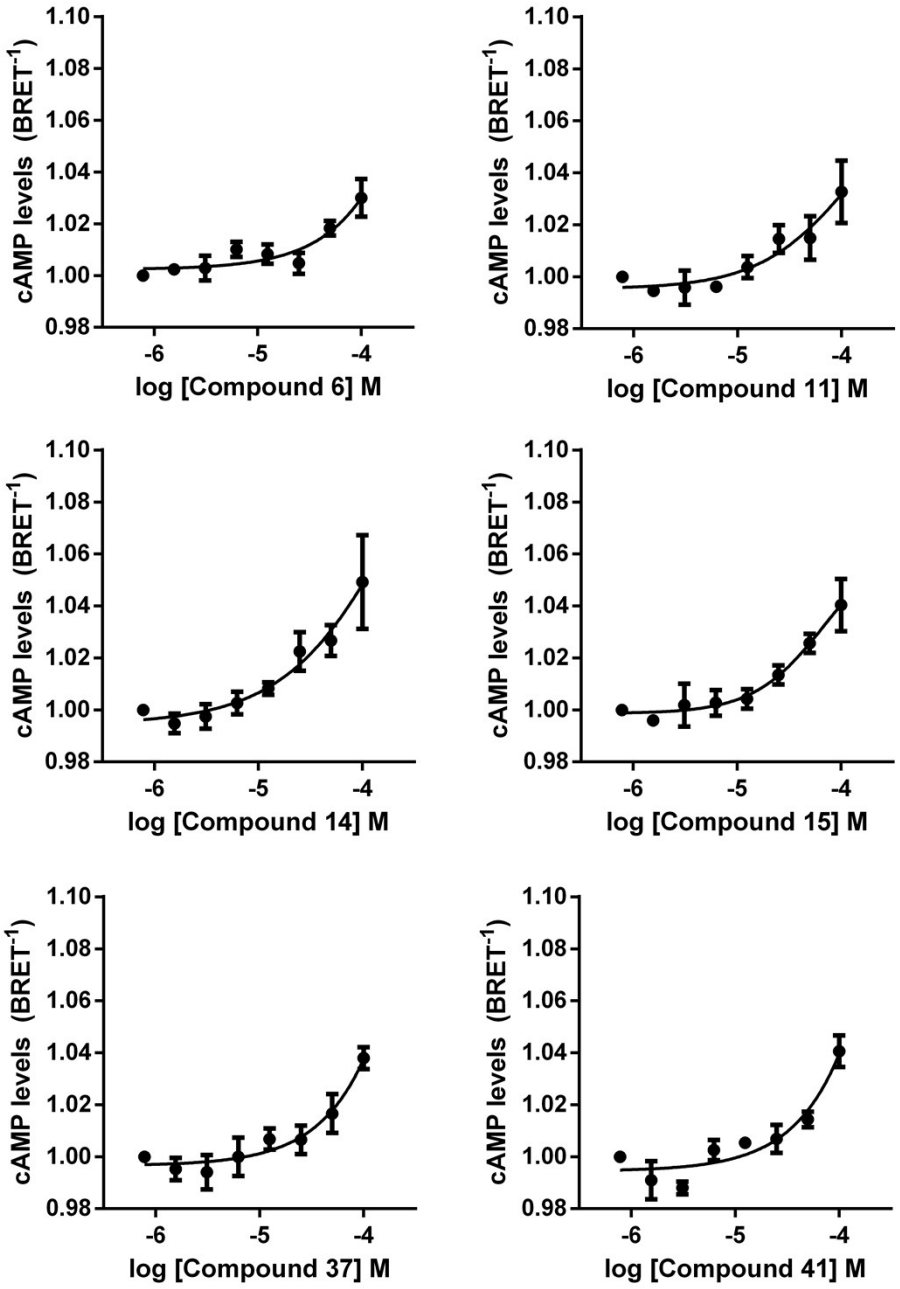
Materials. Plasmids containing cDNA for human β_2TAAR1 and the EPAC BRET biosensor were the same as described in Barak *et al.* 2008.[16] Sources for cell culture reagents and buffers were obtained from Sigma-Aldrich (St. Louis, MO) and Life Technologies (Carlsbad, CA). HEK293T cells were purchased from American Type Culture Collection (Hopkinton, USA). All compounds in this study were obtained from commercial sources, Enamine Ltd (Kiev, Ukraine), Chembridge (San Diego, USA), Tocris Bioscience (Bristol, United Kingdom), Toronto Research Chemicals (Toronto, Canada), and Asinex (Winston-Salem, USA). Compounds were dissolved in DMSO at a concentration of 100 mM and stored at -20˚C.

Cell culture and transfection of cell lines. HEK293T cells were cultured in DMEM supplemented with 10% fetal bovine serum (Sigma-Aldrich) and maintained at 37 ˚C with 5% CO_2 in a humidified atmosphere. Cells were passaged 24 hours prior to transfection to 50% confluency (~2 x 10^6 cells in a 10 cm plate). Transfections were carried out using the polyethylimine (PEI) method described previously.[17] Briefly plasmid DNA and PEI (3µl:1µg PEI:DNA ratio) were added into separate tubes followed by 200 µL of DMEM containing no supplements. The mixtures were allowed to incubate for 5 minutes before the two tubes were mixed. The PEI:DNA mixture was incubated for 30 minutes at room temperature and

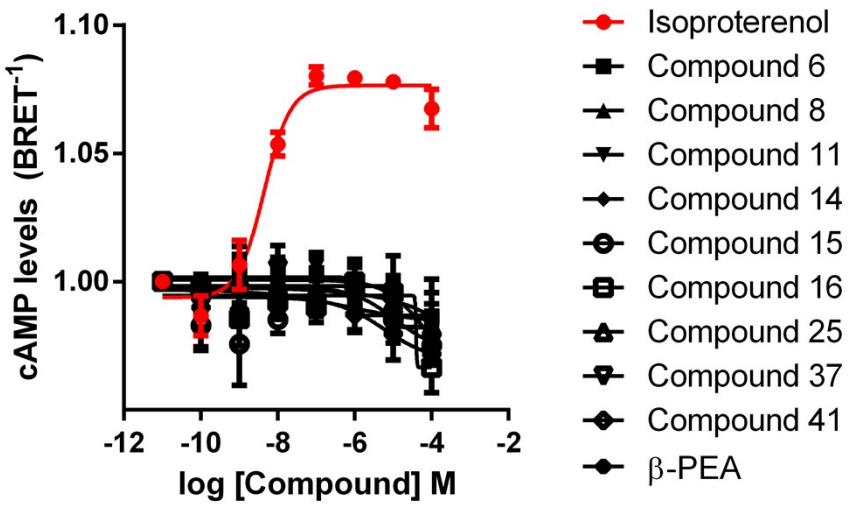
subsequently added drop wise to a 10 cm plate containing HEK293T cells at 50% confluency. For the BRET screening assays, the cells were seeded into poly-D lysine (Sigma-Aldrich) coated white clear bottom 96 well plates six hours post transfection.

BRET Screening. HEK293T cells were transiently transfected with the EPAC sensor and the β_2TAAR1. Six hours post transfection the cells were seeded into poly-D lysine (Sigma-Aldrich) coated white clear bottom 96 well plates (Costar) at a density of 1 x 10^5 cells per well. The BRET assay was carried out within 24 hours after transfection. First the wells were aspirated and washed twice with 100 µL PBS (Mg^{2+} and Ca^{2+}). BRET assays were all done at a final volume of 100 µL and the temperature kept at 25 °C. For agonist testing, the wells were filled with 80 µL of PBS followed by the addition of 10 µL of 50 µM coelenterazine h (5 µM final concentration). After 10 minute incubation, 10 µL of vehicle or agonists were added and the plate placed into a Mithras LB940 instrument (Berthold Technologies, Bad Wildbad, Germany). For antagonist testing, the wells were filled with 70 µL of PBS followed by addition of 10 µL of vehicle or antagonist. Following a 30 minute incubation at 37 °C, 10 µL of 50 µM coelenterazine h (5 µM final concentration) was added. After 10 minute incubation 10 µL of vehicle or β-PEA were added and the plate placed into a Mithras LB940 instrument as above. The BRET signal was determined from the ratio of light emitted at 540 nm to the light emitted at 480 nm. Note for data analyses, the ratios are inverted since in the EPAC biosensor BRET ratios decrease with increasing concentrations of cAMP, therefore the ratios were inversed for the purposes of clarity.

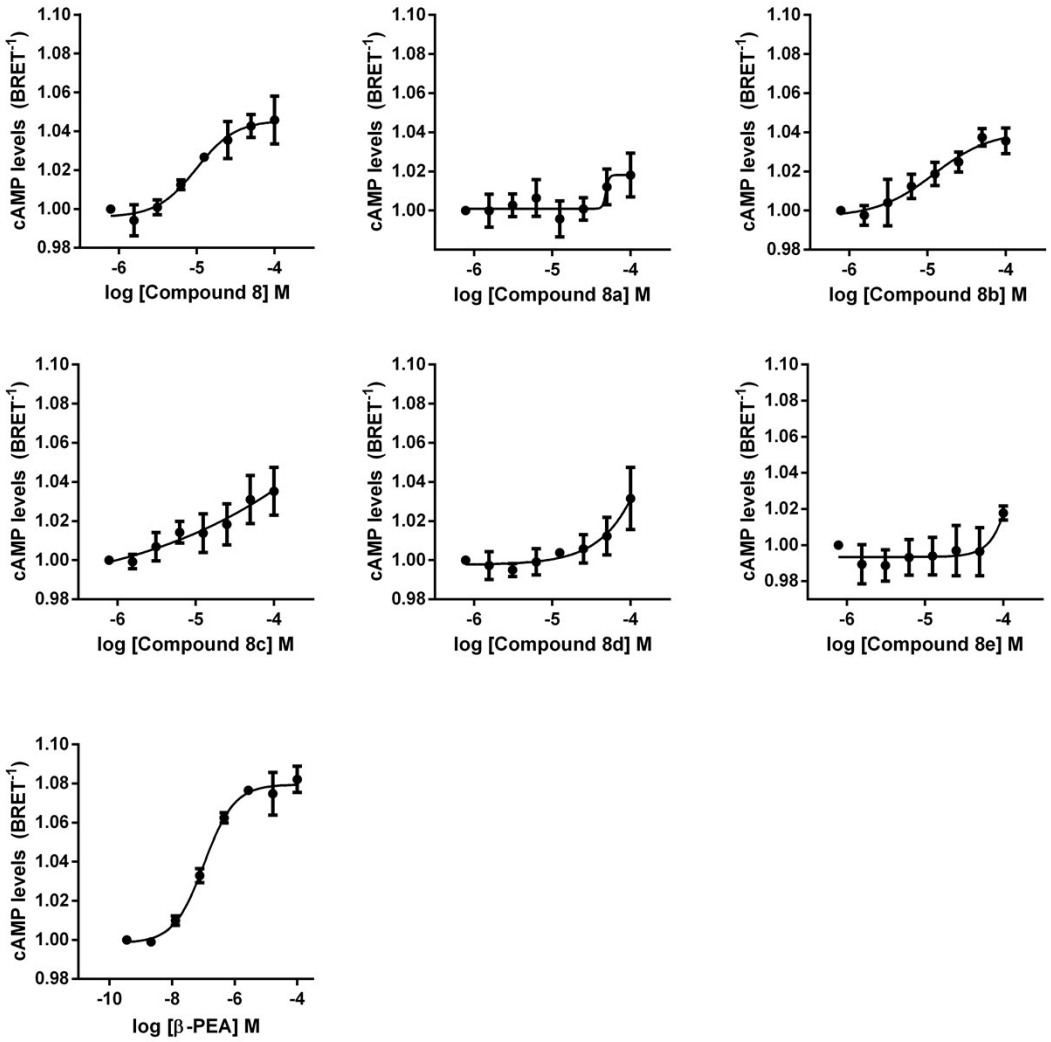
SUPPLEMENTAL FIGURES



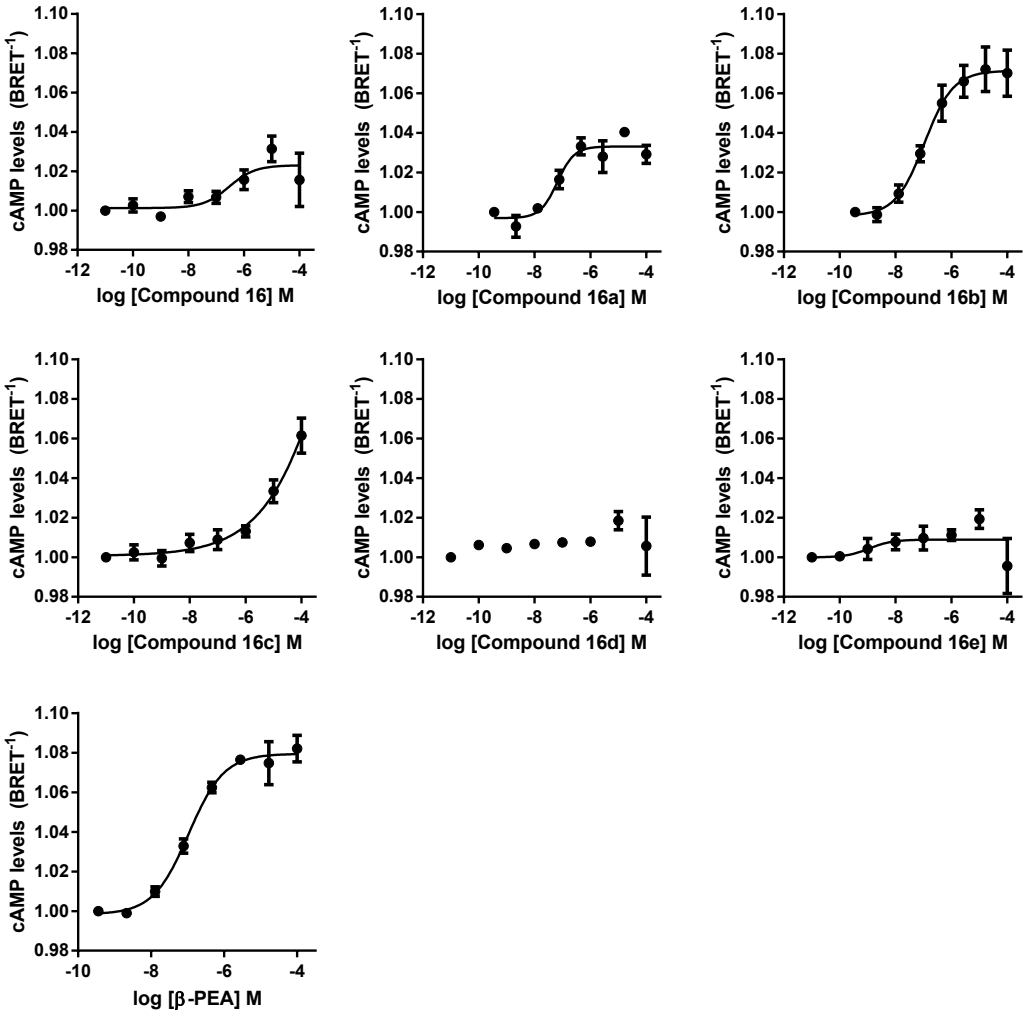
Supplemental Figure 1. Dose response curves for discovered agonists. Dose response curves were generated for agonists by the addition of a range of doses to HEK293T cells transiently expressing both EPAC and β_2-TAAR1. Compounds **6**, **8**, **11**, **15**, **16**, **25**, **37**, and **41** were tested. Error bars represent standard error of mean at N=3.



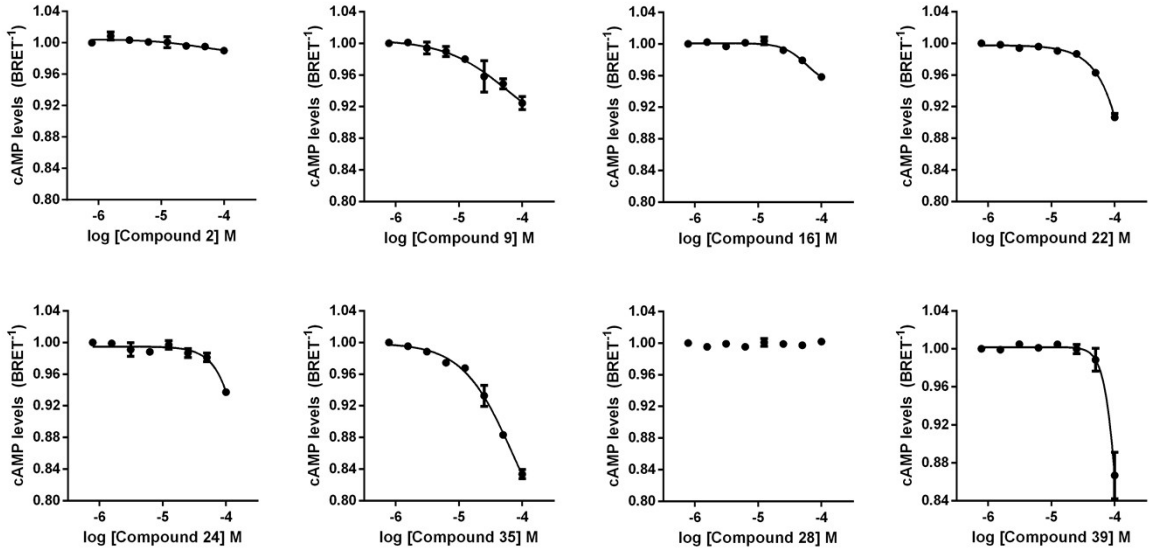
Supplemental Figure 2. Effect of discovered agonist on cells not expressing TAAR1. HEK293 cells stably expressing EPAC were tested with agonists, β-PEA, and the β_2AR full agonist isoproterenol (red). Compounds **6**, **8**, **11**, **14**, **15**, **16**, **25**, **37**, and **41** were tested). Error bars represent standard error of mean at N=3.



Supplemental Figure 3. Dose response curves for analogs of compound **8**. Dose response curves were generated for the analogs by the addition of a range of doses to HEK293T cells transiently expressing both EPAC and β_2-TAAR1. Compounds **8** and analogs **8a-e** were tested. Error bars represent standard error of mean at N=3.



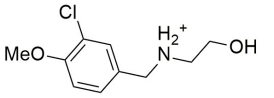
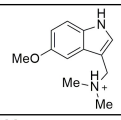
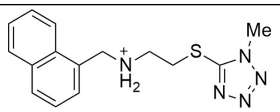
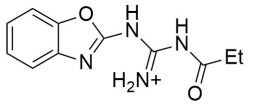
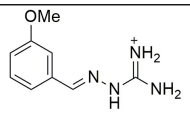
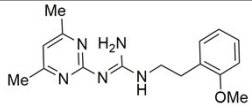
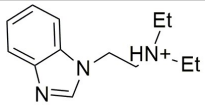
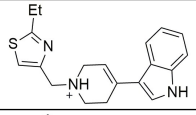
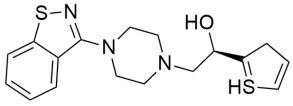
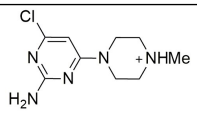
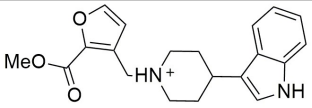
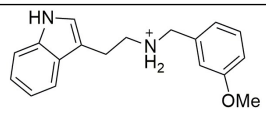
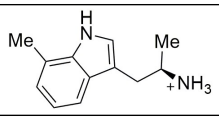
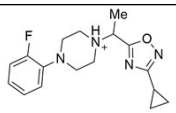
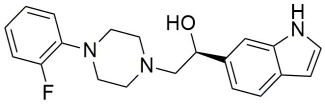
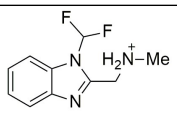
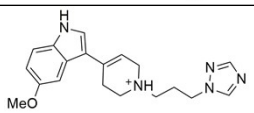
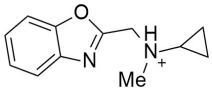
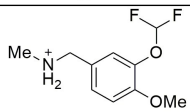
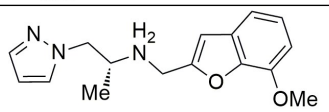
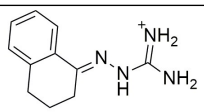
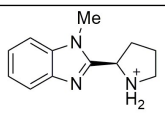
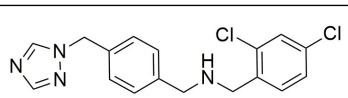
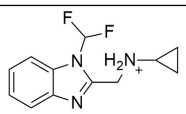
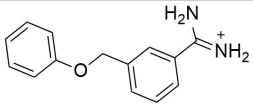
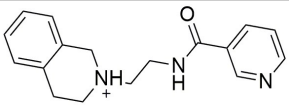
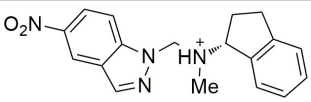
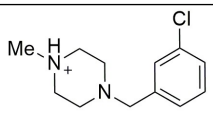
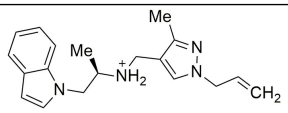
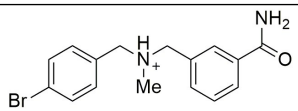
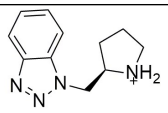
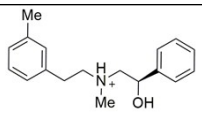
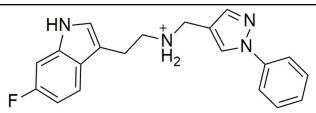
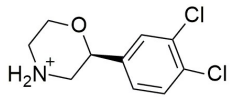
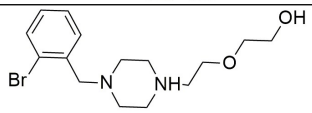
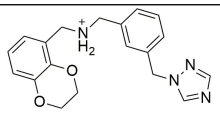
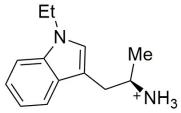
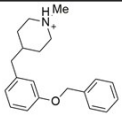
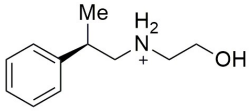
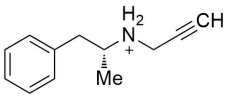
Supplemental Figure 4. Dose responses curves for analogs of compound **16**. Dose response curves were generated for the analogs by the addition of a range of doses to HEK293T cells transiently expressing both EPAC and β_2-TAAR1. Compounds **16** and analogs **16a-e** were tested. Error bars represent standard error of mean at N=3.



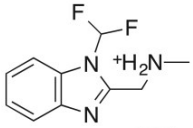
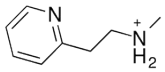
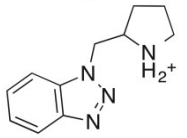
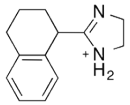
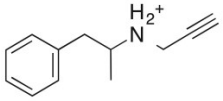
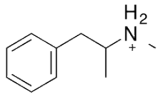
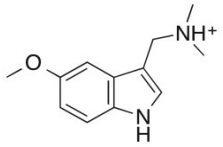
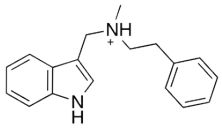
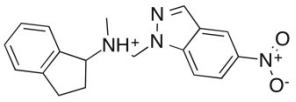
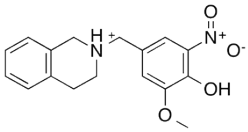
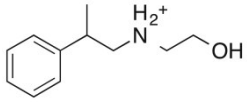
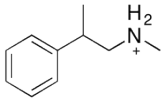
Supplemental Figure 5. Dose response curves for potential TAAR antagonists. Dose response curves for the antagonists were generated by incubating HEK293T cells transiently expressing EPAC and β_2-TAAR1 for 30 minutes and a subsequent addition of 100 nM β-PEA to each well. Compounds **2**, **9**, **16**, **22**, **24**, **35**, **28** and **39** were tested. Error bars represent standard error of mean at N=3.

SUPPLEMENTAL TABLES

Supplemental Table 1: Chemical structures of the compounds 1-42.

1 Frag		**15 Frag**		**29 Lead**	
2 Frag		**16 Frag**		**30 Lead**	
3 Frag		**17 Lead**		**31 Lead**	
4 Frag		**18 Lead**		**32 Lead**	
5 Frag		**19 Lead**		**33 Lead**	
6 Frag		**20 Lead**		**34 Frag**	
7 Frag		**21 Lead**		**35 Frag**	
8 Frag		**22 Lead**		**36 Frag**	
9 Frag		**23 Lead**		**37 Lead**	
10 Frag		**24 Lead**		**38 Lead**	
11 Frag		**25 Lead**		**39 Lead**	
12 Frag		**26 Lead**		**40 Lead**	
13 Frag		**27 Lead**		**41 Frag**	
14 Frag		**28 Lead**		**42 Frag**	

Supplemental Table 2. Functional data for weak agonists discovered by the docking screen against the TAAR1 homology model (EC_{50} > 100 µM).

Cmpd (frag/lead)[a]	2D structure	% activation of β-PEA (10 µM)	Most similar known TAAR1 ligand[b]	T_c[c]
6 (frag)		43.7%		0.29
11 (frag)		58.3%		0.23
14 (frag)		74.6%		0.55
15 (frag)		62.0%		0.40
37 (lead)		54.7%		0.26
41 (frag)		46.1%		0.57

[a] Compound from the fragment- (frag) or lead-like screening library.
[b] Most similar TAAR1 ligand in the ChEMBL19 database.
[c] Tanimoto coefficient, calculated with ECFP4 fingerprints.

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