Acta Pharmacologica Sinica

www.nature.com/aps

ARTICLE

Discovery of β-arrestin-biased β_2-adrenoceptor agonists from 2-amino-2-phenylethanol derivatives

Anthony Yiu-Ho Woo[1,2], Xin-yue Ge[3,4], Li Pan[3,4], Gang Xing[3,4], Yong-mei Mo[3,4], Rui-juan Xing[3,4,9], Xiao-ran Li[1], Yu-yang Zhang[1], Irving W. Wainer[5,6], Mao-sheng Cheng[3,4] and Rui-ping Xiao[2,7,8]

β-Arrestins are a small family of proteins important for signal transduction at G protein-coupled receptors (GPCRs). β-Arrestins are involved in the desensitization of GPCRs. Recently, biased ligands possessing different efficacies in activating the G protein- versus the β-arrestin-dependent signals downstream of a single GPCR have emerged, which can be used to selectively modulate GPCR signal transduction in such a way that desirable signals are enhanced to produce therapeutic effects while undesirable signals of the same GPCR are suppressed to avoid side effects. In the present study, we evaluated agonist bias for compounds developed along a drug discovery project of β_2-adrenoceptor agonists. About 150 compounds, including derivatives of fenoterol, 2-amino-1-phenylethanol and 2-amino-2-phenylethanol, were obtained or synthesized, and initially screened for their β-adrenoceptor-mediated activities in the guinea pig tracheal smooth muscle relaxation assay or the cardiomyocyte contractility assay. Nineteen bioactive compounds were further assessed using both the HTRF cAMP assay and the PathHunter β-arrestin assay. Their concentration-response data in stimulating cAMP synthesis and β-arrestin recruitment were applied to the Black–Leff operational model for ligand bias quantitation. As a result, three compounds (L-2, L-4, and L-12) with the core structure of 5-(1-amino-2-hydroxyethyl)-8-hydroxyquinolin-2(1*H*)-one were identified as a new series of β-arrestin-biased β_2-adrenoceptor agonists, whereas salmeterol was found to be G_s-biased. These findings would facilitate the development of novel drugs for the treatment of both heart failure and asthma.

Keywords: β_2-adrenoceptor agonists; cAMP; β-arrestin; biased agonism; fenoterol; salmeterol; heart failure; asthma

Acta Pharmacologica Sinica (2019) 0:1–11; https://doi.org/10.1038/s41401-018-0200-x

INTRODUCTION

G protein-coupled receptors (GPCRs) represent the largest class of druggable targets [1]. In recent years, functional selectivity has emerged as a major concept for the redevelopment of many traditional GPCR drug targets, such as β-adrenoceptors [2–6]. This concept suggests that a so-called biased ligand could be used to selectively modulate GPCR signal transduction in such a way that desirable signals are enhanced to produce therapeutic effects, whereas undesirable signals of the same GPCR are suppressed to avoid side effects. The terms "functional selectivity" and "biased agonism" are often used interchangeably. Although "biased agonism" is sometimes used in a stricter sense to refer to "β-arrestin-biased agonism" [7], it generally describes the disparity of the efficacies of ligands in activating signals mediated by different downstream effectors, for example, different G protein isoforms, G protein versus β-arrestin or biases from many other signaling readouts. In some cases, the biased ligand could act as an antagonist or an inverse agonist for G protein-dependent signaling but as an agonist for β-arrestin-dependent signaling in a single GPCR.

Ligand biases at both β_1- and β_2-adrenoceptors have been described [6]. For example, carvedilol is an antagonist of both β-adrenoceptors for the G_s pathway, but it is also mildly active in triggering β-arrestin-dependent signaling [8, 9]. It has been suggested that the superior efficacy of carvedilol compared with other β-blockers in heart failure may be due to the activation of the β-adrenoceptor-mediated β-arrestin-dependent signaling [7, 8].

Although it is well-established that long-term stimulation of the β_1-adrenoceptor would lead to cardiomyocyte death and predisposes the heart to failure [10–13], the role of the β_2-adrenoceptor is still unclear. As the β_2-adrenoceptor couples to both G_s and G_i proteins [14] and β_2-adrenoceptor-G_i signaling becomes exaggerated in heart failure [15–17], causing various adverse structural and functional consequences in the heart [17–20], based on the concept of functional selectivity, a G_s-biased β_2-adrenoceptor

[1]Department of Pharmacology, Shenyang Pharmaceutical University, Shenyang 110016, China; [2]State Key Laboratory of Membrane Biology, Institute of Molecular Medicine, Peking University, Beijing 100871, China; [3]Department of Medicinal Chemistry, Shenyang Pharmaceutical University, Shenyang 110016, China; [4]Key Laboratory of Structure-Based Drug Design and Discovery, Ministry of Education, Shenyang Pharmaceutical University, Shenyang 110016, China; [5]Laboratory of Clinical Investigation, National Institute on Aging, NIH, Baltimore, MD 21224, USA; [6]Mitchell Woods Pharmaceuticals, LLC, Shelton, CT 06484, USA; [7]Centers for Life Sciences, Peking University, Beijing 100871, China and [8]Beijing City Key Laboratory of Cardiometabolic Molecular Medicine, Peking University, Beijing 100871, China
Correspondence: Mao-sheng Cheng (mscheng@syphu.edu.cn) or Rui-ping Xiao (xiaor@pku.edu.cn)
[9]Present address: School of Pharmacy, Hebei Medical University, Shijiazhuang 050017, China
These authors contributed equally: Anthony Yiu-Ho Woo, Xin-yue Ge.

Received: 5 July 2018 Accepted: 26 November 2018
Published online: 14 January 2019

agonist, fenoterol (**FEN**), was discovered from screening and subsequently tested on an animal model of heart failure [21]. The efficacy of **FEN**, used alone or in combination with a β_1-adrenoceptor antagonist, in treating heart failure has subsequently been demonstrated in follow-up studies [22–26]. Therefore, we have proposed the use of G_s-biased β_2-adrenoceptor agonists to treat heart failure [20, 21, 27].

Our group is committed to developing β_2-adrenoceptor agonists for clinical use. Characterization of the signal transduction properties of β_2-adrenoceptor agonists is one of our major objectives because it can provide us with experimental compounds with different pharmacological properties, from which we can choose compounds for further research and development. We have synthesized over 90 derivatives of **FEN** [28–31]. Several G_s-biased β_2-adrenoceptor agonists, such as **(*R,R'*)-FEN** (Supplementary Fig. S1), have been discovered from this cohort [32, 33]. We have synthesized other β_2-adrenoceptor agonists including compounds with the classical 2-amino-1-phenylethanol core structure or those containing a novel 2-amino-2-phenylethanol core structure [34–37]. These compounds exhibit β_2-adrenoceptor agonistic activities and tracheal smooth muscle relaxant effects, and many of them are also more selective towards the β_2-adrenoceptor over the β_1-adrenoceptor [34–37]. The aim of the present study is to characterize the β-arrestin-biased agonism at the β_2-adrenoceptor for some of these novel compounds.

MATERIALS AND METHODS

Chemistry

The ^{1}H and ^{13}C spectra (Supplementary Fig. S2) were recorded on a Bruker (Billerica, MA, USA) ARX-300, ARX-400, or ARX-600 NMR spectrometer using DMSO-d_6 as the solvent. Chemical shifts (δ) were reported in parts per million (ppm) relative to tetramethylsilane (internal standard), and coupling constants (J) were reported in Hz. High-resolution mass spectrometry (HRMS) was performed on a Bruker SolariX 7.0T. All reactions were monitored by thin layer chromatography (TLC) using TLC plates (Silica gel60 GF254, Merck) and UV light visualization. When appropriate, crude products were purified by column chromatography using silica gel (200–300 mesh) purchased from the Qingdao Haiyang Chemical Co. Ltd (China). The purity of the final products was determined by high performance liquid chromatography (Waters Corp., Milford, MA, USA) on a C18 column (250 mm × 4.6 mm, 5 μm bead size; Thermo Fisher Scientific, Waltham, MA, USA) with acetonitrile/H_2O (33/67 *v/v*, the pH was adjusted to 5.30 with H_3PO_4 and NaH_2PO_4) as the mobile phase at a 1.0 mL/min flow rate and a detection wavelength of 259 or 254 nm. The structures of newly synthesized compounds were confirmed by ^{1}H-NMR, ^{13}C-NMR, and HRMS, whereas the structures of other compounds were confirmed by ^{1}H-NMR and low-resolution mass spectrometry (MS).

Materials

All solvents and reagents were obtained from commercial suppliers and were used as received. All biochemical reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted.

Compounds

(*R*)-Epinephrine (**(*R*)-EPI**), **FEN**, and (*R*)-isoproterenol (**(*R*)-ISO**) were purchased from Sigma-Aldrich. Salmeterol (**SAL**) was purchased from Adamas-Beta (Shanghai, China). The synthesis of **(*R,R'*)-FEN** and (*R,R'*)-4-methoxy-1-naphthylfenoteol (**(*R,R'*)-MNF**) has been described previously [29–31]. They were kindly provided by Dr. Joseph Kozocas from SRI International in California, USA (Supplementary Fig. S1). Compounds with a 2-amino-1-phenylethanol core structure including **A-17**, **A-18**, **A-23**, **A-31**, **A-32**, **A-33**, and **A-35**, as well as those with a 2-amino-2-phenylethanol core structure including **B-24**, **B-30**, **L-2**, **L-4**, **L-6**, and **L-12** (Table 1) were designed and synthesized at Shenyang Pharmaceutical University by methods adopted from our previous studies [34, 36, 37]. The steps for the syntheses of the target compounds are illustrated in Scheme 1 for **A-17**, **A-18**, **A-23**, **A-31**, **A-32**, and **A-33**; Scheme 2 for **B-24** and **B-30**; and Scheme 3 for **L-2**, **L-4**, **L-6**, and **L-12**. Compounds **A-33**, **B-24**, **L-2**, **L-4**, **L-6**, and **L-12** are novel. The purities of all of the biologically evaluated compounds exceeded 95%. All test compounds are phenylethanolamines. Phenylethanolamine is the basic structure of β-agonists. Stock solutions of the compounds were prepared in dimethylsulfoxide (DMSO). Dilutions were made with buffer solutions. The final concentration of DMSO in the assays did not exceed 1%, and at this concentration, DMSO did not interfere with the assays.

General procedures for 2-amino-1-phenylethanol (A-series) and 2-amino-2-phenylethanol (B-series) compounds

A-series compounds were prepared by combining different substituted 1-(4-aminophenyl)-2-bromoethanone derivatives with various amines. The carbonyl group in each intermediate was then reduced by sodium borohydride to afford the 2-aminoethanol product (Scheme 1). B-series compounds were synthesized by coupling different substituted 2-bromo-1-phenylethanol compounds with various amines after reduction of the ethanone (Scheme 2). Finally, all of the target compounds were treated with a saturated solution of hydrogen chloride in isopropanol to form hydrochlorides.

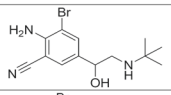
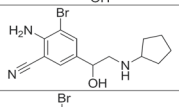
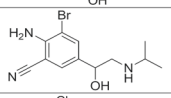
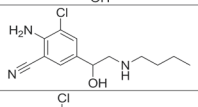
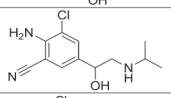
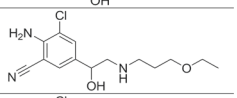
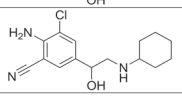
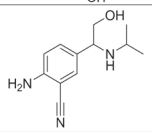
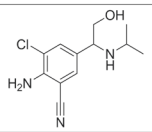
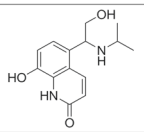
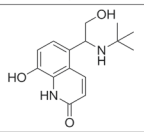
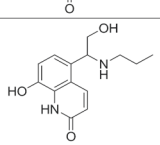
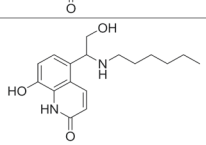
1-(4-Amino-3-chloro-5-cyano-phenyl)-2-(3-ethoxy)propylamino-ethanol hydrochloride (**A-33**)

To a stirred solution of 2-amino-5-(2-bromoacetyl)-3-chlorobenzonitrile (5.00 g, 0.018 mol) in 50 mL of ethanol, 3-ethoxypropylamine (5.63 g, 0.054 mol) was added slowly under an atmosphere of nitrogen at 5 °C. The reaction mixture was stirred at room temperature for 5 h. Following the addition of 4 mL of water, sodium borohydride (0.68 g, 0.018 mol) was added portion-wise, and the reaction mixture was further stirred for 5 h. Aqueous 2 mol/L HCl was then added to adjust the pH to 3, followed by stirring for 30 min. Ammonia water was added to adjust the pH to 10, followed by evaporation under vacuum. The residue was mixed with 50 mL of water, and ammonia water was added again until the pH = 10. The mixture was extracted with ethyl acetate (3 × 50 mL). Organic layers were combined, and the ethyl acetate was evaporated under reduced pressure. The residue was extracted with aqueous 2 mol/L HCl (3 × 60 mL). Aqueous layers were combined, and activated carbon was added. The mixture was heated to reflux for 20 min and then filtered. After cooling to room temperature, the pH was adjusted to 10 with ammonia water. The mixture was extracted with diethyl ether (3 × 50 mL). The combined organic layers were washed with water, dried over anhydrous sodium sulfate, and filtered. A saturated solution of hydrogen chloride in isopropanol was added to the filtrate until the pH = 3. Filtration of the resulting mixture gave **A-33** as a white solid (0.80 g, 14.8%). ^{1}H-NMR (400 MHz, DMSO-d_6, ppm) δ: 8.93 (br, 1H), 8.69 (br, 1H), 7.56 (s, 1H), 7.46 (s, 1H), 6.30 (s, 2H), 6.23(s, 1H), 4.83 (d, J = 3.18 Hz, 1H), 3.38–3.45 (m, 4H), 2.98–3.11 (m, 4H), 1.89 (s, 2H), 1.11 (d, J = 4.17, 3H). ^{13}C-NMR δ: 147.2, 132.5, 130.7, 129.9, 118.9, 117.6, 95.6, 67.5, 67.1, 65.8, 53.1, 45.6, 26.1, 15.5. MS (EI) *m/z* 298, 206, 116, 44. HRMS ($[M + H]^+$) *m/z* calcd for $C_{14}H_{21}ClN_3O_2$ 298.1322; found 298.1329.

2-(4-Amino-3-cyano-phenyl)-2-isopropylamino-ethanol hydrochloride (**B-24**)

To a stirred solution of 2-amino-5-(2-bromo-1-hydroxyethyl)-benzonitrile (2.45 g, 0.016 mol) in 50 mL of ethanol, isopropylamine (2.14 g, 0.036 mol) was slowly added. The mixture was heated to reflux for 13 h. Following the evaporation of most of the ethanol, the residue was extracted with aqueous 2 mol/L HCl (3 × 50 mL). Aqueous layers were combined and washed with

Table 1 Structures of the compounds synthesized and tested

Code	Compound	Structure [a]
A-17	1-(4-Amino-3-bromo-5-cyano-phenyl)-2-*tert*-butylamino-ethanol hydrochloride	
A-18	1-(4-Amino-3-bromo-5-cyano-phenyl)-2-cyclopentylamino-ethanol hydrochloride	
A-23	1-(4-Amino-3-bromo-5-cyano-phenyl)-2-isopropylamino-ethanol hydrochloride	
A-31	1-(4-Amino-3-chloro-5-cyano-phenyl)-2-*N*-butylamino-ethanol hydrochloride	
A-32	1-(4-Amino-3-chloro-5-cyano-phenyl)-2-isopropylamino-ethanol hydrochloride	
A-33	1-(4-Amino-3-chloro-5-cyano-phenyl)-2-(3-ethoxy)propylamino-ethanol hydrochloride	
A-35	1-(4-Amino-3-chloro-5-cyano-phenyl)-2-cyclohexylamino-ethanol hydrochloride	
B-24	2-(4-Amino-3-cyano-phenyl)-2-isopropylamino-ethanol hydrochloride	
B-30	2-(4-Amino-3-chloro-5-cyano-phenyl)-2-isopropylamino-ethanol hydrochloride	
L-2	8-Hydroxy-5-[2-hydroxy-1-(isopropylamino) ethyl]quinolin-2(1*H*)-one hydrochloride	
L-4	8-Hydroxy-5-[2-hydroxy-1-(*tert*-butylamino) ethyl]quinolin-2(1*H*)-one hydrochloride	
L-6	8-Hydroxy-5-[2-hydroxy-1-(*N*-propylamino) ethyl]quinolin-2(1*H*)-one hydrochloride	
L-12	8-Hydroxy-5-[2-hydroxy-1-(*N*-hexylamino) ethyl]quinolin-2(*1H*)-one hydrochloride	

[a] HCl omitted

methyl benzene. Activated carbon was then added. The mixture was heated to reflux for 20 min and then filtered. After cooling to room temperature, ammonia water was added to adjust the pH to 10. The mixture was extracted with diethyl ether (3 × 50 mL). The combined organic layers were washed with water, dried over anhydrous sodium sulfate, and filtered. A saturated solution of hydrogen chloride in isopropanol was added to the filtrate until the pH = 3. Filtration of the resulting mixture gave **B-24** as a white solid (0.50 g, 15.2%). ^{1}H-NMR (300 MHz, DMSO-d_6, ppm) δ: 9.10 (br, 2H), 7.71 (d, J = 1.65 Hz, 1H), 7.56-7.60 (dd, J = 8.69 Hz, 1.73 Hz, 1H), 6.82 (d, J = 8.69 Hz, 1H), 6.28 (s, 2H), 5.55 (s, 1H), 4.20 (d, J = 0.56 Hz, 1H), 3.85 (s, 1H), 3.73 (s, 1H), 2.95–3.00 (m, 1H), 1.27 (d, J = 6.42 Hz, 3H), 1.18 (d, J = 6.42 Hz, 3H). ^{13}C-NMR δ: 152.39, 134.65, 133.51, 121.13, 118.26, 115.94, 93.64, 62.61, 59.66, 47.83, 19.74, 18.00. MS (ESI) *m/z* 219.9 $[M + H]^+$. HRMS ($[M + H]^+$) *m/z* calcd for $C_{12}H_{18}N_3O$ 220.1450; found 219.1441.

General procedures for **L-2**, **L-4**, **L-6**, and **L-12**

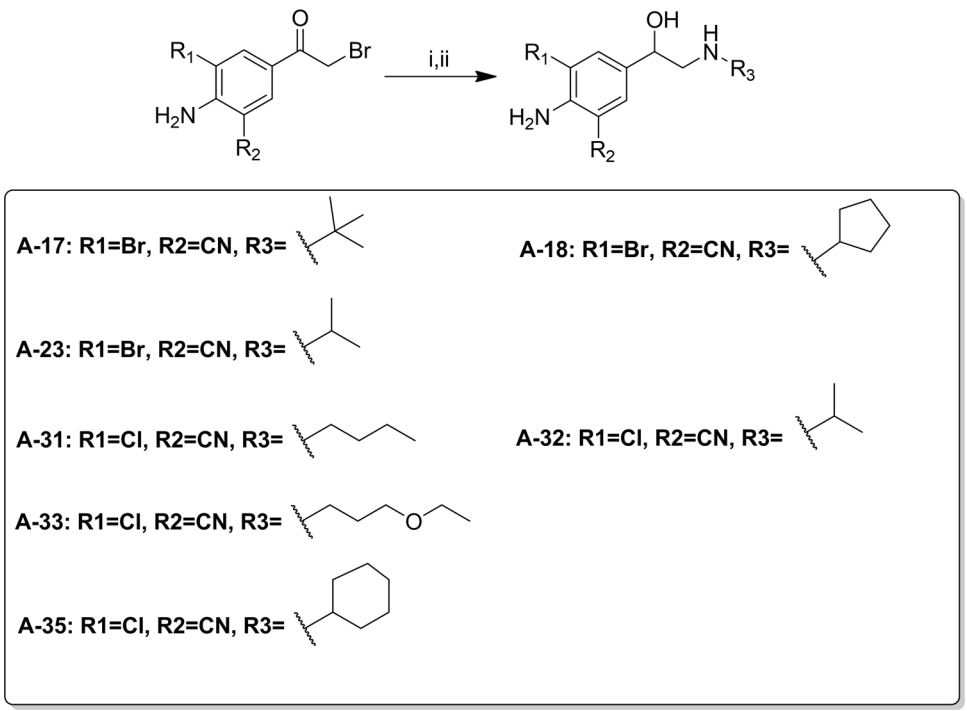
Synthesis of the target compounds **L-2, L-4, L-6**, and **L-12** was achieved through the pathway illustrated in Scheme 3. The starting material 8-hydroxyquinoline (commercially purchased) was reacted with *meta*-chloroperoxybenzoic acid (mCPBA) in dichloromethane (DCM) to give 8-hydroxyquinoline-1-oxide **2**, which was subsequently acetylated to give 8-acetoxyquinolin-2(1*H*)-one **3**. 5-Acetyl-8-hydroxyquinolin-2(1*H*)-one **4** was synthesized from intermediate **3** by a Fries rearrangement with aluminum trichloride and acetyl chloride as catalysts along with 1,2-dichloroethane (DCE) as the solvent. The phenolic hydroxyl group of intermediate **4** was benzyl protected to give 5-acetyl-8-benzyloxyquinolin-2(1*H*)-one **5**, and then, the keto-carbonyl group was brominated to give 8-benzyloxy-5-(2-bromoacetyl)quinolin-2(1*H*)-one **6**. Reduction of **6** with sodium borohydride afforded 8-benzyloxy-5-(2-bromo-1-hydroxyethyl)quinolin-2(1*H*)-one **7**, which was subjected to intramolecular nucleophilic substitution to afford the epoxide 8-benzyloxy-5-(2-oxiranyl)quinolin-2(1*H*)-one **8**. Detailed methods for the synthesis of compounds **1–8** can be found in Supplementary Methods. Intermediates 8-benzyloxy-5-[2-hydroxy-1-(isopropylamino)ethyl]quinolin-2(1*H*)-one hydrochloride **9a**, 8-benzyloxy-5-[2-hydroxy-1-(*tert*-butylamino)ethyl]quinolin-2(1*H*)-one hydrochloride **9b**, 8-benzyloxy-5-[2-hydroxy-1-(*N*-propylamino)ethyl]quinolin-2(1*H*)-one hydrochloride **9c** and 8-benzyloxy-5-[2-hydroxy-1-(*N*-hexylamino)ethyl]quinolin-2(1*H*)-one hydrochloride **9d** were prepared by refluxing **8** with different amines in the presence of zinc chloride as the catalyst and acetonitrile as the solvent. The target compounds **L-2, L-4, L-6**, and **L-12** were synthesized from **9a**, **9b, 9c**, and **9d** by hydrogenation with Pd/C in methanol.

Procedure A: 8-Benzyloxy-5-[2-hydroxy-1-(isopropylamino)ethyl] quinolin-2(1*H*)-one hydrochloride (**9a**)

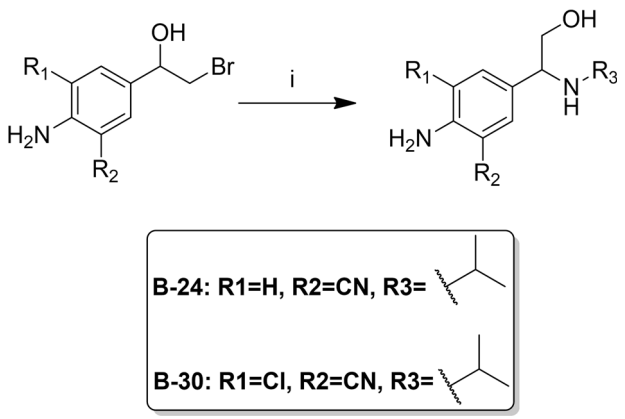
To a stirred solution of intermediate **8** (3.0 g, 0.0102 mol) and zinc chloride (0.35 g, 0.0026 mol) in 150 mL of acetonitrile, isopropylamine (2.63 mL, 0.0307 mol) was added drop-wise. The reaction mixture was heated slowly under reflux for 12 h. Then, the solvent was evaporated. The product was purified by column chromatography (CH_2Cl_2:CH_3OH:NH_4OH = 350:10:1). After evaporation, the solid was dissolved in 50 mL of acetone, followed by acidification with a saturated solution of hydrogen chloride in isopropanol to a pH of 2. Filtration of the resulting mixture gives **9a** as a yellow solid (0.82 g, 20.6%). ^{1}H-NMR (400 MHz, DMSO-d_6, ppm) δ: 10.84 (s, 1H), 9.45 (s, 1H), 9.23 (s, 1H), 8.32–8.33 (d, J = 10.02 Hz, 1H), 7.59–7.61 (m, 3H), 7.38–7.41 (m, 2H), 7.32–7.34 (m, 2H), 6.62–6.63 (d, J = 9.90 Hz, 1H), 5.53 (s, 1H), 5.34 (s, 2H), 4.89–4.92 (m, 1H), 3.87–3.94 (m, 1H), 3.74–3.82 (m, 1H), 3.05–3.12 (m, 1H), 1.29 (d, J = 5.76 Hz, 3H), 1.14 (d, J = 6.12 Hz, 3H). ^{13}C-NMR δ: 161.2, 144.8, 137.0, 136.2, 130.1, 128.9, 128.5, 128.4, 123.7, 123.3, 121.6, 118.5, 112.7, 70.3, 63.3, 55.3, 48.4, 19.6, 18.6. HRMS ($[M + H]^+$) *m/z* calcd for $C_{21}H_{25}N_2O_3$ 353.1865; found 353.1854.

8-Benzyloxy-5-[2-hydroxy-1-(*tert*-butylamino)ethyl]quinolin-2(1*H*)-one hydrochloride (**9b**)

In the presence of zinc chloride (0.35 g, 0.0026 mol), intermediate **8** (3.0 g, 0.0102 mol) was reacted with *tert*-butylamine (2.27 g, 0.0307 mol) in 150 mL of acetonitrile according to procedure A. The crude product was purified by column chromatography (CH_2Cl_2:CH_3OH:NH_4OH = 350:10:1). Evaporation of the solvent and acidification of the product gives **9b** as a white solid (0.64 g, 17.11%). ^{1}H-NMR (600



Scheme 1 Synthesis of 2-amino-1-phenylethanol derivatives. Reagents and conditions: (i) amines, ethanol, 5 °C to room temperature (rt), 5 h; (ii) $NaBH_4$, ethanol and water, rt



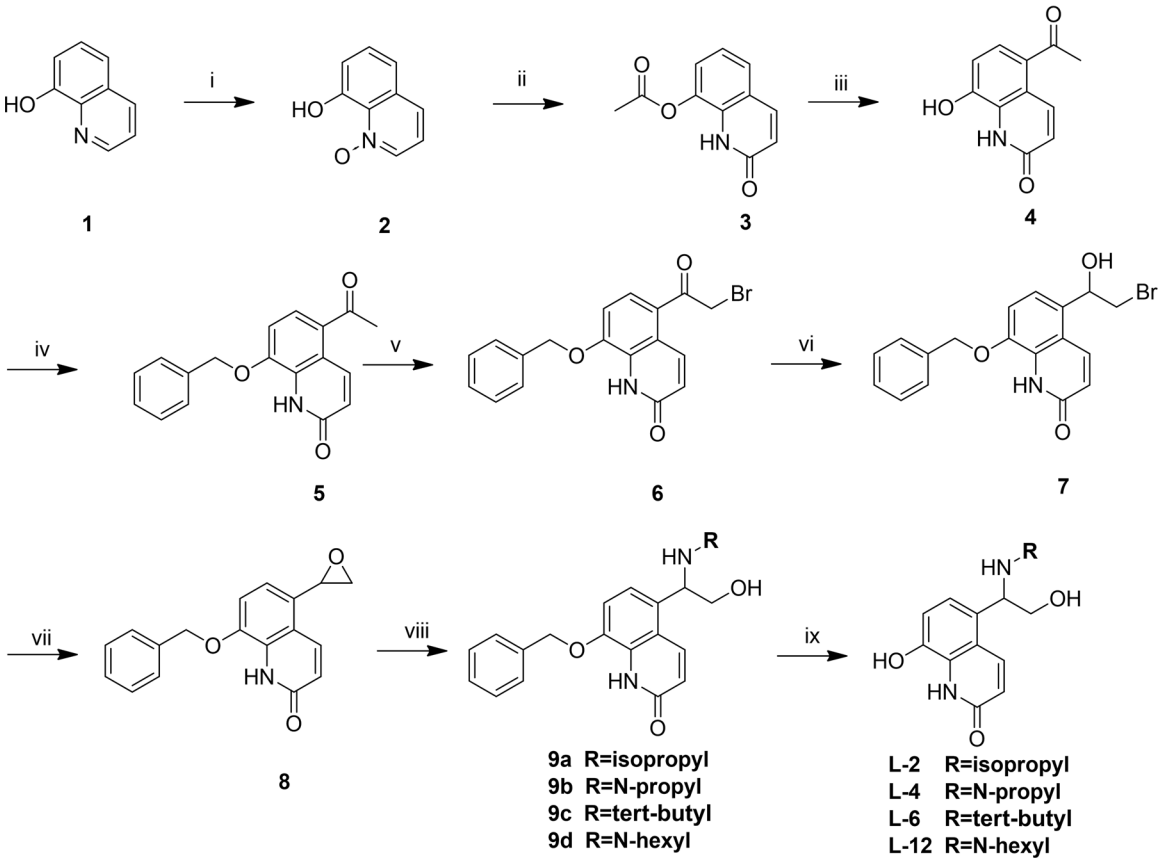
Scheme 2 Synthesis of **B-24** and **B-30**. Reagents and conditions: (i) isopropylamine, ethanol, reflux, 13 h

MHz, DMSO-d_6, ppm) δ: 10.86 (s, 1H), 9.31 (s, 1H), 8.65 (s, 1H), 8.39 (s, J = 7.5 Hz, 1H), 7.60 (d, J = 4.8, 2H), 7.39 (t, J = 4.88 Hz, 2H), 7.32 (t, J = 4.84 Hz, 2H), 6.63 (d, J = 6.60 Hz, 1H), 5.52 (s, 1H), 5.34 (s, 2H), 4.91 (s, 1H), 3.89 (s, 1H), 3.80–3.82 (m, 1H), 1.23 (s, 9H). ^{13}C-NMR δ: 160.1, 143.6, 135.9, 135.3, 128.9, 127.8, 127.4, 127.3, 124.4, 122.5, 121.2, 116.9, 111.5, 69.1, 63.0, 58.1, 53.0, 25.6. HRMS ($[M + Cl]^-$) *m/z* calcd for $C_{22}H_{26}ClN_2O_3$ 401.1632; found 401.1653.

8-Benzyloxy-5-[2-hydroxy-1-(*N*-propylamino)ethyl]quinolin-2(1*H*)-one hydrochloride (**9c**)
In the presence of zinc chloride (0.35 g, 0.0026 mol), intermediate **8** (3.0 g, 0.0102 mol) was reacted with *N*-propylamine (1.8 g, 0.0307 mol) in 150 mL of acetonitrile according to procedure A. The crude product was purified by column chromatography (CH_2Cl_2:CH_3OH:NH_4OH = 350:10:1). Evaporation of the solvent and acidification of the product gives **9c** as a white solid (0.47 g, 13.09%). ^{1}H-NMR (600 MHz, DMSO-d_6, ppm) δ: 10.90 (s, 1 H), 9.47 (s, 1 H), 9.15 (s, 1H), 8.24 (d, J = 10.1 Hz, 1H), 7.60 (d, J = 7.3 Hz, 2H), 7.50 (d, J = 8.5 Hz, 1H), 7.39 (t, J = 7.5 Hz, 2H), 7.33 (s, 2H), 6.63 (d, J = 9.9 Hz, 1H), 5.74–5.46 (m, 1H), 5.35 (s, 2H), 4.99–4.81 (s, 1H), 3.86 (dd, J = 11.6, 6.2 Hz, 1H), 3.79 (dd, J = 11.6, 4.9 Hz, 1H), 2.88 (s, 1H), 2.67 (s, 1H), 1.65 (ddd, J = 27.9, 15.5, 7.4 Hz, 2H), 0.81 (t, J = 7.4 Hz, 3H). ^{13}C-NMR δ: 160.6, 144.2, 136.4, 135.9, 129.5, 128.3, 127.9, 127.8, 123.1, 122.9, 120.8, 188.0, 112.1, 69.6, 62.1, 47.0, 18.8, 10.9. HRMS ($[M + H]^+$) *m/z* calcd for $C_{21}H_{25}N_2O_3$ 353.1865; found 353.1860.

8-Benzyloxy-5-[2-hydroxy-1-(*N*-hexylamino)ethyl]quinolin-2(1*H*)-one hydrochloride (**9d**)
In the presence of zinc chloride (0.35 g, 0.0026 mol), intermediate **8** (3.0 g, 0.0102 mol) was reacted with *N*-hexylamine (2.64 mL, 0.0307 mol) in 150 mL of acetonitrile according to procedure A. The crude product was purified by column chromatography (CH_2Cl_2:CH_3OH:NH_4OH = 350:10:1). Evaporation of the solvent and acidification of the product gave **9d** as a white solid (0.60 g, 13.6%). ^{1}H-NMR (400 MHz, DMSO-d_6, ppm) δ: 10.84 (s, 1H), 9.52 (s, 1H), 9.20 (s, 1H), 8.24 (d, J = 10.02 Hz, 1H), 7.51–7.60 (m, 3H), 7.29–7.41 (m, 4H), 6.62 (d, J = 9.87 Hz, 1H), 5.52 (br, 1H), 5.34 (s, 2H), 4.89 (t, J = 7.68, 1H), 3.75–3.91 (s, 2H), 2.86–2.89 (m, 1H), 2.70 (m, 1H), 1.59–1.62 (m, 2H), 1.17–1.24 (m, 6H), 0.79–0.83 (m, 3H). ^{13}C-NMR δ: 161.2, 144.7, 137.0, 136.5, 130.0, 128.8, 128.4, 128.4, 123.7, 123.4, 121.4, 118.6, 112.6, 70.2, 62.7, 57.7, 46.0, 31.1, 26.1, 25.6, 22.2, 14.2. HRMS ($[M + H]^+$): *m/z* calcd for $C_{24}H_{31}N_2O_3$ 395.2335, found 395.2325.

Procedure B: 8-Hydroxy-5-[2-hydroxy-1-(isopropylamino)ethyl] quinolin-2(1*H*)-one hydrochloride (**L-2**)
To a solution of intermediate **9a** (150 mg, 0.386 mmol) in 50 mL of methanol, 10% Pd/C (20.0 mg) was added. The reaction mixture was stirred under an atmosphere of hydrogen at room temperature for 2 h. The resulting mixture was filtered and concentrated to give **L-2** as a white solid (60 mg, 52.1%). ^{1}H-NMR (400 MHz, DMSO-d_6, ppm) δ: 10.81 (s, 1H), 10.63 (s, 1H), 9.33 (s, 1H), 9.14 (s, 1H), 8.29 (d, J = 9.90 Hz, 1H), 7.50 (d, J = 8.04 Hz, 1H), 7.09 (d, J = 8.10 Hz, 1H), 6.59 (d, J = 9.90 Hz, 1H), 5.58 (s, 1H), 4.93 (s, 1H), 3.89–3.91 (m, 1H), 3.75–3.77 (m, 1H), 3.10 (t, J = 3.88 Hz, 1H), 1.29 (d, J = 3.88 Hz, 3H), 1.14 (d, J = 3.88 Hz, 3H). ^{13}C-NMR δ: 161.1, 144.5, 136.2, 129.2, 123.4, 121.7, 121.3, 118.7, 114.7, 63.5,



Scheme 3 Synthesis of **L-2**, **L-4**, **L-6**, and **L-12**. Reagents and conditions: (i) mCPBA, DCM, 0 °C to rt, 30 min; (ii) Ac_2O, 100 °C, 3 h; (iii) CH_3COCl, $AlCl_3$, DCE, 0 °C to reflux; (iv) $C_6H_5CH_2Br$, K_2CO_3, in *N*,*N*-dimethylformamide, 40 °C, 3 h; (v) Br_2, BF_3Et_2O, DCM, reflux, 15 min; (vi) $NaBH_4$, CH_3OH, DCM, 0 °C, 30 min; (vii) KOH, CH_3OH, DCM, 0 °C, 30 min; (viii) isopropylamine, *N*-propylamine, *tert*-butylamine or *N*-hexylamine, $ZnCl_2$, CH_3CN, reflux, 12 h; (ix) H_2, 10% Pd/C, CH_3OH, rt, 2 h

48.3, 19.6, 18.6. HRMS ($[M + Cl]^-$) *m/z* calcd for $C_{14}H_{18}ClN_2O_3$ 297.1006; found 297.1014.

8-Hydroxy-5-[2-hydroxy-1-(*tert*-butylamino)ethyl]quinolin-2(1*H*)-one hydrochloride (**L-4**)

In the presence of 10% Pd/C (20.0 mg), intermediate **9b** (150 mg, 0.386 mmol) was reacted with H_2 in 50 mL of methanol according to procedure B to give **L-4** as a white solid (117.1 mg, 97.0%). ^{1}H-NMR (600 MHz, DMSO-d_6, ppm) δ: 10.70 (s, 1H) 10.53 (s, 1H), 9.05 (s, 1H), 8.54 (s, 1H), 8.32 (d, J = 9.99 Hz, 1H), 7.45 (d, J = 8.28 Hz, 1H), 7.05 (d, J = 8.28 Hz, 1H), 6.59 (d, J = 9.90 Hz, 1H), 5.50 (s, 1H), 4.87 (s, 1H), 3.79–3.84 (m, 2H), 1.23 (s, 9H). ^{13}C-NMR δ: 160.0, 143.3, 135.3, 128.0, 125.5, 122.3, 121.3, 117.0, 113.4, 63.0, 58.0, 53.2, 25.6. HRMS ($[M + H]^+$) *m/z* calcd for $C_{15}H_{21}N_2O_3$ 277.1552; found 277.1559.

8-Hydroxy-5-[2-hydroxy-1-(*N*-propylamino)ethyl]quinolin-2(1*H*)-one hydrochloride (**L-6**)

In the presence of 10% Pd/C (20.0 mg), intermediate **9c** (150 mg, 0.386 mmol) was reacted with H_2 in 50 mL of methanol according to procedure B to give **L-6** as a white solid (113.0 mg, 98.0%). ^{1}H-NMR (600 MHz, DMSO-d_6, ppm) δ: 10.74 (s, 1H) 10.54 (s, 1H), 9.36 (s, 1H), 9.08 (s, 1H), 8.20 (d, J = 10.08 Hz, 1H), 7.391 (d, J = 8.28 Hz, 1H), 7.06 (d, J = 8.28 Hz, 1H), 6.59 (d, J = 10.08 Hz, 1H), 5.53 (s, 1H), 4.86 (s, 1H), 3.75–3.90 (m, 2H), 2.84 (s, 1H), 2.67 (s, 1H), 1.59–72 (m, 2H), 0.82 (t, d = 7.47 Hz, 3H). ^{13}C-NMR δ: 161.1, 144.5, 136.5, 129.2, 123.2, 121.7, 121.5, 118.9, 114.7, 62.8, 57.7,47.5, 19.4, 11.5. HRMS ($[M + H]^+$) *m/z* calcd for $C_{14}H_{19}N_2O_3$ 263.1396; found 263.1392.

8-Hydroxy-5-[2-hydroxy-1-(*N*-hexylamino)ethyl]quinolin-2(1*H*)-one hydrochloride (**L-12**)

In the presence of 10% Pd/C (20.0 mg), intermediate **9d** (150 mg, 0.386 mmol) was reacted with H_2 in 50 mL of methanol according to procedure B to give **L-12** as a white solid (70 mg, 59.0%). ^{1}H-NMR (400 MHz, DMSO-d_6, ppm) δ: 10.74 (s, 1H) 10.54 (s, 1H), 9.38 (s, 1H), 9.09 (s, 1H), 8.19–8.22 (d, J = 9.99 Hz, 1H), 7.38–7.41 (d, J = 8.25 Hz, 1H), 7.05–7.08 (d, J = 8.25 Hz, 1H), 6.56–6.60 (d, J = 9.84 Hz, 1H), 5.52 (s, 1H), 4.86 (s, 1H), 3.75–3.87 (m, 2H), 2.86 (s, 1H), 2.71 (s, 1H), 1.61 (s, 2H), 1.17 (s, 6H), 0.78–0.83 (t, J = 7.80 Hz, 3H). ^{13}C-NMR δ: 161.1, 144.5, 136.4, 129.2, 123.2, 121.7, 121.5, 118.9, 114.6, 62.8, 31.1, 26.1, 25.7, 22.2, 14.25. HRMS ($[M + Cl]^-$) *m/z* calcd for $C_{17}H_{24}ClN_2O_3$ 339.1475; found 339.1487.

Cell lines

The HEK293 cell line was obtained from Thermo Fisher Scientific and was cultured in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (Beijing YuanHeng ShengMa Biotechnology Research Institute, Beijing, China). The PathHunter Chinese Hamster Ovary-K1 β_2-adrenoceptor β-arrestin (CHO-β_2-β-arr) cell line was purchased from Discoverx (CA, USA) and was cultured in a proprietary medium supplied by Discoverx. All cells were maintained in a humidified incubator at 37 °C in 5% CO_2.

cAMP assay

The homogeneous time-resolved fluorescence (HTRF) cAMP assay was conducted according to the manufacturer's protocol for the cAMP Dynamic 2 kit (Cisbio Bioassays, Codolet, France). Briefly,

HEK293 cells, expressing endogenous β_2-adrenoceptors, were grown to < 80% confluence. Dissociated cells were resuspended in Hank's balanced salt with 20 mmol/L 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid solution (HBSS) supplemented with 0.1% bovine serum albumin and 1 mmol/L 3-isobutyl-1-methylxanthine (IBMX), and then, they were dispensed into 384-well low volume plates (Greiner #784076) at 2000 cells/5 μL per well. The cells were stimulated with compounds diluted in HBSS supplemented with 0.1% bovine serum albumin (5 μL/well) for 30 min at room temperature. Reactions were stopped by the addition of the cAMP-d2 conjugate in lysis buffer (5 μL/well), followed by the addition of the anti-cAMP cryptate conjugate in lysis buffer (5 μL/well). After incubation for 1 h at room temperature, the plates were read in a PerkinElmer (Waltham, MA, USA) 2300 EnSpire multilabel reader for time-resolved fluorescence resonance energy transfer detection with an excitation wavelength of 337 nm and emission wavelengths of 620 nm and 665 nm, with a lag time of 100 μs and an integration time of 400 μs for each emission wavelength. The resultant cAMP concentrations were calculated using Prism 4 (GraphPad Software, CA, USA) by applying the 620/665 nm fluorescence ratios to a standard curve of known cAMP concentrations.

β-Arrestin assay

The PathHunter β-arrestin assay (Discoverx) was carried out with the CHO-β_2-β-arr cell line expressing the Prolink-tagged human β_2-adrenoceptors and the β-arrestin-2-β-galactosidase enzyme fragment fusion proteins. An active β-galactosidase enzyme is formed when a β-arrestin interacts with an activated β_2-adrenoceptor in the cell. The assay was performed according to the manufacturer's protocol (Discoverx). Briefly, CHO-β_2-β-arr cells were grown to 90% confluence. Dissociated cells were resuspended in a cell plating medium (Discoverx) and seeded at 10 000 cells/20 μL per well into 384-well tissue culture plates (with white walls and a clear bottom). After overnight culturing at 37 °C in 5% CO_2, compounds diluted in phosphate-buffered saline (pH = 7.4) supplemented with 0.1% bovine serum albumin (5 μL/well) were added to the cells. The plates were then incubated at 37 °C for 90 min. A β-galactosidase substrate (Discoverx; 12 μL/well) was added. After further incubation for 60 min at room temperature, the plates were read in a PerkinElmer 2300 EnSpire multilabel reader for luminescence detection.

Isolated guinea pig trachea relaxation assay

Male Dunkin–Hartley guinea-pigs weighing 250–350 g were provided by the Experimental Animal Center of Shenyang Pharmaceutical University. The experiment has been approved by the Animal Care and Use Committee of Shenyang Pharmaceutical University. The methods for the isolation of tracheal smooth muscle strips and muscle tone measurements have been described [36]. The strips were equilibrated in Krebs–Henseleit solution (composition in mM: NaCl 118, KCl 5.4, $CaCl_2$ 2.5, $MgSO_4$ 0.6, NaH_2PO_4 2.94, $NaHCO_3$ 25 and *D*-glucose 11.7) and aerated with 95% O_2 and 5% CO_2 for 2 h. Histamine (10 μmol/L) was added to produce maximum contraction. The compound was then added to produce relaxation. The percentage of relaxation was calculated as follows:

$$\text{Relaxation}\,\% = \frac{\text{tension after compound} - \text{tension after histamine}}{\text{tension after histamine}} \times 100\%$$

Curve fitting and statistical analysis

Fitting of the concentration-response curves and statistical analysis of the data were performed using Prism 4. E_{max} and pEC_{50} values were calculated from the curves fitted on the four-parameter logistic model. Ligand bias was quantified as described previously [38]. Briefly, the concentration-response data of all the compounds to be analyzed were fitted into the Black-Leff operational model: [39]

$$\text{Response} = \frac{E_m[A]^n\tau^n}{[A]^n\tau^n + ([A] + K_A)^n} + \text{basal} \quad (1)$$

where [A] is the concentration of the agonist, E_m is the maximal response of the system and n is the transducer slope. The two latter parameters and basal are common for the curve fitting of all of the concentration-response data. Fitting of the functional data to eq. 1 would yield some combinations of τ and K_A. The log transformation of eq. 1 would yield the transduction coefficient, $\log(\tau/K_A)$, which represents the efficiency of a particular agonist in activating a given signaling pathway. The $\Delta\log(\tau/K_A)$ value would, therefore, describe the relative "efficacy" of an agonist for a particular pathway when normalized with a reference agonist, defined as **(*R*)-ISO** in the current analysis. The ligand bias or log bias values, $\Delta\Delta\log(\tau/K_A)$, of a given agonist for different signaling pathways were then evaluated by statistical analysis using Bonferroni's *t*-test against the reference ligand **(*R*)-ISO**. The standard errors (SE) of the $\Delta\Delta\log(\tau/K_A)$ values were calculated as described previously [38].

RESULTS

We initially screened a library of approximately 150 2-amino-1-phenylethanol (A-series) or 2-amino-2-phenylethanol (B-series and L-series) compounds for β-adrenoceptor-mediated activity using the tracheal smooth muscle relaxation assay or the cardiomyocyte contractility assay [32, 33, 36, 37]. Compounds with biological effects were short-listed for subsequent tests. To study ligand bias, the concentration-response profiles of **(*R*)-ISO**, **(*R*)-EPI**, **SAL**, **FEN**, **(*R,R'*)-FEN**, **(*R,R'*)-MNF**, **A-17**, **A-18**, **A-23**, **A-31**, **A-32**, **A-33**, **A-35**, **B-24**, **B-30**, **L-2**, **L-4**, **L-6**, and **L-12** (Table 1 and Supplementary Fig. S1) in activating the β_2-adrenoceptor-G_s-cAMP signaling and the β_2-adrenoceptor-β-arrestin signaling were obtained from the two different assays (Supplementary Fig. S3 and S4). The HTRF cAMP assay (Cisbio Bioassays) was conducted on compound-stimulated HEK293 cells expressing a low level of an endogenous β_2-adrenoceptor [40]. The PathHunter β-arrestin assay (Discoverx) was conducted on compound-stimulated CHO-β_2-β-arr cells. Both of these cell lines have no background expression of other β-adrenoceptor isoforms.

The E_{max} and pEC_{50} values of the concentration-response curves are shown in Table 2. The high $E_{max\ cAMP}$ values of the respective compounds (approximately 90%) suggest that **(*R*)-ISO**, **FEN** and **(*R,R'*)-FEN** are full agonists of the β_2-adrenoceptor in the cAMP assay. The other compounds with lower $E_{max\ cAMP}$ values are partial agonists in this assay. **SAL** and **L-6** are the two compounds with the lowest $E_{max\ cAMP}$ values (15% and 10%, respectively). Similarly, **(*R*)-ISO**, **FEN** and **(*R,R'*)-FEN** are also full agonists of the β_2-adrenoceptor in the β-arrestin assay, and **SAL** and **L-6** also have the lowest efficacies in the β-arrestin assay ($E_{max\ \beta\text{-Arrestin}}$ values are 16% and 14%, respectively). **(*R*)-EPI** has an $E_{max\ cAMP}$ value of 61% and an $E_{max\ \beta\text{-Arrestin}}$ value of 112%. **L-4** has an $E_{max\ cAMP}$ value of 54% and an $E_{max\ \beta\text{-Arrestin}}$ value of 90%. The E_{max} value differences of these two compounds are the greatest among the compounds tested. They are considered full β_2-adrenoceptor agonists in the β-arrestin assay and partial β_2-adrenoceptor agonists in the cAMP assay. For **(*R*)-ISO**, the $pEC_{50\ cAMP}$ value is 7.16 and the $pEC_{50\ \beta\text{-Arrestin}}$ value is 7.28. The difference between these two pEC_{50} values is negligible (< 0.3). This result also holds true for most of the test compounds except **SAL**, **L-12**, **L-4**, **A-35**, and **L-2** (with the absolute differences being 0.87, 0.76, 0.54, 0.53, and 0.40, respectively). The similarity in the efficacies and potencies of most of these compounds across the two assay systems suggests that these two assays are comparable in terms

Table 2 Efficacies (E_{max}) and potencies (pEC_{50}) of the compound-stimulated cAMP responses in HEK293 cells and β-arrestin responses in CHO-β_2-β-arr cells, respectively

Compound	cAMP		β-Arrestin	
	E_{max} [a] (% ISO)	pEC_{50} [a]	E_{max} [b] (% ISO)	pEC_{50} [b]
(*R*)-ISO	91 ± 3	7.16 ± 0.06	104 ± 2	7.28 ± 0.03
(*R*)-EPI	61 ± 2	7.13 ± 0.05	112 ± 2	6.92 ± 0.03
SAL	15 ± 1	8.93 ± 0.07	16 ± 0	8.06 ± 0.03
FEN	94 ± 4	7.11 ± 0.07	91 ± 2	7.18 ± 0.03
(*R,R'*)-FEN	86 ± 4	7.42 ± 0.07	91 ± 2	7.39 ± 0.03
(*R,R'*)-MNF	52 ± 2	7.11 ± 0.05	74 ± 1	6.92 ± 0.02
A-17	36 ± 2	8.21 ± 0.08	29 ± 1	8.37 ± 0.05
A-18	23 ± 1	7.11 ± 0.09	19 ± 0	7.31 ± 0.06
A-23	27 ± 1	7.77 ± 0.07	20 ± 1	7.63 ± 0.07
A-31	39 ± 2	6.81 ± 0.06	33 ± 1	6.89 ± 0.04
A-32	43 ± 2	8.09 ± 0.07	38 ± 1	8.13 ± 0.03
A-33	28 ± 2	6.56 ± 0.08	28 ± 1	6.68 ± 0.05
A-35	25 ± 1	6.02 ± 0.10	25 ± 1	6.55 ± 0.05
B-24	60 ± 3	4.19 ± 0.05	43 ± 3	4.18 ± 0.08
B-30	37 ± 1	5.43 ± 0.07	33 ± 1	5.42 ± 0.06
L-2	44 ± 3	4.72 ± 0.07	54 ± 2	5.12 ± 0.05
L-4	54 ± 3	4.76 ± 0.06	90 ± 3	5.30 ± 0.05
L-6	10 ± 1	4.42 ± 0.15	14 ± 1	4.46 ± 0.05
L-12	36 ± 2	4.45 ± 0.08	32 ± 2	5.21 ± 0.06

The E_{max} value of each compound is the maximal response at saturation expressed as a percentage of the response at 10 μmol/L of **(*R*)-ISO**. Values are means ± SE, $n = 5$ separate experiments with duplicates
[a]E_{max} and pEC_{50} values are derived from the corresponding concentration-response profiles in Supplementary Fig. S3
[b]E_{max} and pEC_{50} values are derived from the corresponding concentration-response profiles in Supplementary Fig. S4

of signal amplification. As the efficacy of **(*R*)-EPI** is just 61% of **(*R*)-ISO** in cAMP induction, **(*R*)-ISO** is the more suitable unbiased β_2-agonist reference for subsequent ligand bias determination.

The $\log(\tau/K_A)$ values of the compounds for cAMP responses and β-arrestin responses (Table 3) were determined after fitting the concentration-response data to the Black–Leff operational model as previously described [38]. Ligand bias or the $\Delta\Delta\log(\tau/K_A)$ values of the compounds (Table 3 and Fig. 1) were then calculated using **(*R*)-ISO** as the reference. Ligand bias is also expressed by the ratio values (bias factors or BFs) in Table 3. The bias factor of **SAL** is 0.14, or in other words, the activation of the G_s compared with the β-arrestin pathway is 7.35-fold. **L-12**, **L-4**, **L-2**, and **A-35** activate the β-arrestin over the G_s pathway with bias factors equal 5.01, 4.32, 2.64, and 2.64, respectively. Statistical analyses of the $\Delta\Delta\log(\tau/K_A)$ values suggest that **SAL** is a G_s-biased β_2-agonist, whereas **L-2**, **L-4**, and **L-12** are β-arrestin-biased β_2-agonists (Fig. 1). The agonist bias of **A-35** towards β-arrestin signaling does not reach statistical significance. Other compounds tested did not show ligand bias in the current analysis.

Compounds were tested for their abilities to produce bronchodilation by the isolated guinea pig trachea relaxation assay. Relaxant effects on airway smooth muscles were observed with the L-series compounds tested at 5 μmol/L (Table 4). In line with the stimulatory effects of the compounds on cellular cAMP production (Table 2), compounds **L-2**, **L-4**, and **L-12** but not **L-6** produced relaxation responses greater than the contractile effect of histamine (10 μmol/L). These effects also appeared less than the relaxant effect produced by **ISO**.

DISCUSSION

Several studies have implicated β-arrestin-biased signaling at the β-adrenoceptor to be cardioprotective [9, 41], leading to the hypothesis that β-arrestin-biased agonism at the β-adrenoceptor may be a novel therapeutic target for heart failure or other cardiovascular diseases [7–9, 41]. Interestingly, our own studies have shown that the G_s-biased β_2-agonist **FEN** is beneficial to survival, cardiac remodeling, and myocardial function in various animal models of heart failure [22–26]. This G_s-biased agonism of **FEN** at the β_2-adrenoceptor describes the ligand bias for preferential G_s signaling over G_s and G_i dual signaling because the β_2-adrenoceptor is capable of coupling to both G_s and G_i proteins [14]. **FEN** and **(*R,R'*)-FEN** have been shown to be G_s-biased [21, 32]. **(*R,R'*)-MNF** is unbiased in terms of β_2-adrenoceptor-G_s/G_i signaling [33]. Here, the signaling biases of **FEN**, **(*R,R'*)-FEN**, and **(*R,R'*)-MNF** for β-arrestin over G_s were investigated. Our results show that the $\Delta\log(\tau/K_A)$ values of **FEN**, **(*R,R'*)-FEN**, and **(*R,R'*)-MNF** for G_s are slightly larger than those for β-arrestin (Table 3), but the ligand biases for these compounds have no difference compared with that of **(*R*)-ISO**, a non-biased agonist of the β_2-adrenoceptor ($P > 0.05$, Fig. 1).

A research group has studied 65 β_2-agonists for biased agonism in the signaling pathways downstream of the β_2-adrenoceptor, namely, G_s, β-arrestin-1, and β-arrestin-2 [42–44]. These series of studies have included many **FEN** derivatives including **(*R,R'*)-FEN** and **(*R,R'*)-MNF**. In one of these studies [44], **(*R*)-SAL** and **(*S*)-SAL** have been shown to be strongly G_s-biased over both β-arrestin-1 and β-arrestin-2. Littmann et al. have also found that many **FEN** derivatives are G_s biased. Specifically, **(*R,R'*)-FEN** exhibits no bias in their study, whereas **(*R,R'*)-MNF** is weakly G_s-biased. Littmann *et al.* have performed no statistical analysis on their ligand bias data, likely because these data were pooled from different studies. Thus, the ligand bias data of the present study for **SAL**, **(*R,R'*)-FEN**, and **(*R,R'*)-MNF** are consistent with those reported in Littmann et al.

On the other hand, the study of Rajagopal et al. [45] on quantifying ligand bias at seven transmembrane receptors

suggests that **SAL** is strongly β-arrestin-biased, whereas **FEN** exhibits no bias towards β-arrestin and G_s at the β_2-adrenoceptor. In addition, Van der Westhuizen et al. [38] have reported that **SAL** is biased towards the extracellular signal-regulated kinase 1/2 (ERK1/2) pathway over the cAMP pathway. ERK1/2 has been widely regarded as a downstream effector of β-arrestin in the field [46], but through G_i or G_s activation, the phosphorylation of ERK1/2 can also be induced [40, 47]. The results of Rajagopal et al. on **SAL**, and possibly those of Van der Westhuizen et al., are in direct contradiction with those of Littmann et al. and the present study. In Littmann et al., **(*R*)-EPI** rather than **(*R*)-ISO** was used as the reference unbiased agonist for ligand bias quantitation. However, this is not the main reason why Littmann et al. obtained a different result on the **SAL** stereoisomers because **EPI** has been shown to be a non-biased β_2-agonist in other studies including the present one. In addition, all of these studies have employed the Black–Leff operational model in quantifying ligand bias. Therefore, the discrepancy in results can only be due to the different techniques used in determining the G_s and β-arrestin activities of the compounds.

Careful examination of the data revealed that the reported $E_{max\ cAMP}$ or $E_{max\ Gs}$ values of **SAL** (or its stereoisomers) in

Table 3 Log(τ/K_A) and Δlog(τ/K_A) values for cAMP responses, log(τ/K_A) and Δlog(τ/K_A) values for β-arrestin responses, ΔΔlog(τ/K_A) values (ΔΔLog) and bias factors (BFs) of the compounds

Compound	cAMP		β-Arrestin		ΔΔLog	BF
	Log(τ/K_A)	ΔLog(τ/K_A)	Log(τ/K_A)	ΔLog(τ/K_A)		
(*R*)-ISO	7.23 ± 0.03	0.00 ± 0.03	7.30 ± 0.02	0.00 ± 0.02	0.00 ± 0.04	1.00
(*R*)-EPI	7.03 ± 0.05	−0.19 ± 0.06	6.99 ± 0.02	−0.31 ± 0.03	−0.12 ± 0.06	0.76
SAL	8.28 ± 0.14	1.06 ± 0.14	7.49 ± 0.07	0.19 ± 0.07	−0.87 ± 0.16*	0.14
FEN	7.25 ± 0.04	0.03 ± 0.05	7.19 ± 0.02	−0.11 ± 0.03	−0.13 ± 0.06	0.74
(*R,R'*)-FEN	7.50 ± 0.04	0.27 ± 0.05	7.40 ± 0.02	0.11 ± 0.03	−0.16 ± 0.06	0.69
(*R,R'*)-MNF	7.02 ± 0.05	−0.21 ± 0.06	6.85 ± 0.03	−0.45 ± 0.03	−0.24 ± 0.07	0.58
A-17	8.05 ± 0.07	0.83 ± 0.08	8.08 ± 0.06	0.78 ± 0.06	−0.05 ± 0.10	0.90
A-18	6.72 ± 0.09	−0.05 ± 0.09	6.88 ± 0.08	−0.41 ± 0.08	0.09 ± 0.12	1.24
A-23	7.42 ± 0.08	0.19 ± 0.08	7.25 ± 0.07	−0.05 ± 0.08	−0.24 ± 0.11	0.57
A-31	6.56 ± 0.06	−0.67 ± 0.07	6.64 ± 0.05	−0.66 ± 0.06	0.01 ± 0.09	1.02
A-32	7.95 ± 0.06	0.73 ± 0.07	7.91 ± 0.05	0.61 ± 0.05	−0.11 ± 0.09	0.77
A-33	6.17 ± 0.08	−1.06 ± 0.08	6.39 ± 0.06	−0.91 ± 0.06	0.15 ± 0.10	1.41
A-35	5.68 ± 0.08	−1.54 ± 0.09	6.18 ± 0.07	−1.12 ± 0.07	0.42 ± 0.12	2.64
B-24	4.01 ± 0.05	−3.22 ± 0.06	4.02 ± 0.05	−3.27 ± 0.05	−0.05 ± 0.08	0.88
B-30	5.22 ± 0.07	−2.00 ± 0.07	5.18 ± 0.05	−2.12 ± 0.06	−0.11 ± 0.09	0.77
L-2	4.51 ± 0.06	−2.72 ± 0.07	5.00 ± 0.04	−2.30 ± 0.04	0.42 ± 0.08*	2.64
L-4	4.59 ± 0.05	−2.64 ± 0.06	5.29 ± 0.03	−2.00 ± 0.03	0.64 ± 0.07***	4.32
L-6	3.63 ± 0.23	−3.60 ± 0.23	3.84 ± 0.09	−3.45 ± 0.09	0.15 ± 0.25	1.40
L-12	4.12 ± 0.07	−3.10 ± 0.07	4.90 ± 0.05	−2.40 ± 0.06	0.70 ± 0.09***	5.01

Log(τ/K_A) values were obtained after fitting the concentration-response data to the operational model for agonism by Black and Leff (1983). ΔLog(τ/K_A) values were calculated by subtracting the log(τ/K_A) value of **(*R*)-ISO** from the log(τ/K_A) value of each compound. ΔΔLog values were calculated by ($\Delta Log_{\beta\text{-Arrestin}} - \Delta Log_{cAMP}$). The significance was determined by comparing each value with that of **(*R*)-ISO** using Bonferroni's *t*-test. *$P < 0.05$, ***$P < 0.001$. BF is an index of the ΔΔlog value with a base of 10. The BF of a compound represents the ratio of β-arrestin activation over G_s activation with respect to **(*R*)-ISO**, an unbiased β_2-agonist. Values are means ± SE, $n = 5$ separate experiments with duplicates. A ligand bias is considered strong if the ΔΔlog value is >1 or < −1

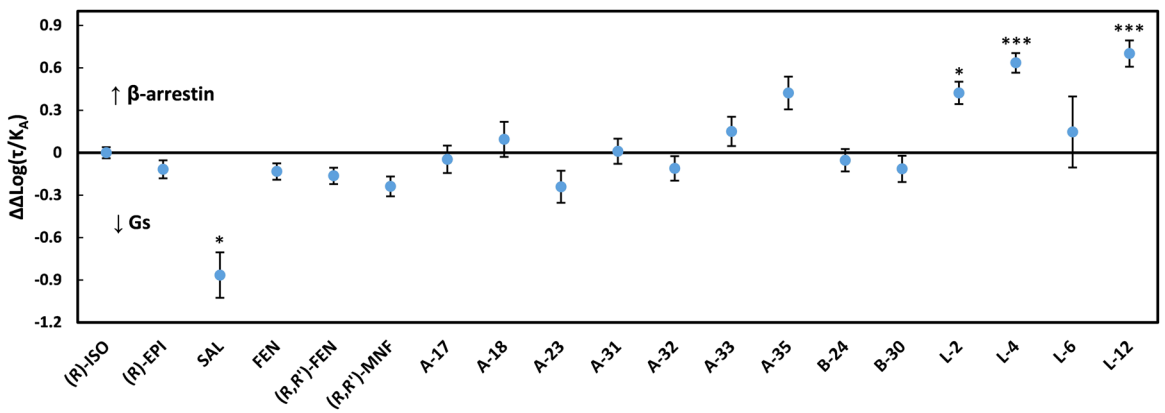


Fig. 1 ΔΔLog(τ/K_A) values of the compounds for β-arrestin signaling over G_s signaling. The significance of the ligand bias was determined by comparing each value with that of **(*R*)-ISO** using Bonferroni's *t*-test. *$P < 0.05$, ***$P < 0.001$ (Error bars are standard errors, $n = 5$)

Table 4 Effects of compounds (5 μmol/L) on relaxation of tracheal smooth muscle strips precontracted with histamine

Compound	Relaxation(%)
ISO	285.57 ± 35.24
L-2	118.34 ± 26.56
L-4	130.48 ± 24.44
L-6	28.16 ± 4.84
L-12	206.30 ± 45.45

Values are means ± SEM, $n = 4$

Littmann et al., Rajagopal et al., and Van der Westhuizen et al. are equivalent to that of **(*R*)-ISO**, but the $E_{max\ \beta\text{-Arrestin}}$ values of **SAL** are only 29% or less relative to **(*R*)-ISO**. The reported $E_{max\ ERK1/2}$ value of **SAL** in Van der Westhuizen et al. is approximately 83% that of saturated **(*R*)-ISO**. A major finding of the present study different from those previous ones is that both the $E_{max\ cAMP}$ and the $E_{max\ \beta\text{-Arrestin}}$ values of **SAL** are approximately 16% of saturated **(*R*)-ISO**. **SAL** is regarded as a partial agonist of the β_2-adrenoceptor compared with the natural ligand **(*R*)-EPI**, a full β_2-agonist for G_s activation in relieving bronchoconstriction [48, 49]. In view of the low efficacies of **SAL** in our determination, the data reported in the present investigation may be more akin to data derived from physiological measurements compared with other studies.

Moreover, the incubation time of compound stimulation for the cAMP assay was 5 min and that for the Tango β-arrestin assay was 14–20 h in Rajagopal et al., whereas the incubation times of compound-stimulation for our cAMP and β-arrestin assays were 30 and 90 min, respectively. Therefore, kinetic differences between the assays would complicate the interpretation of our data to a lesser extent than those of Rajagopal et al. In addition, the incubation time of compound stimulation for the ERK1/2 assay in Van der Westhuizen et al. was <5 min. However, it has been shown that the G protein-dependent ERK1/2 activation at the β_2-adrenoceptor has a sharp peak between 2 and 5 min, whereas the β-arrestin-dependent ERK1/2 activation persists from 5 to 30 min [50]. Therefore, the ERK1/2 signals detected by Van der Westhuizen et al. may represent more of the signals of the G protein pathway rather than the signals of the β-arrestin pathway. More recent data have provided evidence that carvedilol signaling from the β_2-adrenoceptor is not merely β-arrestin-mediated but requires G_i [51]. Moreover, β-arrestins do not mediate ERK1/2 phosphorylation in the absence of functional G proteins [52]. These new findings suggest that ERK1/2 activity is not a good parameter for β-arrestin recruitment and cannot be used to access β-arrestin bias. In conclusion, our data and those of Littmann et al. support the notion that sustained bronchodilation with the chronic use of **SAL** is due to a defect in the desensitization of the **SAL**-stimulated β_2-adrenoceptor in a β-arrestin-dependent manner [49].

In the same manner as ERK1/2 activity is to β-arrestin recruitment, cAMP accumulation is widely used to represent the activity of the G_s pathway, as the G_s-adenylyl cyclase-cAMP-protein kinase A signaling cascade is accepted as a dogma in the field of receptor pharmacology. Applying the cAMP assay in ligand bias quantitation, however, is not necessarily error-free. One should note that most β_2-agonists including **ISO** and **EPI** stimulate the β_2-adrenoceptor to activate both G_s and G_i proteins, and the selectivity for the G_i versus G_s pathways to varying degrees may affect the determination of β-arrestin-biased agonism by altering the cAMP production. To address this issue, we tested whether pertussis toxin (PTX), a disruptor of G_i signaling, may cause a strong enhancement in the cAMP responses of the compounds. We used zinterol as the positive control compound because it has exhibited very substantial β_2-adrenoceptor-G_i signaling and a sensitivity towards PTX-treatment in our previous studies using rodent cardiomyocytes [14, 21, 33]. As expected, treatment with PTX increased the $E_{max\ cAMP}$ values of zinterol (from 21.4% without PTX to 26.4% with PTX, 123% increase, $P < 0.001$) and **(*R*)-ISO** (from 33.7% without PTX to 36.3% with PTX, 110% increase, $P > 0.05$) without causing substantial changes in the EC_{50} values of either compounds (Supplementary Fig. S5A, B). Treatment with PTX also increased the cAMP responses of most of the ligands tested at a saturated concentration and a sub-saturated concentration (Supplementary Fig. S5C). The greatest changes occurred in **(*R*)-EPI** (126% increase) and **L-6** (127% increase) at their respective saturating concentrations, whereas the PTX-induced changes in the maximal cAMP responses for **SAL**, as well as the G_s-selective β_2-adrenoceptor agonists **FEN** and **(*R,R'*)-FEN** were 96%, 110%, and 116%, respectively. The PTX-induced changes in the compound-stimulated responses were generally small but definite. Statistical significance in some of the comparisons occurred only by chance and is not an indication of exceptional G_i activity for any particular compound(s). These results suggest that the HTRF cAMP assay is not particularly sensitive for detecting β_2-adrenoceptor-G_i signaling compared with other assays, such as the rat cardiomyocyte contractility assay. Thus, the selectivity of the β_2-adrenoceptor ligands to different G_s/G_i pathways should not be a confounding factor in the determination of β-arrestin bias in the present study.

The trachea relaxation assay results suggest that these compounds are bronchodilators like other β_2-adrenoceptor agonists and that their activities (presumably those of cAMP induction) rather than their ligand bias status are associated with the bronchodilator effects of the compounds. The correlation of E_{max} in the cAMP assay with relaxation is fairly good except for **L-12**, which shows more potent relaxation than **L-2** and **L-4**, suggesting that the smooth muscle relaxant effect of **L-12** might involve additional mechanisms other than stimulation of the β_2-adrenoceptor. One possible mechanism is the inhibition of phosphodiesterase, which causes an accumulation of cAMP in the cytosol. This effect, if it exists, will be masked in the cAMP assay because the cAMP assay buffer contained IBMX, a phosphodiesterase inhibitor, in an excess amount.

BI-167107 (Supplementary Fig. S1), a high affinity and selective β_2-adrenoceptor agonist previously used in X-ray crystallographic studies of the active conformations of the β_2-adrenoceptor [53], has been shown to be partially biased for β-arrestin over G_s [54]. As the L-series compounds possess a different head group and a different core structure compared with BI-167107, the present study has discovered a new scaffold for the design of novel β-arrestin-biased β_2-adrenoceptor agonists. Additionally, compounds **L-2**, **L-4**, and **L-12** have much lower potencies in inducing cAMP compared with **(*R*)-ISO** (Table 2). This result may suggest a low binding affinity. In summary, these compounds may better serve as tool reagents or as lead compounds for further development.

CONCLUSION

In the present study, we characterized a cohort of phenylethanolamines for β-arrestin-biased agonism at the β_2-adrenoceptor. Our data show that three 2-amino-2-phenylethanol derivatives, namely, **L-2**, **L-4**, and **L-12**, are partial β_2-adrenoceptor agonists with weak ligand biases for β-arrestin over G_s. The present identification of β-arrestin-biased β_2-adrenoceptor agonists with a new 5-(1-amino-2-hydroxyethyl)-8-hydroxyquinolin-2(1*H*)-one core structure will facilitate the discovery of novel biased agonists with potential usefulness in the treatment of diseases of the heart and airway.

ACKNOWLEDGEMENTS

We thank Dr. Joseph Kozocas from SRI international, California, USA for providing **(*R,R'*)-FEN** and **(*R,R'*)-MNF** for the analysis and Dr. Wei Liang and his team from WuXi AppTec, Shanghai, China for technical assistance in the cAMP assay. This work was supported by the Ministry of Science and Technology of the People's Republic of China under the National Key Research and Development Program of China [grant number 2018YFA0507603 (to R-PX)], the National Science and Technology Major Project [grant numbers 2013ZX09507001-001002 (to AY-HW), 2013ZX09301305-001 (to LP), 2013ZX09508104 (to R-PX), 2018ZX09739009 (to M-SC)], the National Basic Research Program [grant number 2012CB518000 (to R-PX)], the National Natural Science Foundation of China [grant numbers 81673355 (to AY-HW), 81872752 (to M-SC), 31521062, 81630008, 81790621 (to R-PX)], Beijing Municipal Science & Technology Commission [grant number Z171100000417006 (to R-PX)], and the intramural research program of the NIH, USA (to IWW).

AUTHOR CONTRIBUTIONS

AYHW designed the research, performed the research, analyzed the data, and wrote the paper; XYG performed the research, analyzed the data, and wrote the paper; and YYZ analyzed the data. LP performed the research and contributed new reagents or analytical tools; XRL, YMM, GX and RJX performed the research; IWW contributed new reagents or analytical tools; and MSC and RPX contributed new reagents or analytical tools and supervised the research.

ADDITIONAL INFORMATION

The online version of this article (https://doi.org/10.1038/s41401-018-0200-x) contains supplementary material, which is available to authorized users.

Competing interests: The authors declare no competing interests.

REFERENCES

1. Overington JP, Al-Lazikani B, Hopkins AL. How many drug targets are there? Nat Rev Drug Discov. 2006;5:993–6.
2. Kenakin T. Collateral efficacy in drug discovery: taking advantage of the good (allosteric) nature of 7TM receptors. Trends Pharmacol Sci. 2007;28:407–15.
3. Urban JD, Clarke WP, von Zastrow M, Nichols DE, Kobilka B, Weinstein H, et al. Functional selectivity and classical concepts of quantitative pharmacology. J Pharmacol Exp Ther. 2007;320:1–13.
4. Mailman RB. GPCR functional selectivity has therapeutic impact. Trends Pharmacol Sci. 2007;28:390–6.
5. Seifert R, Dove S. Functional selectivity of GPCR ligand stereoisomers: new pharmacological opportunities. Mol Pharmacol. 2009;75:13–18.
6. Evans BA, Sato M, Sarwar M, Hutchinson DS, Summers RJ. Ligand-directed signalling at beta-adrenoceptors. Br J Pharmacol. 2010;159:1022–38.
7. Violin JD, Lefkowitz RJ. β-Arrestin-biased ligands at seven-transmembrane receptors. Trends Pharmacol Sci. 2007;28:416–22.
8. Wisler JW, DeWire SM, Whalen EJ, Violin JD, Drake MT, Ahn S, et al. A unique mechanism of beta-blocker action: carvedilol stimulates beta-arrestin signaling. Proc Natl Acad Sci U S A. 2007;104:16657–62.
9. Kim IM, Tilley DG, Chen J, Salazar NC, Whalen EJ, Violin JD, et al. A beta-blockers alprenolol and carvedilol stimulate beta-arrestin-mediated EGFR transactivation. Proc Natl Acad Sci U S A. 2008;105:14555–60.
10. Engelhardt S, Hein L, Wiesmann F, Lohse MJ. Progressive hypertrophy and heart failure in β1-adrenergic receptor transgenic mice. Proc Natl Acad Sci U S A. 1999;96:7059–64.
11. Bisognano JD, Weinberger HD, Bohlmeyer TJ, Pende A, Raynolds MV, Sastravaha A, et al. Myocardial-directed overexpression of the human β1-adrenergic receptor in transgenic mice. J Mol Cell Cardiol. 2000;32:817–30.
12. Zhu WZ, Zheng M, Koch WJ, Lefkowitz RJ, Kobilka BK, Xiao RP. Dual modulation of cell survival and cell death by β_2-adrenergic signaling in adult mouse cardiac myocytes. Proc Natl Acad Sci U S A. 2001;98:1607–12.
13. Zhu WZ, Wang SQ, Chakir K, Yang D, Zhang T, Brown JH, et al. Linkage of β1-adrenergic stimulation to apoptotic heart cell death through protein kinase A-independent activation of Ca^{2+}/calmodulin kinase II. J Clin Invest. 2003;111:617–25.
14. Xiao RP, Ji X, Lakatta EG. Functional coupling of the β_2-adrenoceptor to a pertussis toxin-sensitive G protein in cardiac myocytes. Mol Pharmacol. 1995;47:322–9.
15. Böhm M, Eschenhagen T, Gierschik P, Larisch K, Lensche H, Mende U, et al. Radioimmunochemical quantification of Giα in right and left ventricles from patients with ischaemic and dilated cardiomyopathy and predominant left ventricular failure. J Mol Cell Cardiol. 1994;26:133–49.
16. Feldman AM, Cates AE, Veazey WB, Hershberger RE, Bristow MR, Baughman KL, et al. Increase of the 40,000-mol wt pertussis toxin substrate (G protein) in the failing human heart. J Clin Invest. 1988;82:189–97.
17. Zhu W, Petrashevskaya N, Ren S, Zhao A, Chakir K, Gao E, et al. Gi-biased β2AR signaling links GRK2 upregulation to heart failure. Circ Res. 2012;110:265–74.
18. Sato M, Gong H, Terracciano CM, Ranu H, Harding SE. Loss of β-adrenoceptor response in myocytes overexpressing the Na^+/Ca^{2+}-exchanger. J Mol Cell Cardiol. 2004;36:43–48.
19. Xiao RP, Balke CW. Na^+/Ca^{2+} exchange linking beta2-adrenergic Gi signaling to heart failure: associated defect of adrenergic contractile support. J Mol Cell Cardiol. 2004;36:7–11.
20. Zhu W, Zeng X, Zheng M, Xiao RP. The enigma of beta2-adrenergic receptor Gi signaling in the heart: the good, the bad, and the ugly. Circ Res. 2005;97:507–9.
21. Xiao RP, Zhang SJ, Chakir K, Avdonin P, Zhu W, Bond RA, et al. Enhanced Gi signaling selectively negates β_2-AR– but not β_1-AR–mediated positive inotropic effect in myocytes from failing rat hearts. Circulation. 2003;108:1633–9.
22. Ahmet I, Krawczyk M, Heller P, Moon C, Lakatta EG, Talan MI. Beneficial effects of chronic pharmacological manipulation of β-adrenoceptor subtype signaling in rodent dilated ischemic cardiomyopathy. Circulation. 2004;110:1083–99.
23. Ahmet I, Lakatta EG, Talan M. Pharmacological stimulation of β_2-adrenergic receptors (β_2AR) enhances therapeutic effectiveness of β_1AR blockade in rodent dilated ischemic cardiomyopathy. Heart Fail Rev. 2005;10:289–96.
24. Ahmet I, Krawczyk M, Zhu W, Woo AY, Morrell C, Poosala S, et al. Cardioprotective and survival benefits of long-term combined therapy with β_2AR agonist and β_1AR blocker in dilated cardiomyopathy post-myocardial infarction. J Pharmacol Exp Ther. 2008;325:491–9.
25. Ahmet I, Morrell C, Lakatta EG, Talan MI. Therapeutic efficacy of a combination of a beta1-adrenoreceptor (AR) blocker and beta2-AR agonist in a rat model of postmyocardial infarction dilated heart failure exceeds that of a beta1-AR blocker plus angiotensin-converting enzyme inhibitor. J Pharmacol Exp Ther. 2009;331:178–85.
26. Chakir K, Deprу C, Dimaano VL, Zhu WZ, Vanderheyden M, Bartunek J, et al. Galphas-biased beta2-adrenergic receptor signaling from restoring synchronous contraction in the failing heart. Sci Transl Med. 2011;3(100):100ra88.
27. Woo AY, Song Y, Xiao RP, Zhu W. Biased β_2-adrenoceptor signalling: pathophysiology and drug discovery. Br J Pharmacol. 2015;172:5444–56.
28. Beigi F, Bertucci C, Zhu W, Chakir K, Wainer IW, Xiao RP, et al. Enantioselective separation and online affinity chromatographic characterization of *R,R*- and *S,S*-fenoterol. Chirality. 2006;18:822–7.
29. Jozwiak K, Khalid C, Tanga MJ, Berzetei-Gurske I, Jimenez L, Kozocas JA, et al. Comparative molecular field analysis of the binding of the stereoisomers of fenoterol and fenoterol derivatives to the β_2 adrenergic receptor. J Med Chem. 2007;50:2903–15.
30. Jozwiak K, Woo AY, Tanga MJ, Toll L, Jimenez L, Kozocas JA, et al. Comparative molecular field analysis of fenoterol derivatives: a platform towards highly selective and effective β_2-adrenergic receptor agonists. Bioorg Med Chem. 2010;18:728–36.
31. Plazinska A, Pajak K, Rutkowska E, Jimenez L, Kozocas J, Koolpe G, et al. Comparative molecular field analysis of fenoterol derivatives interacting with an agonist-stabilized form of the β_2-adrenergic receptor. Bioorg Med Chem. 2014;22:234–46.
32. Woo AY, Wang TB, Zeng X, Zhu W, Abernethy DR, Wainer IW, et al. Stereochemistry of an agonist determines coupling preference of β_2-adrenoceptor to different G proteins in cardiomyocytes. Mol Pharmacol. 2009;75:158–65.
33. Woo AY, Jozwiak K, Toll L, Tanga MJ, Kozocas JA, Jimenez L, et al. Tyrosine 308 is necessary for ligand-directed Gs protein-biased signaling of β_2-adrenoceptor. J Biol Chem. 2014;289:19351–63.
34. Xing RJ, Pan L, Wen X, Ge DD, Zhang YY, Cheng MS. Synthesis and anti-asthma activities of phenylethanolamine derivatives. Chin J Med Chem. 2009;19:94–98. (Chinese).
35. Pan H, Li Q, Pan L, Liu X, Pan L, Zhang X, et al. Stereoselective activity of 2-(4-amino-3-chloro-5-trifluomethyl-phenyl)-2-tert-butylamino-ethanol hydrochloride to improve the pulmonary function in asthma. Biomed Rep. 2014;2:539–44.
36. Ge X, Woo AY, Xing G, Lu Y, Mo Y, Zhao Y, et al. Synthesis and biological evaluation of β(2)-adrenoceptor agonists bearing the 2-amino-2-phenylethanol scaffold. Eur J Med Chem. 2018;152:424–35.
37. Ge X, Mo Y, Xing G, Ji L, Zhao H, Chen J, et al. Synthesis, biological evaluation and molecular modeling of 2-amino-2-phenylethanol derivatives as novel β(2)-adrenoceptor agonists. Bioorg Chem. 2018;79:155–62.
38. Van der Westhuizen ET, Breton B, Christopoulos A, Bouvier M. Quantification of ligand bias for clinically relevant β2-adrenergic receptor ligands: implications for drug taxonomy. Mol Pharmacol. 2014;85:492–509.

39. Black JW, Leff P. Operational models of pharmacological agonism. Proc R Soc Lond B Biol Sci. 1983;220:141–62.
40. Friedman J, Babu B, Clark RB. β2-Arenergic receptor lacking the cyclic AMP-dependent protein kinase consensus sites fully activates extracellular signal-regulated kinase 1/2 in human embryonic kidney 293 cells: lack of evidence for Gs/Gi switching. Mol Pharmacol. 2002;62:1094–102.
41. Noma T, Lemaire A, Naga Prasad SV, Barki-Harrington L, Tilley DG, Chen J, et al. β-arrestin-mediated β1-adrenergic receptor transactivation of the EGFR confers cardioprotection. J Clin Invest. 2007;117:2445–58.
42. Reinartz MT, Kälble S, Littmann T, Ozawa T, Dove S, Kaever V, et al. Structure-bias relationships for fenoterol stereoisomers in six molecular and cellular assays at the β2-adrenoceptor. Naunyn-Schmiedeberg's Arch Pharmacol. 2015;388:51–65.
43. Reinartz MT, Kälble S, Wainer IW, Seifert R. Interaction of fenoterol stereoisomers with β2-adrenoceptor-Gsα fusion proteins: antagonist and agonist competition binding. Naunyn-Schmiedeberg's Arch Pharmacol. 2015;388:517–24.
44. Littmann T, Göttle M, Reinartz MT, Kälble S, Wainer IW, Ozawa T, et al. Recruitment of β-arrestin 1 and 2 to the β2-adrenoceptor: analysis of 65 ligands. J Pharmacol Exp Ther. 2015;355:183–90.
45. Rajagopal S, Ahn S, Rominger DH, Gowen-MacDonald W, Lam CM, Dewire SM, et al. Quantifying ligand bias at seven-transmembrane receptors. Mol Pharmacol. 2011;80:367–77.
46. Lefkowitz RJ, Shenoy SK. Transduction of receptor signals by β-arrestins. Science. 2005;308:512–7.
47. O'Hayre M, Eichel K, Avino S, Zhao X, Steffen DJ, Feng X, et al. Genetic evidence that β-arrestins are dispensable for the initiation of β2-adrenergic receptor signaling to ERK. Sci Signal. 2017;10:pii:eaal3395.
48. Gimenez LE, Baameur F, Vayttaden SJ, Clark RB. Salmeterol efficacy and bias in the activation and kinase-mediated desensitization of β2-adrenergic receptors. Mol Pharmacol. 2015;87:954–64.
49. Moore RH, Millman EE, Godines V, Hanania NA, Tran TM, Peng H, et al. Salmeterol stimulation dissociates beta2-adrenergic receptor phosphorylation and internalization. Am J Respir Cell Mol Biol. 2007;36:254–61.
50. Shenoy SK, Drake MT, Nelson CD, Houtz DA, Xiao K, Madabushi S, et al. β-arrestin-dependent, G protein-independent ERK1/2 activation by the β2 adrenergic receptor. J Biol Chem. 2006;281:1261–73.
51. Wang J, Hanada K, Staus DP, Makara MA, Dahal GR, Chen Q, et al. Gα(i) is required for carvedilol-induced β1 adrenergic receptor β-arrestin biased signaling. Nat Commun. 2017;8:1706.
52. Grundmann M, Merten N, Malfacini D, Inoue A, Preis P, Simon K, et al. Lack of beta-arrestin signaling in the absence of active G proteins. Nat Commun. 2018;9:341.
53. Rasmussen SG, DeVree BT, Zou Y, Kruse AC, Chung KY, Kobilka TS, et al. Crystal structure of the beta2 adrenergic receptor-Gs protein complex. Nature. 2011;477:549–55.
54. Weiss DR, Ahn S, Sassano MF, Kleist A, Zhu X, Strachan R, et al. Conformation guides molecular efficacy in docking screens of activated β-2 adrenergic G protein coupled receptor. ACS Chem Biol. 2013;8:1018–26.