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Similarity- and Substructure-Based Development of β_2-Adrenergic Receptor Ligands Based on Unusual Scaffolds

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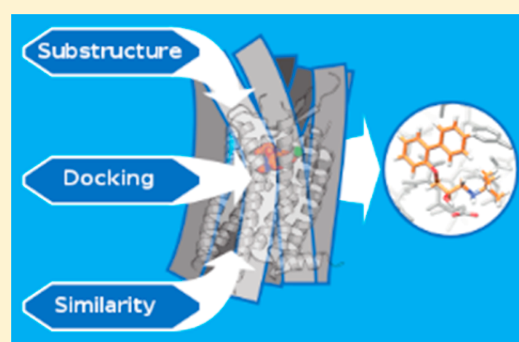
Supporting Information

ABSTRACT: The β_2-adrenergic receptor (β_2AR) is a G protein-coupled receptor (GPCR) and a well-explored target. Here, we report the discovery of 13 ligands, ten of which are novel, of this particular GPCR. They have been identified by similarity- and substructure-based searches using multiple ligands, which were described in an earlier study, as starting points. Of note, two of the molecules used as queries here distinguish themselves from other β_2AR antagonists by their unique scaffold. The molecules described in this work allow us to explore the ligand space around the previously reported molecules in greater detail, leading to insights into their structure−activity relationship. We also report experimental binding and selectivity data and putative binding modes for the novel molecules.



KEYWORDS: *β_2-adrenergic receptor, similarity searches, docking, SAR-by-catalog*

The membrane receptors of the G protein-coupled receptor (GPCR) family are flexible heptahelical bundles transferring signals from the outside to the inside of a cell. This is achieved by a conformational change of the receptor upon binding of a signaling molecule to a cavity located at the extracellular end between the seven helices. GPCRs are expressed in almost all tissues,[1] and it is thus not surprising that approximately 1/3 of present-day drugs interact with a GPCR.[2] Among these receptors, the β_2-adrenergic receptor (β_2AR) is considered a prototypical representative and has been investigated for more than 60 years. It was also the first pharmacologically relevant GPCR to succumb to crystallization in 2007.[3,4]

In a previous work,[5] we have identified six ligands (originally labeled **1−6**, and referred to as **Q1−Q6** in this work to avoid confusion, Chart S1) of the β_2AR through *in silico* docking studies, with affinities ranging from 9 nM to 3.2 μM. Notably, these included two molecules (**5** and **6** in ref 5, denoted as **Q5** and **Q6**, respectively, in the following) that did not follow the classical adrenaline-based scaffold.[6] This was remarkable, as nobody had discovered these scaffolds earlier, despite more than six decades of medicinal chemistry in this area. Building upon the discovery of the six ligands, we wanted to expand chemical space around them. In particular, we wanted to investigate the two ligands with unusual scaffolds by employing *in silico* similarity and substructure searches in the ZINC[7] database. Candidate molecules identified in either way were then docked into the β_2AR, in order to ascertain that their binding modes were consistent. Here we report the results of this combined ligand- and structure-based screen, which also provides insights into the structure−activity relationship (SAR) of molecules **Q5** and **Q6** and their derivatives.

The similarity screen among the 8.5 million molecules of the ZINC database resulted in 6363 molecules, which were distributed across the six query molecules as shown in Table S1. From the substructure-based screen, approximately 653 000 hits emerged. Duplicates were removed from both sets. After docking, 5838 and 587 099 molecules remained, respectively, and the top-scoring 500 of each run were visually inspected. After weeding out molecules with artificially inflated scores due to the absence of corrective terms in present-day scoring functions, e.g., unfavorable desolvation contributions or unsatisfied hydrogen-bond donors, during this inspection, we were left with eight and nine molecules from the similarity and substructure searches, respectively. These were acquired from their respective vendors for further experimental testing (Table S5). Three compounds (**1**, **2**, and **3**) contained a biaryl moiety and a charged amine and thus resembled the classical motif of a β_2AR binder. Indeed, a thorough literature search revealed that these compounds had been described before (Table 1; by the time of selection, these compounds had not been annotated in ChEMBL[8]). To analyze the selectivity of the compounds, we also evaluated them against the closely related β_1AR. The

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Table 1. Affinity (pK_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; Values Are Mean ± SEM of n Separate Experiments

ID	Structure	β2AR pK_D		n	β1AR pK_D		n	β2-selectivity[a]
1[c]		5.42	±0.14	5	4.34	±0.07	4	12.0
2[c]		5.58	±0.06	6	4.56	±0.06	6	10.5
3[d]		10.45	±0.05	8	9.01	±0.04	5	27.5
4		4.63	±0.07	5	4.01[b]	±0.05	5	4.2
5		4.41	±0.08	3	3.59[b]	±0.1	3	6.6
6		4.76	±0.09	5	4.58	±0.03	5	1.5
7		4.66	±0.16	5	4.35	±0.04	4	2
8		4.60[b]	±0.11	4	4.33[b]	±0.05	4	1.9
9		4.84[b]	±0.13	4	4.42[b]	±0.11	4	2.6
10		6.05	±0.11	6	5.51	±0.07	6	3.5
11		5.31	±0.12	6	4.86	±0.05	5	2.8
12		4.75[b]	±0.12	5	n.c.[e]		4	
13		5.26	±0.06	6	4.45	±0.04	5	6.5
ICI 118551		9.61	±0.05	5	6.74	±0.01	5	741
CGP 20712A		5.84	±0.10	5	8.96	±0.13	4	0.0008

[a]Selectivity: $\beta_1/\beta_2 = K_D(\beta_1)/K_D(\beta_2)$ [b]Apparent K_D values: here the maximum concentration of the compound was not sufficient to fully inhibit specific binding; however, the majority of specific binding was inhibited allowing an apparent measure of affinity. [c]US 20090163545. [d]Antiarrythmic

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Table 1. continued

pharmaceutical (Bipranol/Berlafenone), Arzneimittel-Forschung **1992**, 42, 289−291. [e]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.).

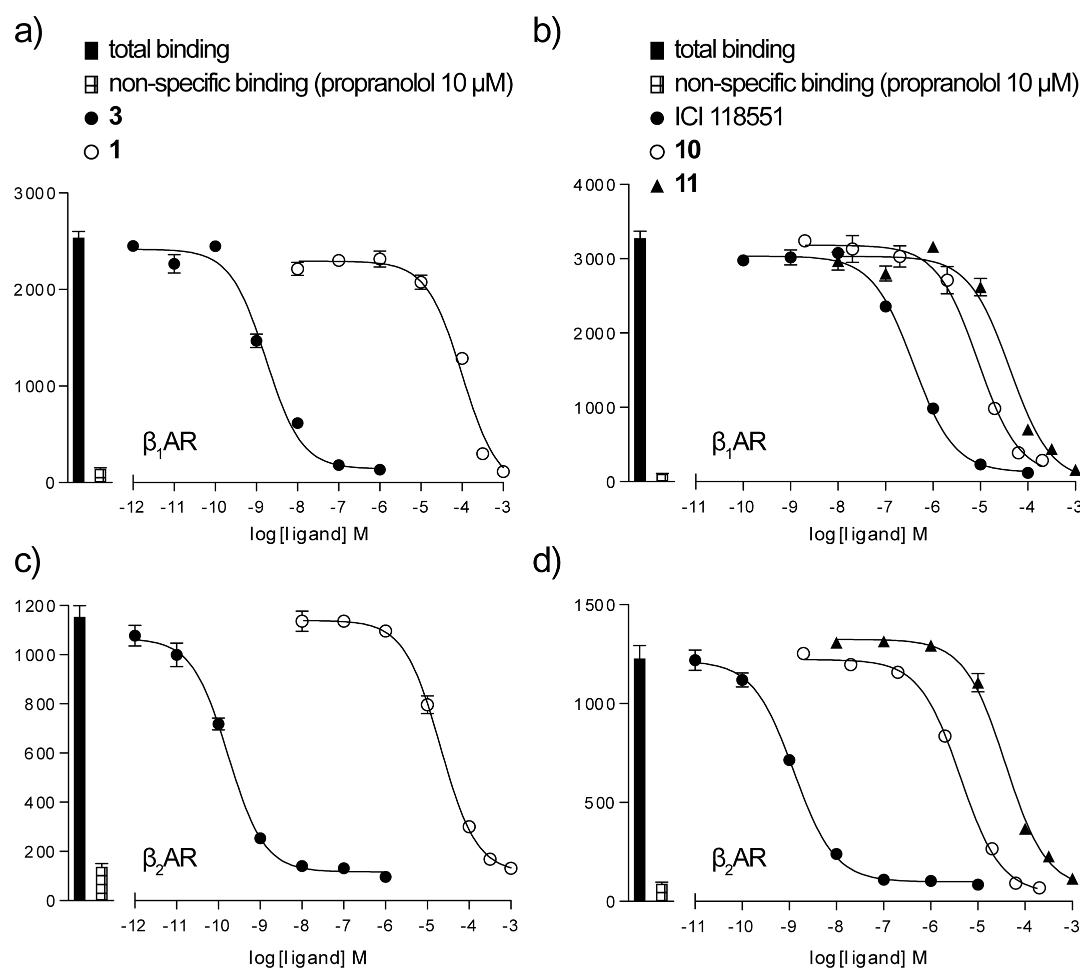

Figure 1. Inhibition of [^{3}H](−)CGP 12177 whole cell binding to (a,b) CHO-β_1 cells and (c,d) CHO-β_2 cells in response to (a,c) **3** and **1** and (b,d) ICI 118551, **10**, and **11**. Bars represent total and nonspecific binding, and data points are mean ± SEM of triplicate determinations. The concentration of [^{3}H](−)CGP 12177 used in these experiments was (a,c) 0.58 nM and (b,d) 0.44 nM, and they are representative of (a) 4, (b) 5, (c) 5, and (d) 5 separate experiments.

efficacy of all compounds was further evaluated in a functional assay.

Several of the compounds identified in this work inhibited [^{3}H](−)CGP 12177 whole cell binding (Table 1; see Supporting Information for assay validation and Table S3 for inactive compounds). This assay also demonstrated that compound **3** had very high affinity (pK_D 9.01 at β_1AR and pK_D 10.45 at β_2AR) and was therefore 28-fold β_2-selective (Figure 1a,c, Table 1). While the remaining compounds had relatively poor affinity in comparison to **3**, many of them, e.g., **1**, **2**, **10**, **11** and **13**, inhibited [^{3}H](−)CGP 12177 binding to yield measurable affinity values (Figure 1b,d, Table 1).

Next, characteristics of ligands were examined in a functional assay, namely, CRE-gene transcription. The ability of ligands to stimulate a response (intrinsic efficacy) was assessed, but also, given that the affinity of many of the ligands to inhibit [^{3}H](−)CGP 12177 binding were at the very limit of the *binding* assay, the ability of ligands to inhibit *functional* responses was also evaluated, thus giving a totally independent measure of affinity from that achieved in the binding assay.

Except for compound **3**, no other compound stimulated a measurable response (n = 4−5 for each compound) in this assay (see Supporting Information for more details and assay validation). However, several compounds antagonized the cimaterol response to give a parallel shift of the cimaterol concentration response curve and thus yield measurable K_D values (Figure S1, Table S2). For some compounds, e.g., **1**, **2**, and **13**, this gave selectivity values similar to those obtained in the binding assay. For other compounds, e.g., **16** and **17**, no rightward shift of the cimaterol response was observed, suggesting no inhibition at the maximum concentration possible (100 μM in each case). For few of the ligands, the highest concentration possible caused a marked fall in CRE-SPAP production to below basal in a manner more consistent with toxicity, cell death, or assay interference, rather than receptor-mediated inverse agonism (see Supporting Information for full details). In these instances, compound concentrations used to inhibit cimaterol responses were reduced until such a time as the reduction in basal was minimal. An example of this was compound **10**, which reduced basal at the maximum

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concentration of 20 μM but not at 2 μM (see Supporting Information). At 2 μM, **10** was still able to cause a rightward shift of the cimaterol concentration response curve at the β_2AR, but not the β_1AR, consistent with its β_2-selectivity. The fall from maximum of the concentration response to cimaterol (most likely because the assay is at the limit of its capability) means that an apparent K_D is reported (calculated from the shift of the lower part of the curve where the lines are parallel), this apparent K_D is however similar to the K_D values obtained from the binding assay, confirming that this is receptor-mediated and β_2-selective.

Compound **3** on its own stimulated a partial agonist response at both the β_1- and β_2AR. This response was inhibited by CGP 20712A in the CHO-β_1-cells with high affinity and by ICI 118551 in the CHO-β_2-cells (Figure S2, Table S4). Furthermore, **3** was able to inhibit the cimaterol responses in both cell lines in a manner consistent with that of a partial agonist (Figure S2, Table S2). Finally, **3** inhibited the response to fixed concentrations of cimaterol in both cell lines in a manner consistent with competition at a single receptor conformation[9] (Figure S2 and Supplementary Procedures for full details).

Altogether, the high affinity of CGP 20712A and ICI 118551 for the CHO-β_1 and CHO-β_2 cells confirm the presence of the β_1- and β_2AR in the respective cell lines. Several of the compounds (e.g., **16** and **17**) did not interact with the receptors in either the binding assay or functional assay up to the maximum concentration possible for the compounds (20–100 μM). Of the molecules with novel scaffolds, **10** and **11** show the highest affinities at pK_D values of 6.05 and 5.31, respectively, for the β_2AR and are thus in a range comparable to those of the established compounds **1** and **2**. These compounds did not induce a functional response in the receptor and are therefore neutral antagonists. However, we emphasize that the outcome of a virtual screening campaign in the manner conducted here is the prediction of binding, not efficacy. Of the novel compounds, **13** exhibited affinity in the binding as well as in the functional assay with low micromolar activity.

The more traditional biaryl compounds **1**, **2**, and **3** display the highest affinities at the β_2AR, as was to be expected. In particular, compound **3** was confirmed as a very high affinity partial agonist at both receptors, but with some β_2AR selectivity. At the β_2AR, the affinity measured by binding (pK_D 10.45) and the affinity measured as antagonism of the cimaterol response (pK_D 10.74) are very similar, confirming the very high affinity ligand–receptor interaction. The partial agonist was itself antagonized by ICI 118551 (yielding a similar pK_D for ICI 118551 as that for antagonism of the cimaterol response), confirming that signaling is indeed occurring via the β_2AR. Compound **3** is therefore a very high affinity, weak partial agonist of the human β_2AR. Moreover, **3** was found to be a partial agonist of the β_1AR, with the agonist response occurring through the primary catecholamine conformation of the receptor (see Supplementary Results).

These three molecules, **1**, **2**, and **3**, were selected by similarity to compounds **Q2**, **Q3**, and **Q4**, all of which contain a biaryl moiety. Not unexpectedly, these hits not only show high affinities but also highest similarities to known (again exclusively biaryl-containing) compounds that are annotated in the ChEMBL database (Table S6). This is encouraging with respect to the performance of similarity screening methods and the value of docking in identifying such compounds. However, it also strongly emphasizes the need for methods that allow for scaffold-hopping to fully explore the ligand space of a target.

By reducing the biaryl scaffold to a 2-ethoxy-ethylamine (**S6** in Chart S2) for the substructure search, two more substances, **4** and **14**, were identified. Compound **4** showed two-digit micromolar affinity, whereas the inhibition by **14** was so weak that no reliable affinity value could be calculated. Interestingly, in **14** the nitrogen matched in the substructure search is the one in the benzoxazine portion, not the exocyclic amine.

Turning to the hits derived from reference molecules **Q5** and **Q6**, we note that they show a much lower Tanimoto similarity of approximately 0.3 and below (when compared to molecules from the ChEMBL database using ECFP4 fingerprints) than the other hits reported in ref 5 (Table S6). This is in line with the fact that these compounds are not based on the classical propanolamine scaffold and underlines the structural novelty of these two scaffolds.

Starting from the benzothiazole-based compound **Q5**, four molecules were identified with benzothiazole (**5**, **10**, **11**, **15**) and two with benzimidazole (**16**, **17**) motifs. Of these, all benzothiazole-containing molecules except **15** show affinity toward the β_2AR in the micromolar range. Docking poses indicate that the orientation of the benzothiazole ring is comparable to the one of **Q5**, with a polarized methyl group interacting with Asp113$^{3.32}$ (Figures S5 and S6). The benzimidazole compounds **16** and **17** show no activity in our assay. These compounds might be more sterically hindered in the vicinity of the positively charged nitrogen atom, in particular compound **16**. Furthermore, the different polarity of the ring system, owing to the variation of the heteroatoms, might render the predicted interaction with Asp113$^{3.32}$ less likely.

Six additional compounds could be identified on the basis of the parent molecule **Q6**. All these molecules (**6**, **7**, **8**, **9**, **12**, and **13**) share a benzofuran-based moiety, independent of whether they originated from the substructure or the similarity search. This moiety, namely, a 3-oxo-4-methyl-6-hydroxy-benzofuran, is present in the parent molecule **Q6**, too, and can thus be considered a "stable scaffold" in terms of SAR. All molecules display affinity, with pK_D values varying between 5.26 and 4.6. Interestingly, **8**, which is the substance with the weakest affinity in this set, differs from **7** only by a methoxy group, which is absent in **8**. This methoxy group could act as an acceptor, which is also present in all remaining molecules of this series as (benzo-)furan or methoxy group. The role of this group is not clearly evident from the docking predictions, but an interaction with Thr195^{ECL2} seems to be the most likely explanation (Figures S5 and S6). Furthermore, the docking poses indicate a binding mode of this scaffold, which resembles the key interactions seen in biaryl-based compounds. The benzofuran scaffold forms interactions with Phe193$^{45.52}$, Phe289$^{6.51}$, Phe290$^{6.52}$, and Val114$^{3.33}$. The hydroxy group at position 6 forms an additional hydrogen bond to Asp113$^{3.32}$, while the ketone serves as acceptor for a hydrogen bond from Ser203$^{5.42}$. A second aromatic moiety is attached at position 2, interacting with Tyr199$^{5.38}$, Tyr308$^{7.35}$, and, presumably, Thr195^{ECL2}. An increased size of the aromatic system appears to be detrimental for affinity (methoxyphenyl in **13** vs benzofuran in **9**). The charged amine in the pyrrolidine moiety is expected to form a salt bridge with Asp113$^{3.32}$.

We have elaborated on six previously identified novel binders of the β_2AR through SAR-by-catalog. Using similarity and substructure searches followed by a docking assessment of the

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interactions of each compound and the receptor, 13 ligands of the β_2AR were verified experimentally. Ten of these molecules are indeed novel ligands for the receptor, while the remaining three turned out to have been described before. Based on this data, several conclusions can be drawn.

First, the benzofuran scaffold of compound **Q5** and the benzothiazole scaffold of compound **Q6** in ref 5 indeed constitute novel chemotypes with derivatization potential for this receptor. Especially the benzofuran series showed a consistent SAR that is in agreement with the predicted binding modes. This study can thus also provide retrospective evidence that the predicted binding modes are indeed very likely correct. The affinities of the novel compounds are not comparable with those of highly optimized adrenaline- or biaryl-based scaffolds. The latter are exemplified by **Q1** with an affinity of 9 nM and **3** with its pK_D of 10.74. However, the novel compounds can serve as unprecedented starting points for further optimization.

Second, that the combination of similarity- and substructure-based searches with protein-structure-based docking constitutes a powerful combination. This is manifest in the quite high hit rate (more than 75% of the molecules bind with an affinity below 100 μM) and the fact that we (re)discovered a molecule with an affinity of only 35 pM. This compound is also known as *bipranol* or *berlafenone*, an antiarrythmia drug.

In terms of selectivity, most of the compounds displaying an affinity are mildly selective toward the β_2AR. Again, **3** takes the lead here at 28-fold selectivity for the β_2AR. While other compounds such as **1** and **2** still have at least 10-fold preference toward the β_2AR, all values are far below 100-fold, which fis considered a ratio that is significant enough to call a compound "selective". Moreover, highly optimized compounds such ICI 118551 show affinity ratios that are closer to 1000-fold. Interestingly, the top three compounds in terms of selectivity all belong to the biaryl cluster of molecules.

Not unexpectedly, most of the compounds with measurable affinity (with the exception of **3**), turned out to be neutral antagonists in the functional assay. This is consistent with what we have seen in our previous study[5] and the fact that we have been docking to an inactive conformation of the receptor.[3,4]

Future studies will show to which affinities the novel scaffolds can be optimized. It is also encouraging to have confirmed that unbiased computational methods can present us with novel molecules, even for target proteins as well-investigated as the β_2AR.

■ EXPERIMENTAL PROCEDURES

Substructure queries (Chart S2) were manually derived from the original hits. Substructure and similarity searches were run on the ZINC database[7] and docked to the β_2AR (PDB 2RH1), as previously described.[5] [^{3}H](−)CGP 12177 whole cell binding and CRE-SPAP production assays were run using CHO-K1 cells expressing either the human β_1AR or the human β_2AR as previously described.[10,11] See Supporting Information for detailed descriptions of experimental procedures.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.6b00363.

Tables of similar compounds, SMILES codes for all compounds, detailed experimental methods, Supplementary Figures and Charts (PDF)

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Author Contributions

P.K. did the original similarity and substructure searches and docking calculations. D.S. and J.G. acquired compounds, prepared assay-ready formats, and supervised initial affinity measurements. J.G.B. performed pharmacological experiments and data analysis. P.K., D.S., and J.G. discussed SAR, and D.S., J.G., J.G.B., and P.K. wrote the manuscript.

Notes

The authors declare no competing financial interest.

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