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Beta-Adrenergic Receptor Signaling by Enantiomeric Drugs in Retinal Endothelial Cells and Müller cells

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Supplementary Data

Experimental Section

General:
All reagents were purchased from Sigma-Aldrich Chemical Co., Alfa Aesar (Ward Hill, MA), and AK Scientific (Mountain View, CA) and were used without further purification. The solvents for moisturesensitive reactions were freshly distilled, and the reactions were carried out under an argon atmosphere. Routine thin layer chromatography (TLC) was performed on aluminum-backed Uniplates (Analtech, Newark, DE). Melting points were measured with Fisher-Johns melting point apparatus (uncorrected). NMR spectra were obtained on a Varian Inova-500 spectrometer spectrometer. Chemical shifts are reported as parts per million (ppm) relative to TMS in $CDCl_3$. Mass spectra were collected on a Bruker ESQUIRE electrospray/ ion trap instrument in positive and negative ion modes. IR spectra were recorded with an FT-IR spectrometer between 400 and 4000 cm^{-1} in KBr pallets.

Preparation of *S-1*:
Enantioselective synthesis of catechol amines were synthesized by Sharpless asymmetric dihydroxylation. Pradeep et al [1] reported an efficient asymmetric synthesis of enantiomerically single (*R*)-isoproterenol, (*R*)-norfluoxetine and (*R*)-fluoxetine using Sharpless asymmetric dihydroxylation. Using this approach, *S*- (+)-isoproterenol (***S-1***) was synthesized. Rotation of both ***S-1*** and ***R-1*** are $[\alpha]_D$ = + 20.7 (*c* 0.13, MeOH), $[\alpha]_D$ = - 21.2 (*c* 0.13, MeOH), respectively.

Preparation of 4:
Commercially available CH_3PPh_3Br (7.5 g, 22 mmol) was taken in dry THF (150 mL) and KO^{t}Bu (2.36 g, 22 mmol) was added to this under an argon atmosphere. This reaction mixture was stirred for 20 min and a solution of compound **3** (5.0 g, 16.3 mmol) in 100 mL of dry THF was added slowly using dropping funnel. The reaction mixture was further stirred at room temperature for 4 h. The reaction mixture was filtered and the filtrate was evaporated and purified by silica gel column chromatography (10 % ethyl acetate in hexanes) to afford 4.8 g (96% yield) of **4**; mp 47-52 °C. ^{1}H NMR (500MHz,

$CDCl_3$): δ 7.47-7.28 (m, 10H), 7.043 (s, 1H), 6.93-6.87 (m, 2H), 6.59 (dd, *J*=11.0 Hz, *J*=17.0 Hz 1H), 5.55 (d, *J*=17.5 Hz, 1H), 5.17-5.16 (m, 4H), 5.118 (d, *J*=10.7 Hz, 1H). ν_{max} (KBr)/cm^{-1}: 3033, 1579, 1509, 1130, 1003. MS (ESI): calculated for $C_{22}H_{20}O_2$, 316.39; found, 339.30 ($[M + Na]^+$).

Preparation of 5:
To a mixture of $K_3Fe(CN)_6$ (30.07 g, 91.33 mmol) and K_2CO_3 (12.62 g, 91.33 mmol) and $(DHQD)_2PHAL$ (0.24 mg, 0.304 mmol) in t-BuOH–H_2O (1:1, 152 mL) cooled to 0 °C was added osmium tetroxide (1.3 mL, 0.1 M solution in water). After stirring for 5min at 0 °C, olefin **4** (5.0 g, 30.45 mmol) in acetone was added in one portion. The reaction mixture was stirred at 0 °C for 48h and then quenched with solid sodium sulfite (7 g). The stirring was continued for an additional 45 min and then solution was extracted with ethyl acetate (2x50 mL). The combined organic phases were dried (Na_2SO_4) and concentrated. Silica gel column chromatography of the crude product using petroleum ether–EtOAc (40:60) as eluent gave **5** (5.85g, 90%) as a white solid; mp 57-61 °C. ^1H NMR (500MHz, $CDCl_3$): δ 7.45-7.28 (m, 10H), 6.98 (s, 1H), 6.97-6.85 (m, 2H), 5.16-5.15 (m, 4H), 4.70 (m, 1H), 3.69-3.57 (m, 2H), 2.42 (s, 1H, OH), 1.97 (s, 1H, OH). ν_{max} (KBr)/cm^{-1}: 3306, 3034, 1514, 1251, 1135, 1003. MS (ESI): calculated for $C_{22}H_{22}O_4$, 350.41; found, 373.20 ($[M + Na]^+$).

Preparation of 6:
To a solution of diol **5** (2.9 g, 14.63 mmol), in dry dichloromethane (30 mL) was added dibutyltin oxide (8.0 mg, 0.2mol% of diol) followed by the addition of *p*-toluensulfonyl chloride (3.03 g, 15.93 mmol) and triethylamine (2.2 mL, 15.70mmol) and the reaction was stirred at room temperature under Ar. The reaction was monitored by TLC. After completion of reaction (45 min), the solution was quenched by adding water. The solution was extracted with dichloromethane (2x25 mL) and then combined organic phases were washed with water, dried (Na_2SO_4) and concentrated. Silica gel column chromatography of crude product using petroleum ether-EtOAc (70:30) as eluent afforded monotosyl compound **6** (4.88 g, 95%) as a viscous liquid. ^1H NMR (500MHz, $CDCl_3$): δ 7.75 (m, 2H), 7.43-7.31 (m, 12H), 6.92 (s, 1H), 6.88-6.79 (m, 2H), 5.14 (s, 2H), 5.11(s, 2H), 4.87 (m, 1H), 4.13-3.96 (m, 2H), 2.44 (s, 3H). ν_{max} (KBr)/cm^{-1}: 3306, 3034, 1514, 1251, 1135, 1003. MS (ESI): calculated for $C_{29}H_{28}O_6S$, 504.60; found, 527.1 ($[M + Na]^+$).

Preparation of 10:
To a solution of monotosyl compound **6** (3.5 g, 6.9 mmol) in dry DMF (30 mL) was added NaN_3 (4.5 g, 60.9 mmol) and the reaction was refluxed under argon. The reaction was monitored by TLC. After completion of reaction, water was added to the reaction mixture and extracted with Ethyl acetate (2x25 mL). Combined organic phases were dried (Na_2SO_4) and concentrated. Silica gel column chromatography of crude product using petroleum ether-EtOAc (75:25) as eluent afforded azide (2.2 g, 85%) as a viscous liquid. ^1H NMR (500MHz, $CDCl_3$): δ 7.45-7.28 (m, 10H), 6.97 (s, 1H), 6.90 (d, *J*=8.3 Hz, 1H), 6.85-6.83 (m, 1H), 5.15-5.13 (m, 4H), 4.75-4.73 (m, 1H), 3.44-3.31 (m, 2H), 2.31 (s, 1H, OH). ν_{max} /cm^{-1}: 3424, 3032, 2097, 1509, 1259, 1132, 1010, 733. MS (ESI): calculated for $C_{22}H_{21}N_3O_3$, 375.42; found, 398.1 ($[M + Na]^+$).

Above azide (2.2 g, 5.9 mmol) dissolved in THF (20 mL) was added to Lithium Aluminum Hydride (0.9 g, 23.5 mmol) in THF at 0 °C and the solution was refluxed for 3 hours. After completion of the reaction, filtered, dried over Na_2SO_4 and concentrated yielded amine (1.54 g, 75%). Amine **7** (0.8 g, 2.3 mmol) in dry CH_2Cl_2 was added morpholine-4-carbonyl chloride (0.27ml, 2.3 mmol) and NEt_3 (0.96 ml, 6.8 mmol) at 0 °C under Ar. The reaction was stirred at room temperature for 12h. After completion of reaction, water was added to the mixture extracted with CH_2Cl_2 (1x50 mL) and then combined organic phases were dried (Na_2SO_4) and concentrated and then simultaneously debenzylated with catalytic amount of H_2 / $Pd(OH)_2$ in EtOAC (20 ml). After completion of reaction, mixture was filtered through celite. Filtrate was concentrated and purified by silica gel column chromatography (6% MeOH in ethyl acetate) to afford of **10** as solid (0.42 g, 65%, overall yield); mp 96-100 °C; $[\alpha]_D$ = -10.1 (*c* 0.2, MeOH). ^{1}H NMR (500MHz, $CDCl_3$): δ 7.12 (bs, 1H, OH), 7.10 (bs, 1H, OH), δ 6.82 (s, 1H), 6.87 (d, *J*=8.1 Hz, 1H), 6.77-6.75 (m, 1H), 6.26 (t, *J* = 5.1 Hz, 1H, NH), 4.79 (m, 1H), 3.9 (m, 1H), 3.69 (m, 4H), 3.61 (m, 1H), 3.30 (m, 4H). ν_{max} /cm^{-1}: 3319, 2856, 1593, 1517, 1258, 1233, 1136, 1018, 763. MS (ESI): calculated for $C_{13}H_{18}N_2O_5$, 282.29; found, 283.1 ($[M + H]^+$).

Synthesis of Compound 11:
To a solution of monotosyl compound **6** (0.5 g, 0.9 mmol) and 3-amino propyl morpholine (1.5 ml, 9.9 mmol) was stirred for overnight at room temperature. After completion of reaction, 1M HCl (20 ml) was added to the mixture and extracted with ethyl acetate (2x100 mL) and then combined organic phases were dried (Na_2SO_4) and concentrated afforded **8** (0.34 g, 72%). Compound **8** was further used for debenzylation with catalytic amount of H_2 / $Pd(OH)_2$ in EtOAC (20 ml). After completion of reaction, the mixture was filtered through celite. The filtrate was concentrated and purified by silica gel column chromatography (10% MeOH in ethyl acetate) to afford **11** as solid (0.1 g, 44 %). The solid was dissolved in MeOH (1 ml), upon cooling 2M HCl in ether was added and stirred for 1h. Methanol was removed and appeared white solid; mp 170- 174 °C; $[\alpha]_D$ = - 6.1 (*c* 0.1, MeOH). ^{1}H NMR (500MHz, d6-DMSO): δ 11.42 (bs, 1H, OH), 9.28 (bs, 1H, OH), 9.10 (bs, 2H, NH_2), 8.80 (bs, 1H, OH), 6.89 (s, 1H), 6.85 (d, *J*=8.3 Hz, 1H), 6.80 (m, 1H), 4.80 (m, 1H), 3.95-3.88 (m, 4H), 3.18-2.91 (m, 10H), 2.18 (m, 2H). ν_{max} /cm^{-1}: 3344, 2963, 2920, 2856, 1600, 1528, 1421, 1260, 1068, 762. MS (ESI): calculated for $C_{15}H_{24}N_2O_4$, 296.36; found, 297.1 ($[M + H]^+$).

Preparation of Compound 9:
To a mixture of monotosyl compound **6** (1.0 g, 1.98 mmol) and ethelenediamine (0.2 ml, 2.18 mmol) was stirred for overnight at room temperature. After completion of reaction, water was added to the mixture and extracted with CH_2Cl_2 (2x100 mL). Combined organic phases were dried (Na_2SO_4) and concentrated to afford an amine (0.39 g, 50%). An amine was dissolved in dry CH_2Cl_2 was added morpholine-4-carbonyl chloride (0.46 ml, 3.96 mmol) and NEt_3 (0.83 ml, 5.9 mmol) at 0 °C under Ar. The reaction was stirred at room temperature for 12h. The reaction mixture was washed with water (100 mL), and brine (50 mL). The organic layer was dried (Na_2SO_4) and evaporated, and the residue was purified by column chromatography (silica gel, 70% ethyl acetate in petroleum ether) to afford **9** (0.41 g, 66%) as liquid. ^{1}H NMR (500MHz, $CDCl_3$): δ 7.45-7.28 (m, 10H), 7.11 (s, 1H), 6.91 (d, *J*=8.6 Hz, 1H), 6.86 (m, 1H), 5.59 (t, *J* = 5.0 Hz, 1H, NH), 5.14 (m,

4H), 4.89 (m, 1H), 3.70-3.67 (m, 9H), 3.50-3.40 (m, 13H). MS (ESI): calculated for $C_{34}H_{42}N_4O_7$, 618.72; found, 641.3 ($[M + Na]^+$).

Preparation of Compound 12:
Compound **9** (0.15 g, 0.24 mmol) was dissolved in (10ml) MeOH and added catalytic amount of $Pd(OH)_2$ and stirred for under H_2 atmosphere for 12h. After completion of reaction, the mixture was filtered through celite. Filtrate was concentrated and purified by silica gel column chromatography (2 % MeOH in $CHCl_3$) to afford 0.1 g of **12** as white solid (0.07 g, 70%); mp 70-74 °C; $[\alpha]_D$ = - 4.2 (*c* 0.4, MeOH). ^{1}H NMR (500MHz, $CDCl_3$): δ 7.62 (bs, 1H, OH), 7.5 (bs, 1H, OH), 6.78 (d, *J*=8.6 Hz, 1H), 6.70 (m, 1H), 6.51-6.47 (m, 1H), 6.25 (bs, 1H, NH), 3.67 (m, 8H), 3.40-3.39 (m, 10H), 3.20-3.18 (m, 4H), 2.71 (m, 2H). ν_{max} /cm^{-1}: 3356, 3179, 2956, 2811, 2451, 1602, 1519, 1358, 1260, 1008, 758. MS (ESI): calculated for $C_{20}H_{30}N_4O_7$, 438.47; found, 439.2 ($[M + Na]^+$).

Binding assay of Compound 12
Retinal endothelial cells and retinal Müller cells were cultured on 10 cm-culture plates, washed twice with 10 ml ice-cold PBS, then scraped from the plates and pelleted by centrifugation at 2,000g_{av} for 10 min. The cell pellets were suspended in 10 ml of hypotonic buffer composed of 20 mM HEPES, pH 7.4, 2 mM $MgCl_2$, 1 mM EDTA and 1 mM 2-mercaptoethanol supplemented with 10 µg/ml leupeptin and 10 µg/ml aprotinin (with or without 1 mM phenylmethyl sulfonyl fluoride) for 10 min on ice. The cells were lysed by 40 up-and-down strokes in a Dounce glass-glass homogenizer (Kimble Chase, Vineland NJ) then centrifuged at 2,500 g_{av} for 5 min. The supernatant was re-centrifuged at 15,000 g_{av} for 20 min to pellet the membranes.

Binding of the highly selective β-adrenergic receptor antagonist [^{125}I] iodocyanopindolol (ICYP) to 0.5 µg of membranes was measured in 50 mM Tris-HCl, pH 7.4 plus 10 mM $MgCl_2$ binding buffer containing 0.1 mM ascorbic acid for 2 h at 25˚C. For saturation binding experiments, ICYP concentrations ranging between 5 and 300 pM were used to calculate the K_D and the B_{max} for ICYP binding by parametric fitting of the data using the Prism 4 software (Graphpad Software, La Jolla, CA).

For competition binding experiments, ICYP 70 pM was competed with 24 increasing concentrations of Compound 12 ranging from 0.01 nM to 10 µM. The IC_{50} (high) and IC_{50} (low) values for Compound 12 were be derived from two-compartment competition to the –GTP data, using Prism 4 software. The IC_{50}'s were converted to the corresponding K_I (high) and K_I (low) values using the equation:

$$K_I = \frac{IC_{50}\ (nM)}{[1+ \text{concentration of ICYP})/K_D]}$$

Each saturation and competition experiment was performed in triplicate and each was replicated between 3 to 5 times to determine the mean ± SEM. Unfortunately, following these methods, we did not find any specific binding of Compound 12 to β-adrenergic receptors in either retinal endothelial cells or retinal Müller cells.

References:

1. P. Kumar, R. Kumar Upadhyay and R. K. Pandey, *Tetrahedron: Asymmetry*., 2004, **15**, 3955