Allosteric "beta-blocker" isolated from a DNA-encoded small molecule library

Seungkirl Ahn[a,1], Alem W. Kahsai[a,1], Biswaranjan Pani[a], Qin-Ting Wang[b], Shuai Zhao[b], Alissa L. Wall[a,2], Ryan T. Strachan[c], Dean P. Staus[a,d], Laura M. Wingler[a,d], Lillian D. Sun[a,3], Justine Sinnaeve[a,4], Minjung Choi[e], Ted Cho[f,5], Thomas T. Xu[a], Gwenn M. Hansen[g,6], Michael B. Burnett[g,7], Jane E. Lamerdin[h], Daniel L. Bassoni[h], Bryant J. Gavino[h], Gitte Husemoen[i], Eva K. Olsen[i], Thomas Franch[i], Stefano Costanzi[j], Xin Chen[b,8], and Robert J. Lefkowitz[a,d,e,8]

[a]Department of Medicine, Duke University Medical Center, Durham, NC 27710; [b]Department of Medicinal Chemistry, School of Pharmaceutical Engineering and Life Science, Changzhou University, Changzhou 213164, Jiangsu, China; [c]Department of Pharmacology, University of North Carolina, Chapel Hill, NC 27599; [d]Howard Hughes Medical Institute, Duke University Medical Center, Durham, NC 27710; [e]Department of Biochemistry, Duke University Medical Center, Durham, NC 27710; [f]Department of Biology, Duke University Medical Center, Durham, NC 27710; [g]Lexicon Pharmaceuticals, Inc., The Woodlands, TX 77381; [h]DiscoverX Co., Fremont, CA 94538; [i]Nuevolution A/S, 2100 Copenhagen, Denmark; and [j]Department of Chemistry, American University, Washington, DC 20016

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The β_2-adrenergic receptor (β_2AR) has been a model system for understanding regulatory mechanisms of G-protein–coupled receptor (GPCR) actions and plays a significant role in cardiovascular and pulmonary diseases. Because all known β-adrenergic receptor drugs target the orthosteric binding site of the receptor, we set out to isolate allosteric ligands for this receptor by panning DNA-encoded small-molecule libraries comprising 190 million distinct compounds against purified human β_2AR. Here, we report the discovery of a small-molecule negative allosteric modulator (antagonist), compound 15 [([4-((2*S*)-3-(((*S*)-3-(3-bromophenyl)-1-(methylamino)-1-oxopropan-2-yl)amino)-2-(2-cyclohexyl-2-phenylacetamido)-3-oxopropyl)benzamide], exhibiting a unique chemotype and low micromolar affinity for the β_2AR. Binding of 15 to the receptor cooperatively enhances orthosteric inverse agonist binding while negatively modulating binding of orthosteric agonists. Studies with a specific antibody that binds to an intracellular region of the β_2AR suggest that 15 binds in proximity to the G-protein binding site on the cytosolic surface of the β_2AR. In cell-signaling studies, 15 inhibits cAMP production through the β_2AR, but not that mediated by other Gs-coupled receptors. Compound 15 also similarly inhibits β-arrestin recruitment to the activated β_2AR. This study presents an allosteric small-molecule ligand for the β_2AR and introduces a broadly applicable method for screening DNA-encoded small-molecule libraries against purified GPCR targets. Importantly, such an approach could facilitate the discovery of GPCR drugs with tailored allosteric effects.

G-protein–coupled receptor | β_2-adrenergic receptor | allosteric modulator | DNA-encoded small-molecule library | drug discovery

G-protein–coupled receptors (GPCRs), also known as seven transmembrane receptors, represent the largest family of cellular receptors and the most common therapeutic drug targets. Accordingly, GPCRs are the subject of intensive research, both in academia and the pharmaceutical industry, aimed at elucidating their structures, detailed mechanisms of action, and discovery of novel ligands with therapeutic potential (1–3). To date, the overwhelming majority of GPCR drugs target the orthosteric site on the receptors. This is the binding site of endogenous ligands, which generally faces the extracellular surface of the receptor (4, 5). However, in recent years, functionally active allosteric ligands, which bind outside the orthosteric site, have also been discovered. Allosteric ligands that augment or reduce the binding affinity and/or functional responses of orthosteric ligands are referred to as positive or negative allosteric modulators (PAMs or NAMs), respectively (5). Such allosteric ligands hold great therapeutic promise due to their enhanced selectivity among receptor subtypes compared with orthosteric drugs targeting the same subtype. The first approved allosteric drugs for GPCRs target chemokine CCR5 (6) and calcium-sensing receptors (7) to treat HIV infections and hyperparathyroidism, respectively, with many more modulators in preclinical development. Allosteric modulators also have great utility as tool compounds in biophysical studies as they are able to lock receptors into specific conformations by virtue of their cooperative interactions with orthosteric ligands (4, 5).

In the past, screening for GPCR ligands, either allosteric or orthosteric, has been cumbersome and labor-intensive. Such screens have generally been based on the functional ability of compounds to either stimulate or block receptor-mediated activities in whole-cell–based settings (8). A more rapid and efficient approach is to use interaction-based methods for initial screening wherein large libraries of molecules are panned against the target receptor. However, until recently, such libraries have consisted only of macromolecules such as phage-displayed antibodies (9, 10)

Significance

The present study reports the discovery of a small-molecule negative allosteric modulator for the β_2-adrenergic receptor (β_2AR) via in vitro affinity-based iterative selection of highly diverse DNA-encoded small-molecule libraries. Characterization of the compound demonstrates its selectivity for the β_2AR and that it negatively modulates a wide range of receptor functions. More importantly, our findings establish a generally applicable, proof-of-concept strategy for screening DNA-encoded small-molecule libraries against purified G-protein–coupled receptors (GPCRs), which holds great potential for discovering therapeutic molecules.

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[1]S.A. and A.W.K. contributed equally to this work.

[2]Present address: Department of Internal Medicine, University of Michigan, Ann Arbor, MI 48109.

[3]Present address: Cleveland Clinic Lerner College of Medicine, Case Western Reserve University, Cleveland, OH 44195.

[4]Present address: Department of Cell and Developmental Biology, Vanderbilt University School of Medicine, Nashville, TN 37240.

[5]Present address: School of Medicine, Georgetown University, Washington, DC 20007.

[6]Present address: Library Discovery Team, Nurix, Inc., San Francisco, CA 94158.

[7]Present address: Bethyl Laboratories, Inc., Montgomery, TX 77356.

[8]To whom correspondence may be addressed. Email: lefko001@receptor-biol.duke.edu or xinchen@cczu.edu.cn.

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or RNA aptamers (11). Another approach, which has not yet been widely applied, is screening of DNA-encoded small-molecule libraries (DELs). In this approach, remarkably large combinatorial libraries consisting of up to billions of small-molecule compounds are displayed on DNA fragments that serve as barcodes for their subsequent identification (12, 13). In the past few years, application of the DEL screening technology for soluble proteins has produced inhibitors against cancer and immune disorders and against therapeutic targets. These are protein kinases such as Src, MK2, Akt3, Pim1, Aurora A kinase, p38 mitogen-activated protein (MAP) kinase, and antiapoptotic protein Bcl-xL (14, 15). Expanding such technology to GPCRs has the potential to yield both orthosteric and allosteric ligands. However, its application to GPCR screening has been challenging, largely because of difficulties associated with preparing appropriate receptor targets as well as the membrane-bound nature of the receptors, which can lead to nonspecific interactions. To date, identification of a compound that inhibits the NK_3 tachykinin receptor by screening against the receptor expressed in whole cells represents the only successful application of DEL technology against GPCRs (16).

The β_2-adrenergic receptor (β_2AR) has served as the model system for molecular studies of ligand-binding GPCRs for over 40 y (1) and plays a significant role in cardiovascular and pulmonary diseases. So-called "β-blockers," which are orthosteric antagonists of the receptor, are mainstays of cardiovascular medicines used to treat a wide variety of illnesses (17–19). On the other hand, β-agonists have proven very effective against asthma (20). However, all known β-adrenergic ligands act orthosterically; thus, it is possible that allosteric modulators would possess enhanced therapeutic efficacy, selectivity, or even unique therapeutic properties such as signaling bias. Such ligands would also facilitate the isolation and characterization of specific receptor conformations for biophysical studies. Accordingly, here we set out to isolate allosteric ligands for the β_2AR using DELs. We report isolation of a small-molecule negative allosteric modulator (antagonist) for the β_2AR and provide a detailed characterization of its pharmacological properties and interaction with the receptor.

Results

Isolation of Compound 15 from DELs. To identify unique chemotypes that bind at structurally relevant sites on the surface of β_2AR, we screened DELs against purified, unliganded β_2AR maintained in the detergent *n*-dodecyl-β-D-maltoside (DDM) (Fig. 1*A* and Fig. S1). A similar beads-only selection was performed in parallel as a control. In total, we screened three libraries together containing approximately a total of 190 million unique compounds synthesized using a DNA-tagged, split-and-pool combinatorial chemical synthesis approach (Chemetics; Nuevolution). In each library, ~5 × 10^{13} molecules in total were used as input, and 1–7 × 10^7 molecules remained after screening. Relative quantification of the recovered compounds was achieved by a combination of PCR amplification and next-generation sequencing of eluted DNA barcodes, followed by computational decoding approaches. To refine the output and eliminate potential nonspecific binders, compounds that displayed less than a 260- to 470-fold increase in frequency from baseline as well as those that were observed in bead-only control selections were filtered from the dataset, leaving a total of 394 potential β_2AR binders for further analysis (Table S1). These compounds were then clustered based on their structural similarity, and 16 putative hits were selected as representatives for these clusters. DNA-tagged versions of these 16 hits were resynthesized and screened individually to evaluate their influence on the binding affinity of orthosteric agonists in radioligand binding assays with membranes obtained from β_2AR-overexpressing cells. One compound [4-((2*S*)-3-(((*S*)-3-(3-bromophenyl)-1-(methylamino)-1-oxopropan-2-yl)amino)-2-(2-cyclohexyl-2-phenylacetamido)-3-oxopropyl)benzamide], designated as compound "**15**" (Fig. 1*B*), markedly decreased orthosteric agonist binding to the receptor and was thus selected for further characterization.

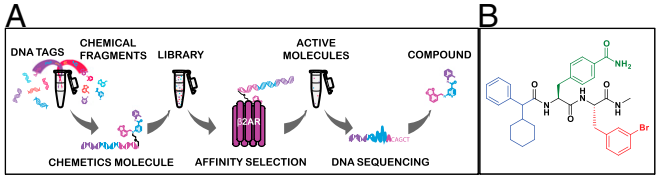

Fig. 1. Screening of the DEL and the chemical structure of **15**. (*A*) Schematic illustration of the screening of a DNA-encoded compound library. DNA-encoded library molecules, synthesized using a DNA-tagged, split-and-pool combinatorial chemical synthesis approach, were mixed with a target (purified β_2AR) immobilized on a matrix. Target binding (active) molecules were collected through affinity-based selection, and the encoding DNA tags were sequenced to identify the binding molecules. (*B*) Compound **15** is composed of three building blocks: methybenzamide (green), bromo-benzyl (red), and cyclohexylmethyl-benzene (blue). The amide backbone is shown in black.

Characterization of Compound 15 for Its Binding to the β_2AR. To further characterize the pharmacological properties of **15**, we synthesized it in its DNA-free form (Fig. 1*B*). We first performed competition binding experiments with the radioiodinated antagonist cyanopindolol (^{125}I-CYP) to evaluate the influence of **15** on the binding ability of orthosteric ligands to the β_2AR reconstituted into high-density lipoprotein (HDL) (also known as nanodisc) particles (21). We found that, although **15** had little to no influence on binding of ^{125}I-CYP, which is a neutral orthosteric antagonist of the β_2AR, it robustly decreased the binding affinity of the agonist isoproterenol for the receptor in a dose-dependent fashion (Fig. 2*A*). Compound **15** caused the isoproterenol competition curve to shift to the right by close to one log (ninefold) at the maximal concentration tested. The half-maximal concentration of **15** for this shift was ~1.9 μM (Fig. 2*B*). On the other hand, when the inverse agonist [^{3}H]-ICI-118,551 was used as an orthosteric ligand tracer, **15** significantly increased (18 ± 1% max) its binding to the β_2AR in a dose-dependent manner (Fig. 2*C*) with an EC_{50} value of 0.48 μM, which is close to that obtained in Fig. 2*B* (1.9 μM). This reflects positive cooperativity between **15** and the orthosteric antagonist [^{3}H]-ICI-118,551 for binding to the receptor. To validate the direct binding of **15** to the β_2AR, we performed isothermal titration calorimetry (ITC). By this technique, we found that the equilibrium dissociation constant (K_d) of **15** for the receptor is 1.7 ± 0.8 μM, and the stoichiometry of the interaction is 1, suggesting that **15** binds to one site on the β_2AR (Fig. 2*D*). The K_d value obtained by ITC is in good agreement with the half-maximal concentration of the shift obtained in the radioligand competition binding experiment (Fig. 2*B*) as well as the EC_{50} value obtained from the **15** titration curve for [^{3}H]-ICI-118,551 binding (Fig. 2*C*).

To further confirm that **15** favors an inactive conformational state of the receptor, we assessed the extent of binding of an inactive conformation-specific β_2AR single domain antibody [nanobody-60 (Nb60)] that binds to an intracellular region of the receptor (10, 22). Contrary to the positive cooperativity predicted to occur between a negative allosteric modulator and Nb60 (22), we found a decrease in Nb60 binding to the receptor in the presence of **15** (Fig. 2*E*). This suggests that **15** competes with Nb60 for binding to the receptor, and thus that their binding sites at least partially overlap, indicating that **15** binds to an intracellular region of the β_2AR.

It has been well appreciated that allosteric transducers such as heterotrimeric G proteins or β-arrestins promote high-affinity agonist binding (10, 23, 24). We also observed that **15** inhibited the high-affinity binding of a radiolabeled β_2AR-agonist, [^{3}H](*R*,*R*′)-4-methoxyfenoterol (^{3}H-Fen) (25) that is promoted by either heterotrimeric Gs protein or β-arrestin1 (Fig. 2*F*). This finding also suggests that **15** negatively modulates activation of the β_2AR by agonists. Furthermore, the dose-dependent effects of **15** in ^{3}H-Fen binding assays conducted in the presence of either G protein or

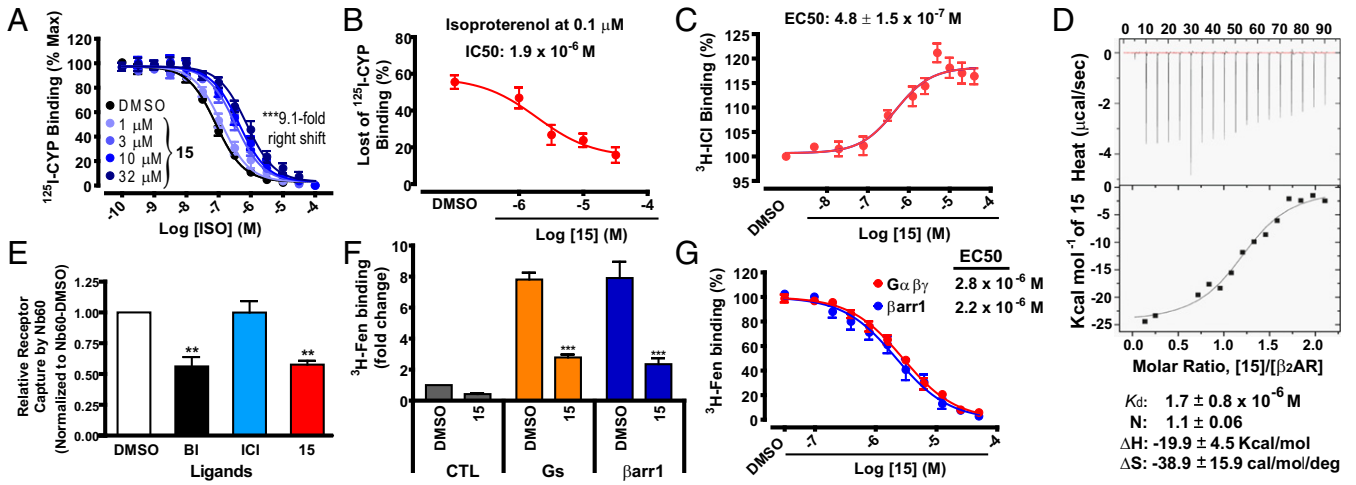

Fig. 2. Characterization of **15** for its binding to the β_2AR. (*A*) Dose–response curves of isoproterenol (ISO) competition binding to the β_2AR reconstituted in nanodiscs with ^{125}I-CYP were obtained in the presence of various concentrations of **15** as indicated. Values were expressed as percentages of the maximal ^{125}I-CYP binding level obtained from a one-site competition binding-log IC_{50} curve fit of the vehicle [0.9% dimethyl sulfoxide (DMSO)] control data. Points on curves represent mean ± SEM obtained from at least three independent experiments done in duplicate. (*B*) The half-maximal concentration of **15** in the changes of isoproterenol competition binding was obtained from a dose–response curve replotted with the data set at 0.1 μM isoproterenol with various doses of **15** in *A*. (*C*) Dose-dependent increases in inverse agonist ^{3}H-ICI-118,551 (ICI) binding to the β_2AR in nanodiscs. Points on the curve represent normalized values as percentages of the ^{3}H-ICI-118,551 binding amount in the absence of **15** and mean ± SEM obtained from at least four independent experiments done in duplicate. (*D*) Characteristics of **15** for its physical interaction with the β_2AR were determined by the isothermal titration calorimetry (ITC) analysis with the detergent-solubilized, purified receptor. The thermogram (*Top*) and binding isotherm with the best titration curve fit (*Bottom*) shown are representatives of three independent experiments. Values represent mean ± SEM. (*E*) Extent of nanobody-60 (Nb60) binding to the β_2AR determined by ELISA in the presence of different ligands including **15**. Values were expressed as ratios of the level of Nb60 binding in the vehicle (0.5% DMSO) control sample and represent mean ± SEM obtained from three independent experiments done in duplicate. BI, BI-167107. (*F*) The levels of ^{3}H-Fen binding to the β_2AR upon treatment with the vehicle control (0.5% DMSO) or **15** at 50 μM in the absence or presence of transducers, either trimeric $G_{\alpha\beta\gamma}$ protein or β-arrestin1 (β-arr1) together with Fab30. Values were expressed as fold changes of the level of ^{3}H-Fen binding in the vehicle (DMSO) control sample without the transducer and represent mean ± SEM obtained from three independent experiments done in duplicate. (*G*) Compound **15** dose-dependent decreases in the level of ^{3}H-Fen high-affinity binding to the β_2AR promoted by either $G_{\alpha\beta\gamma}$ protein or β-arr1 together with Fab30. Values were expressed as percentages of the maximal ^{3}H-Fen binding level promoted by each transducer in the vehicle control (0.5% DMSO) and represent mean ± SEM obtained from at least three independent experiments done in duplicate. All of the statistical analyses in the figure were performed, as described in *Materials and Methods*.

β-arrestin were similar to each other (Fig. 2*G*). We used Fab30, which stabilizes an active conformation of β-arrestin1 (26, 27), to enhance the weak high-affinity ^{3}H-Fen binding signal induced by β-arrestin to levels comparable to those observed with G protein. The EC_{50} values obtained from the **15** dose–response curves for the two transducer-promoted signals were comparable (2.8 vs. 2.2 μM, respectively, for G protein and β-arrestin; Fig. 2*G*). This suggests that **15** displays no strong "bias" between its inhibitory activities in the two transducer-induced high-affinity ^{3}H-Fen binding signals. The EC_{50} values of **15** obtained here (Fig. 2*G*) are also in good agreement with its K_d measured by ITC (Fig. 2*D*). Taken all together, the results in Fig. 2 show that **15** behaves as a negative allosteric modulator for the β_2AR, and suggest that it binds at the cytoplasmic surface of the receptor.

Functional Modulation of β_2AR Activity by Compound 15. Next, we investigated the effects of **15** on β_2AR function in cells by measuring G-protein–mediated cAMP production (28, 29) and β-arrestin recruitment to the receptor (29, 30). Due to the high signal amplification of the G-protein activation assay compared with β-arrestin recruitment, to achieve comparable signaling outputs between the two, we used endogenously expressed β_2AR in the reporter cells to measure cAMP production, but we used stably overexpressed β_2V_2R for monitoring β-arrestin recruitment. The β_2V_2R, a chimeric receptor with a V_2R tail at the C terminus, displays stronger and more stable agonist-promoted β-arrestin binding than the native β_2AR while retaining the pharmacological properties of the native β_2AR (31). Compound **15** decreased the isoproterenol-stimulated responses in both assays (Fig. 3 *A* and *B*). We observed rightward shifts of the EC_{50} values, as well as decreases in the maximal level of the stimulated responses in the presence of increasing concentrations of **15**, indicating that **15** inhibits β_2AR agonist-induced functional responses. Additionally, the extent of rightward shift of isoproterenol potency promoted by **15** was similar for both G-protein activation and β-arrestin recruitment to activated receptor. On the other hand, the decreases in the maximal response induced by **15** were more robust in β-arrestin recruitment than in cAMP production (Fig. 3 *A* and *B*). This likely is attributed to the differences in the sensitivity between

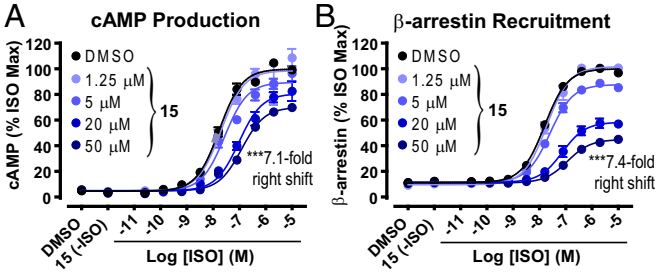

Fig. 3. The effect of **15** on β_2AR-mediated functional activities. After pretreatment with **15** for 20 min at various concentrations as indicated, the β_2AR-mediated activities in cells were measured upon stimulation with isoproterenol (ISO) in a dose-dependent manner: (*A*) cAMP production by the endogenously expressed β_2AR and (*B*) β-arrestin recruitment to the exogenously expressed β_2V_2R. Values were expressed as percentages of the maximal level of the isoproterenol-induced activity in the vehicle (0.5% DMSO) control. Points on curves represent mean ± SEM obtained from four independent experiments done in duplicate. All of the statistical analyses in the figure were performed as described in *Materials and Methods*.

the assays, as described before (29), rather than to any biased activity of **15**. Such unbiased inhibition of β_2AR activity by **15** is consistent with the finding that 15 similarly inhibits the high-affinity binding of ^{3}H-Fen promoted by either transducer (G protein or β-arrestin) (Fig. 2*G*). These results confirm that **15** modulates β_2AR by antagonizing its agonist-induced activity.

We further validated that the inhibitory effect of **15** in functional assays was a result of specific inhibition at the receptor level and not due to nonspecific effects by testing **15** in other cell-based assays that have distinct readouts from the luminescence-based assays shown in Fig. 3. We monitored the effect of **15** on cAMP production upon stimulation of the overexpressed β_2AR using a fluorescence resonance energy transfer (FRET)-based biosensor, ICUE2 (32). In this experiment, **15** caused a rightward shift in the EC_{50} for isoproterenol-stimulated cAMP production (Fig. S2*A*), consistent with that shown in Fig. 3*A*, albeit without changing the maximal response. This may be attributed to the increased amplification of the signal due to overexpression of the β_2AR in this assay compared with the signal by the receptor expressed at endogenous levels in Fig. 3*A*. Similarly, we also confirmed the inhibitory effect of **15** on agonist-stimulated β-arrestin recruitment to the β_2AR by assessing FRET signals between β_2AR-YFP and CFP-β-arrestin2 (Fig. S2*B*). We further confirmed the inhibitory effect of **15** on β_2AR activation using an in vitro GTPase activity assay with the β_2AR reconstituted into HDL particles together with purified Gs protein (Fig. S2*C*). The **15**-induced shift in the EC_{50} for isoproterenol obtained in this in vitro assay is comparable to that observed in the cell-based assays, suggesting that **15** must readily penetrate cell membranes to bind to an intracellular region of the β_2AR, which is suggested by the result in Fig. 2*E*.

Selective Inhibition of β_2AR-Mediated Activities by Compound 15. To confirm the selectivity of **15** for the β_2AR, we performed functional assays to evaluate whether **15** inhibited activation of other members of the GPCR family closely or distantly related to the β_2AR. First, we compared the extent of **15** blockade of agonist-stimulated activities of β_2AR with two other endogenously expressed Gs-coupled receptors in HEK-293 cells, the prostaglandin E2 (PGE2) and vasoactive intestinal peptide (VIP) receptors. Notably, **15** had no effect on cAMP production following stimulation of these receptors (Fig. S3 *A* and *B*). Additionally, we looked at the specificity of **15** for the β_2AR by assessing its inhibitory effect on β-arrestin recruitment to other receptors besides the chimeric β_2AR (β_2V_2R). Here, we used the parental cell line stably expressing β-arrestin2 alone, and transiently expressed receptors indicated in Fig. 4. First, we confirmed that the extent of **15**-mediated inhibition of agonist-stimulated β-arrestin recruitment to the wild-type β_2AR (Fig. 4*A*) is comparable to that obtained in the transiently expressed chimeric receptor, β_2V_2R (Fig. 4*B*). On the other hand, following stimulation of the β_1AR, a receptor closely related to the β_2AR, **15** substantially inhibited the maximal response as well as the basal activity in a concentration-dependent manner, whereas no significant changes were observed in the EC_{50} value (Fig. 4*C*). We also observed significant, but much reduced, inhibitory effects of **15** on β-arrestin recruitment to the vasopressin V_2 receptor (V_2R), which is also a Gs-coupled receptor (Fig. 4*D*). In contrast to this, **15** only minimally inhibited agonist-induced β-arrestin recruitment to the VIPR, another Gs-coupled receptor. We observed only minimal decreases in the maximal response to stimulation with VIP without any change in the EC_{50} value induced by **15** (Fig. 4*E*), consistent with the result obtained in cAMP accumulation (Fig. S3*B*). Furthermore, no significant inhibition by **15** was detected in β-arrestin recruitment to the Gq-coupled angiotensin II type 1 receptor (AT_1R) (Fig. 4*F*). To further assess the extent of the **15** inhibitory activities among different receptors, we quantified the level of **15**-mediated decreases in the maximal response as well as shifts of the EC_{50} value exhibited as fold shifts (Table S2). These results demonstrate that the inhibitory effect of **15** on agonist-stimulated responses is greatest for the β_2AR and is substantially diminished in even closely related receptors such as β_1AR.

To obtain further insights into the specificity of **15** for the β_2AR, we examined its inhibitory activity on agonist-induced β-arrestin internalization. Unlike "class B" receptors, including the V_2R and the AT_1R whose tight interactions with β-arrestin allow for their cointernalization, "class A" receptors such as the β_2AR have weaker β-arrestin interactions and are not cointernalized with β-arrestin (33). Therefore, we examined the effect of **15** on this functional activity with the transiently expressed β_2V_2R (Fig. S4*A*), V_2R (Fig. S4*B*), and AT_1R (Fig. S4*C*). The extent of β-arrestin internalization was monitored by measuring the amount of β-arrestin targeted to endosomes (34). Results obtained in this assay are consistent with the inhibitory effects of

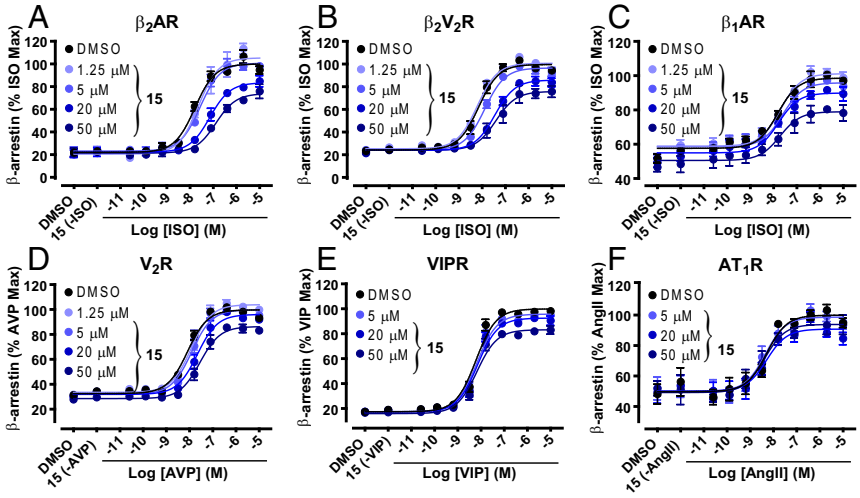

Fig. 4. Specificity of **15** inhibition for β_2AR-mediated activity. Various receptors were transiently expressed to monitor β-arrestin recruitment, including (*A*) β_2AR, (*B*) β_2V_2R, (*C*) β_1AR, (*D*) V_2R, (*E*) VIPR, and (*F*) AT_1R. After pretreatment with **15** at different concentrations as indicated for 20 min, the extent of agonist-induced β-arrestin recruitment to these receptors was determined in a dose-dependent manner. Values were expressed as percentages of the maximal level of the activity induced by the agonist of each receptor in the vehicle (0.5% DMSO) control. Points on graphs represent mean ± SEM obtained from at least three independent experiments done in duplicate. AngII, angiotensin II; AVP, arginine vasopressin; ISO, isoproterenol; VIP, vasoactive intestinal peptide.

15 on β-arrestin recruitment to activated receptors as shown in Fig. 4, which are summarized in Table S2, further confirming the specificity of the modulating activity of **15** for the β_2AR.

We also investigated whether the NAM activity of **15** at the β_2AR was dependent on the presence of a specific agonist at the orthosteric site (i.e., probe dependence). We performed this by monitoring Gs-mediated cAMP production and β-arrestin recruitment to the receptor in the presence of orthosteric probes ranging from full to weak partial agonists, including epinephrine, fenoterol, and clenbuterol (Fig. S5) as done with isoproterenol (Fig. 3). We also compared competition binding of these agonists to the β_2AR with ^{125}I-CYP in the presence of different concentrations of **15** (Fig. 2*A* and Fig. S5), which allowed us to assess the probe dependence of **15** among the agonists in the absence of transducer coupling. Table S3 shows the summary of quantified values in each assay, including the extent of **15**-mediated decreases in the maximal response and shifts of the EC_{50} value exhibited as fold shifts. Overall, **15** appears to display no significant probe dependence among the tested agonists. We observed that the extent of the EC_{50} value shift by **15**, which is consistent among the tested assays, follows the efficacy of the tested agonists. On the other hand, the magnitude of **15** inhibition of the maximal response is negatively correlated with the efficacy of these agonists.

Structure–Activity Relationships of Compound 15 Analogs at the β_2AR. To discern the structure–activity relationship (SAR) pattern for the allosteric modulation of **15** at the β_2AR, we designed and synthesized a series of **15** derivatives (Table S4). We assessed the ability of these derivatives to modulate β_2AR functions in two different types of experimental settings. These were cell-based activity assays, including G-protein–mediated cAMP production and β-arrestin recruitment to the activated β_2AR, as well as high-affinity binding of the agonist ^{3}H-Fen to the receptor induced by transducers, Gs or β-arrestin. To assist our SAR analyses, **15** was divided into three structural subunits, the methylbenzamide (region I), bromo-benzyl (region II), and cyclohexylmethyl-benzene (region III) regions, into each of which we introduced modifications. We found that the formamide group in region I (methylbenzamide) is an important determinant of functional properties of **15**. Removal of this group on the phenyl ring (A1) led to a dramatic decrease in the inhibitory activity of **15** down to about 20% or less of its original activity. The same, but less severe, trend was observed when the position of this formamide group was changed from its original *para*-position to a *meta*-position (A2), which resulted in a ∼60% reduction of its original activity. In the case of region II (bromo-benzyl), removal of the electronegative atom bromine (A3) also caused variable but substantial attenuation of the inhibitory activity of **15** down to about 10–55%. Two other modifications in this region, replacement of bromine with fluorine, an atom of comparable electronegativity but smaller radius (A4), and introduction of additional bromine at the *meta*-position of the phenyl ring (A5) modestly decreased the functional effects of **15**. Next, we evaluated the activity of derivatives with modifications on the aromatic ring in region III (cyclohexylmethyl-benzene). Interestingly, addition of a hydroxyl group to this ring at the *para*-position (A6) led to dramatic loss of inhibitory activity, while replacing the hydroxyl group at this position with a slightly hydrophobic methoxy group (A7) partially restored the inhibitory activity. This strongly suggests that the hydrophobic nature of this region is another important determinant for efficient interaction of **15** with the presumably hydrophobic portion of the putative binding site of **15** on the β_2AR.

Discussion

We report here the discovery and characterization of a small molecule, compound **15**, as an allosteric β-blocker. Compound **15** was derived from an in vitro affinity-based screening of DELs against the purified human β_2AR. Compound **15** shares no structural or chemical similarities with known β_2AR orthosteric ligands, and it does not compete with radiolabeled β_2AR ligands for binding at the orthosteric site. On the other hand, it binds allosterically to the β_2AR with low micromolar affinity. The compound negatively modulates the binding of agonists to the β_2AR while it clearly displays positive cooperativity with an orthosteric inverse agonist. In addition, in cell-based functional assays, **15** displays robust inhibition of β_2AR agonist-promoted, Gs-mediated cAMP generation as well as β-arrestin recruitment to the receptor. Together, these characteristics demonstrate that **15** allosterically binds to and stabilizes an inactive conformation of the β_2AR, which are the classic hallmarks of a negative allosteric modulator (5, 35).

A large number of orthosteric ligands for the β_2AR have been developed, whereas before this study, no allosteric small-molecule β_2AR ligand had been identified. The affinity-based screening strategy is an ideal way to identify allosteric ligands for a receptor, and DEL screening is an innovative strategy to perform affinity-based selections against targets that are isolated or expressed on whole cells (12). Although this technique enables an unprecedented increase in the size of libraries that can be screened compared with conventional activity-based screening formats, its use has mostly been limited to soluble protein targets (12, 13). Due to the inherent difficulty in isolating functional membrane proteins, this technique has been only rarely used to obtain ligands for GPCRs. To date, there has been only one report describing the discovery of a ligand for a GPCR from a DEL (16). There, the recombinant NK_3 tachykinin receptor expressed on HEK-293 cells was used as a target in a whole-cell selection format to identify an inhibitor for the receptor (16). Here, we have demonstrated that the DEL screening strategy can be successfully applied to the isolation of small-molecule ligands using a purified GPCR. Although our study was focused on isolating and characterizing β_2AR allosteric ligands, this strategy could be used as well to isolate ligands that target orthosteric sites of GPCRs. Despite the power of this approach, predicting the functional outcomes of the isolated compounds remains empirical. However, in our in vitro purified receptor target system, it is highly feasible to bias the selections through differential display of the receptor in unique conformations (e.g., agonist vs. antagonist vs. no ligand in the orthosteric site) or in complex with signaling partners such as G proteins or β-arrestins. This should provide allosteric modulators with distinct properties (e.g., NAMs, PAMs, or even biased molecules for coupling to transducers, leading to signaling bias).

To date, pharmacological studies of GPCR allosteric modulation have been restricted to a few receptor families including muscarinic acetylcholine, adenosine, chemokine, and metabotropic glutamate receptors (5, 36). Compared with orthosteric ligands, drugs targeting allosteric sites often display greater receptor subtype selectivity and therefore potentially reduce adverse side effects (4, 5). This is presumably due to decreased evolutionary pressure at allosteric sites than at the orthosteric site of GPCRs (35), leading to their greater divergence within a family. Moreover, multiple allosteric sites can exist on a given receptor (37). In addition, allosteric GPCR modulators may have greater potential than orthosteric ligands to engender biased signaling through selective modulation of specific signaling pathways, for example, G-protein versus β-arrestin pathways (4).

Our SAR studies provide insights into key regions of **15** that must engage in contacts with the allosteric binding site on the β_2AR to allow its functional modulation. We found some alterations, including complete deletions of the formamide group in region I and bromine in region II, lead to dramatic decreases in the functional activities of the parent compound, **15**. We also observed a positive association between increased polarity of region III and loss of functional activity. This suggests that this region of the molecule might interface deep within the β_2AR allosteric site to establish contacts with core hydrophobic residues.

As with other GPCRs, several putative allosteric sites on the β_2AR have been recently proposed based on crystal structures (37). Some are located at the intracellular face of the receptor; these are relatively large and can accommodate a wide range of compound sizes. Interestingly, most of the currently reported non–small-molecule β_2AR allosteric modulators, such as nanobodies (10, 22, 38) and RNA aptamers (11), bind to intracellular cavities that overlap with the G-protein binding site (39). This appears to be true as well for **15** because it competes for β_2AR binding with a nanobody (Nb60) that favors an inactive conformation and that binds to this intracellular region of the β_2AR (22). Although our findings suggest that **15** binds to the intracellular region of the β_2AR, further SAR and structural studies at atomic-level resolution will be required to precisely define the site and mechanism of action, by which **15** acts as a NAM.

In summary, our study reports the discovery via in vitro affinity selection of a DEL against purified receptors and functional characterization of a β_2AR-selective negative allosteric modulator. Our findings suggest that targeting GPCR allosteric sites with such combinatorial small-molecule libraries provides a powerful and efficient approach for developing highly selective ligands that can modulate a wide range of receptor's functional activities. Furthermore, our findings establish a proof-of-concept strategy using the DEL screening technique, which can be broadly applied to discover small molecules for other GPCRs.

Materials and Methods

Complete details and descriptions of the materials used; cell culture and transfections; expression and purification of the β_2AR; purified β_2AR-based DEL selection, quantitative PCR, and next-generation sequencing analysis; reconstitution of the β_2AR into HDL particles; radioligand binding; ITC; ELISA; measurement of cAMP accumulation and in vitro GTPase activity; β-arrestin recruitment and β-arrestin endocytosis assays; data analyses; and synthesis and characterization of compounds are provided in *SI Materials and Methods*.

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