Supporting Information

Minor Structural Modifications Convert the Dual TP/CRTH2 Antagonist Ramatroban into a Highly Selective and Potent CRTH2 Antagonist

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Table of Contents:

Synthetic procedures and characterization of compounds

General Comments. Melting points were determined on an Electrothermal 9200 melting point apparatus. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AMX 300 spectrometer, operating at 300.13 and 75.47 MHz respectively. Spectra were calibrated relative to tetramethylsilane internal standard or residual solvent peak. Infrared spectra were recorded from films on a NaCl plate or from KBr disks on a Perkin Elmer Spectrum One FT-IR spectrophotometer. High resolution mass spectra (HRMS) were obtained on a JEOL-SX 102 in FAB+ mode with NBA matrix. Analytical HPLC was performed on an Agilent 1100-series instrument equipped with UV-detector and a VL mass detector operating under electrospray ionisation conditions in positive (ESI^+) or negative (ESI^-) mode. Conditions: column: XTerra MS C18; flow: 1.0 mL/min; gradient: 0-5 min: 15-100% acetonitrile in water, 5-7½ min: 100% acetonitrile, modifier: 5 mM ammonium formate. Elemental analyses were performed by Johannes Theiner at Mikroanalytisches Laboratorium, University of Vienna, Austria, and results are within 0.4% of calculated values, unless otherwise stated. All reactions were conducted under an atmosphere of argon, unless otherwise stated. Starting materials and reagents were purchased from Aldrich. Dry solvents were purchased from Fluka and used as obtained.

4-Fluoro-*N*-methyl-*N*-(2,3,4,9-tetrahydro-1H-carbazol-3-yl)benzenesulfonamide (4). A solution of 1,2,4,9-tetrahydrocarbazole-3-one[1] (800 mg, 4.32 mmol) in dry MeOH (80 mL) was added methylamine (2M in THF, 15 mL, 30 mmol), acetic acid (1.72 mL, 30 mmol) and dry $MgSO_4$ (5 g). The reaction mixture was stirred at room temperature for 15 min before $NaBH_3CN$ (534 mg, 8.5 mmol) was added, and stirring was continued for 12 h. The reaction was added EtOAc, solids were filtered off and washed with EtOAc. The organic phase was washed with 1 M Na_2CO_3 (2x) and brine (1x), dried ($MgSO_4$) and concentrated to give methyl-(2,3,4,9-tetrahydro-1H-carbazol-3-yl)-amine as a yellow oil (750 mg), which was used directly in the next step.

The methyl-(2,3,4,9-tetrahydro-1H-carbazol-3-yl)-amine (3.8 g, 19 mmol) in CH_2Cl_2 (100 mL) was added Et_3N (4.2 mL, 30 mmol) and fluorobenzenesufonyl chloride (5.8 g, 30 mmol), and the reaction was stirred at room temperature. After 12 h the reaction

mixture was diluted with CH_2Cl_2 and the organic phase was washed with 3% HCl (2x), 1 M NaOH (2x) and brine (1x), dried ($MgSO_4$) and concentrated. The residue was purified by flash chromatography (SiO_2, EtOAc:heptane, 1:2), and the product was recrystallized from MeOH to give 4.6 g (68%) of **4** as yellow crystals: mp: 211-212 ^oC; ^1H NMR ($CDCl_3$): δ 1.83 (m, 1H), 2.02 (ddd, *J* = 17.7, 11.9, 5.8 Hz, 1H), 2.63-2.80 (m, 2H), 2.81-3.02 (m, 2H), 2.92 (s, 3H), 4.33 (m, 1H), 7.09 (m, 1H), 7.16 (m, 1H), 7.20-7.31 (m, 3H), 7.34 (d, *J* = 4.1 Hz, 1H), 7.76 (br s, 1H), 7.90 (m, 2H); ^{13}C NMR ($CDCl_3$): δ 23.1, 24.5, 27.6, 29.2, 54.6, 108.4, 110.8, 116.6 (d, $^2J_{CF}$ = 22 Hz), 117.8, 119.7, 121.9, 127.6, 129.9 (d, $^3J_{CF}$ = 9 Hz),132.4, 136.5, 165.4 (d, $^1J_{CF}$ = 253 Hz); IR ν_{max} 3400, 1592, 1492, 1333, 1236, 1166, 1154, 1088, 959, 840, 741 cm^{-1}; MS ESI^+ *m/z* 358.9 $[M + H]^+$.

3-{3-[(4-Fluorobenzenesulfonyl)methylamino]-1,2,3,4-tetrahydrocarbazol-9-yl}-propionic acid (5). The indole **4** (339 mg, 0.95 mmol) in dry DMF (18 mL) was added NaH (60% in mineral oil, 44 mg, 1.1 mmol) and the reaction mixture was stirred under argon at room temperature. After 60 min ethyl 3-bromopropionate (0.25 mL, 2.0 mmol) was added, and stirring at room temperature was continued. After 12 h the reaction mixture was added EtOAc. The organic phase was washed with water (3x) and brine (1x), dried ($MgSO_4$) and concentrated. The crude product was purified by flash chromatography (SiO_2, EtOAc:heptane, 1:2) to give 365 mg (87%) white foam, consisting of a 10:7 mixture of the alkylated product and the starting material (**4**). The mixture was inseparable on TLC, and was used directly in the next step: TLC (EtOAc:heptane, 1:2): R_f 0.20.

The product mixture containing the ethyl ester in THF (15 mL) was added $LiOH \cdot H_2O$ (65 mg, 1.6 mmol) in water (6 mL). After 12 h the reaction was acidified with 3% HCl and extracted with CH_2Cl_2 (2x). The extract was washed with brine (1x), dried ($MgSO_4$) and concentrated. The residue was purified by flash chromatography (SiO_2, EtOAc:heptane, 1:1, then [MeOH w/10%AcOH]:EtOAc, 1:9) to give 186 mg (47% over 2 steps) of **5** as a white solid: mp 148-151 ^oC; TLC (SiO_2, [MeOH w/10%AcOH]:EtOAc, 1:9) R_f 0.5; ^1H NMR ($CDCl_3$): δ 1.83-2.01 (m, 2H), 2.60-2.75 (m, 2H), 2.79 (t, *J* = 7.1 Hz, 2H), 2.80-3.00 (m, 2H), 2.89 (s, 3H), 4.25-4.44 (m, 1H), 4.34 (td, *J* = 7.1, 3.4 Hz, 2H), 7.04-7.11 (m, 1H), 7.14-7.29 (m, 4H), 7.33 (d, *J* = 7.5 Hz, 1H), 7.88 (m, 2H); ^{13}C

NMR ($CDCl_3$): δ 22.0, 24.5, 27.6, 29.2, 34.5, 38.6, 54.4, 108.1, 109.0, 116.6 (d, $^2J_{CF}$ = 22 Hz),118.1, 119.7, 121.7, 127.3, 129.9 (d, $^3J_{CF}$ = 9 Hz), 133.4, 136.5, 165.2 (d, $^1J_{CF}$ = 253 Hz), 176.2; IR ν_{max} 1712, 1592, 1493, 1467, 1337, 1167, 958, 840, 737 cm^{-1}; HRMS calcd for $C_{22}H_{23}FN_2O_4S$ $[M]^+$: 430.1363, found 430.1353; LC/MS ESI^- R_t 3.23 min, *m/z* 428.8 $[M - H]^-$, purity: >95% by UV-trace, >95 by MS-trace. Anal. ($C_{22}H_{23}FN_2O_4S$·¼H_2O) C, H, N, S.

Ethyl (3-oxo-1,2,3,4-tetrahydrocarbazol-9-yl)-acetate glycol ketal (6). The indol **2** (4.53 g, 19.8 mmol) in dry DMF (225 ml) under argon at room temperature was added NaH (60% in mineral oil, 948 mg, 23.7 mmol) in one portion. After 60 min ethyl bromoacetate (4.4 mL, 40 mmol) was added and stirring was continued. After 12 h the reaction mixture was added EtOAc and the organic phase was washed with water (3x) and brine (1x), dried ($MgSO_4$) and concentrated. The crude product was recrystallized from EtOAc:heptane (1:2), and the crystals were washed with EtOAc:heptane (1:5) to give 4.71 g white powder. The residue was concentrated and purified by flash chromatography (SiO2, EtOAc:hepante, 1:2), and the product was recrystallized from Et_2O to provide another 0.77 g white powder, in total 5.48 g (88%) of **6** as white fine crystals: mp 112-113 ^oC; TLC (SiO_2, EtOAc:heptane, 1:2) Rf 0.2; ^1H NMR ($CDCl_3$): δ 1.26 (t, *J* = 7.0 Hz, 3H), 2.11 (t, *J* = 6.4 Hz, 2H), 2.88 (t, *J* = 6.4 Hz, 2H), 2.99 (s, 2H), 4.06 (m, 4H), 4.20 (q, *J* = 7.1 Hz, 2H), 4.72 (s, 2H), 7.05-7.19 (m, 3H), 7.42 (d, *J* = 7.9 Hz, 1H); ^{13}C NMR ($CDCl_3$): δ 14.4, 20.6, 31.9, 32.1, 45.1, 61.8, 64.9, 108.5, 108.6, 109.2, 118.1, 119.6, 121.6, 127.6, 133.6, 137.7, 169.0; IR ν_{max} 1751, 1468, 1198, 1115, 1055, 1017, 740 cm^{-1}; MS ESI^+ *m/z* 315.9 $[M + H]^+$.

Ethyl (3-oxo-1,2,3,4-tetrahydrocarbazol-9-yl)-acetate (7). Ketal **6** (1.43 g, 4.54 mmol) in THF (40 mL) was added 15% aq. HCl (10 mL), and the homogenous reaction mixture was stirred at room temperature under inert atmosphere. After 16 h the reaction was added water and extracted with CH_2Cl_2 (3x). The extract was washed with brine (1x), dried ($MgSO_4$) and concentrated to give 1.32 g red oil consisting of a mixture of the title compound and the corresponding carboxylic acid. The crude product was dissolved in dry CH_2Cl_2 (40 mL) and added EDC (600 mg, 4 mmol). The mixture was stirred at

room temperature under argon for 15 min, and dry EtOH (10 mL) was added. Stirring was continued for 3 h. The reaction was concentrated, added CH_2Cl_2, washed with 3% HCl, and brine, dried ($MgSO_4$) and concentrated. The residue was subject to flash chromatography (SiO_2, EtOAc:heptane, 1:2) to provide 1.0 g (83%) of compound **7** as white fine crystals: mp 92-94 ^oC; TLC (SiO_2, EtOAc:heptane, 1:2): Rf 0.2; ^1H NMR ($CDCl_3$): δ 1.27 (t, *J* = 7.2 Hz, 3H), 2.83 (t, *J* = 7.0 Hz, 2H), 3.12 (t, *J* = 7.0 Hz, 2H), 3.64 (s, 2H), 4.22 (q, *J* = 7.2 Hz; 2H), 4.78 (s, 2H), 7.11-7.18 (m, 1H), 7.20-7.24 (m, 2H), 7.44 (d, *J* = 7.7 Hz, 1H); ^{13}C NMR ($CDCl_3$): δ 14.4, 21.7, 36.6, 38.6, 45.0, 62.0, 107.7, 108.9, 118.9, 120.2, 122.4, 126.9, 133.9, 137.7, 168.7, 209.3; IR ν_{max} 1748, 1714, 1467, 1200, 1179, 741 cm^{-1}; MS ESI^+ *m/z* 272.0 $[M + H]^+$.

Ethyl [3-(4-fluorobenzenesulfonylamino)-1,2,3,4-tetrahydrocarbazol-9-yl]acetate (10). Ketone **7** (136 mg, 0.5 mmol) in EtOH (3 mL) was added ammonium formate (385 mg, 5 mmol) and sodium cyanoborohydride (31 mg, 0.5 mmol). The reaction was stirred under argon at room temperature. After 24 h the reaction mixture was concentrated and the residue was partitioned between CH_2Cl_2 and 1 M Na_2CO_3. The organic phase was dried ($MgSO_4$) and concentrated to give amine **8** as a green oil, which was used in the next step without purification: LC/MS: R_t 2.59 min, *m/z* 272.9 $[M + H]^+$.

The crude amine **8** in dry CH_2Cl_2 (8 mL) was added triethylamine (0.08 mL, 0.6 mmol) and 4-fluorophenylsulfonyl chloride (117 mg, 0.6 mmol). The reaction was stirred under argon at room temperature. After 16 h the reaction mixture was added CH_2Cl_2 and washed with 3% HCl (2x), 1 M Na_2CO_3 (1x) and brine (1x), dried ($MgSO_4$) and concentrated. The residue was purified by flash chromatography (SiO_2, EtOAc:heptane, 1:2) to give 56 mg (26% yield over 2 steps) **10** as a white amorphous solid: TLC(SiO_2, EtOAc:heptane, 1:2): R_f 0.15; ^1H NMR ($CDCl_3$): δ 1.27 (t, *J* = 7.0 Hz, 3H), 1.98-2.07 (m, 2H), 2.52 (dd, *J* = 15.6, 6.0 Hz, 1H), 2.68-2.78 (m, 2H), 2.89 (dd, *J* = 15.5, 4.7 Hz, 1H), 3.82 (m, 1H), 4.20 (q, *J* = 7.0 Hz, 2H), 4.70 (s, 2H), 4.86 (d, *J* = 8.1 Hz, 1H), 7.04-7.11 (m, 1H), 7.11-7.22 (m, 4H), 7.30 (d, *J* = 7.0 Hz, 1H), 7.89 (m, 2H); ^{13}C NMR ($CDCl_3$): δ 14.4, 19.2, 28.6, 29.3, 44.8, 49.4, 61.9, 106.9, 108.7, 116.6 (d, $^2J_{CF}$ = 22 Hz), 118.1, 119.9, 122.0, 127.4, 129.9 (d, $^3J_{CF}$ = 9 Hz), 134.0, 137.3, 137.4, 165.2 (d, $^1J_{CF}$ = 253 Hz),

168.9; IR ν_{max} 3286, 2931, 1740, 1592, 1468, 1166, 1153, 839, 740 cm^{-1}; MS ESI^+ *m/z* 430.8 $[M + H]^+$.

[3-(4-Fluorobenzenesulfonylamino)-1,2,3,4-tetrahydrocarbazol-9-yl]acetic acid (12). The ester **10** (15 mg, 0.034 mmol) in THF (1 mL) was added $LiOH \cdot H_2O$ (5 mg, 0.1 mmol) in water (0.6 mL). The homogenous reaction mixture was stirred at room temperature. After 1 h the reaction was added 3% HCl until pH <1 and the mixture was extracted with CH_2Cl_2 (2x). The extract was washed with brine (1x), dried ($MgSO_4$) and concentrated to give 13 mg (95%) of acid **12** as a white foam: TLC(SiO_2, [MeOH w/5% AcOH]:EtOAc, 1:9) R_f ~0.25; ^1H NMR ($CDCl_3$): δ 1.95-2.06 (m, 2H), 2.50 (dd, *J* = 15.6, 6.0 Hz, 1H), 2.59-2.78 (m, 2H), 2.85 (dd, *J* = 15.6, 4.9 Hz, 1H), 3.76-3.86 (m, 1H), 4.69 (s, 2H), 5.00 (d, *J* = 7.7 Hz, 1H), 7.04-7.21 (m, 5H), 7.29 (d, *J* = 7.5 Hz, 1H), 7.81-7.90 (m, 2H); ^{13}C NMR ($CDCl_3$): δ 19.1, 28.6, 29.1, 44.2, 49.4, 107.2, 108.7, 116.6 (d, $^2J_{CF}$ = 22 Hz), 118.2, 120.1, 122.1, 129.9 (d, $^3J_{CF}$ = 9 Hz), 134.0, 137.2, 173.2; IR ν_{max}3273, 2928, 1732, 1592, 1494, 1468, 1166, 1153, 910, 839, 736 cm^{-1}. HRMS calcd for $C_{20}H_{19}FN_2O_4S$ $[M]^+$: 402.1050, found 402.1042. LC/MS ESI^- R_t 2.56 min, *m/z* 400.8 $[M - H]^-$, purity: >97% by UV-trace, >97 by MS-trace.

Ethyl {3-[(4-fluorobenzenesulfonyl)methylamino]-1,2,3,4-tetrahydrocarbazol-9-yl}-acetate (11). The carbazolone **7** (900 mg, 3.3 mmol) in dry EtOH (60 mL) was added acetic acid (1.32 mL, 23 mmol), methyl amine (33 wt% in EtOH, 2.86 mL, 23 mmol) and 3Å molecular sieves (7 g). The reaction mixture was stirred for 15 min before sodium cyanoborohydride (600 mg, 7 mmol) was added. Stirring was continued over night. The reaction was then added EtOAc, solids were filtered off and washed with EtOAc. The organic phase was washed with 1 M Na_2CO_3 (2x) and brine (1x), dried ($MgSO_4$) and concentrated to give 0.9 g of methylamine **9** as a green solid foam, which was used directly in the next step.

The crude methylamine **9** (0.9 g) in CH_2Cl_2 (20 mL) was added Et_3N (0.81 mL, 5.8 mmol) and fluorobenzenesufonyl chloride (1.23 g, 5.8 mmol), and the reaction mixture was stirred at room temperature. After 12 h the reaction mixture was diluted with CH_2Cl_2 (250 mL), washed with 3% HCl (2x), 1 M NaOH (2x) and brine (1x), dried ($MgSO_4$) and

concentrated. The residue was purified by flash chromatography (SiO_2, EtOAc:heptane, 1:2) to give 0.92 g (54%) of **11** as a white foam: TLC (SiO_2, EtOAc:heptane, 1:2) R_f 0.2; ^{1}H NMR ($CDCl_3$): δ 1.26 (t, *J* = 7.2 Hz, 3H), 1.82-2.06 (m, 2H), 2.61-2.91 (m, 4H), 2.90 (s, 3H), 4.20 (q, *J* = 7.1 Hz, 2H), 2.26-2-39 (m, 1H), 4.69 (s, 2H), 7.03-7.12 (m, 1H), 7.12-7.27 (m, 4H), 7.33 (d, *J* = 7.7 Hz, 1H), 7.84-7.92 (m, 2H); ^{13}C NMR ($CDCl_3$): δ 14.4, 21.8, 24.4, 27.5, 29.2, 44.8, 54.3, 61.9, 108.4, 108.7, 116.6 (d, $^2J_{CF}$ = 22 Hz), 118.1, 119.9, 121.9, 127.3, 129.9, 133.8, 136.48, 136.52, 137.3, 165.2 (d, $^1J_{CF}$ = 253 Hz), 166.9, 168.8; IR ν_{max} 1749, 1591, 1493, 1467, 1338, 1195, 1167, 1088, 958, 840, 740 cm^{-1}; MS ESI$^+$ *m/z* 444.6 $[M + H]^+$.

{3-[(4-Fluorobenzenesulfonyl)methylamino]-1,2,3,4-tetrahydrocarbazol-9-yl}acetic acid (13). The ester **11** (0.56 g, 1.26 mmol) in THF (20 mL) was added $LiOH{\cdot}H_2O$ (105 mg, 2.5 mmol) in water (8 mL) and the reaction was stirred at room temperature. After 12 h the reaction was added 3% HCl until pH <1 and the mixture was extracted with CH_2Cl_2 (2x). The extract was washed with brine (1x), dried ($MgSO_4$) and concentrated to give 0.49 g (94%) orange solid foam. The crude product was subject to flash chromatography (SiO_2, EtOAc:Hep, 1:1, then [MeOH w/10%AcOH]:EtOAc, 1:9). The residue was dissolved in CH_2Cl_2 and washed with 3% HCl (1x) and brine (1x), dried (MgSO4) and concentrated to give 0.30 g (57%) solid as orange foam. The product was recrystallized from CH_2Cl_2 to give 178 mg (34%) of **13** as white crystals: mp 165-173 ^{o}C; TLC (SiO_2, [MeOH w/5% AcOH]:EtOAc, 1:9) R_f 0.40; ^{1}H NMR (DMSO-d_6): δ 1.48-1.58 (m, 1H), 1.79-1.98 (m, 1H), 2.45-2.59 (m, 1H), 2.60-2.85 (m, 3H), 2.83 (s, 3H), 4.12 (m, 1H), 4.84 (s, 2H), 6.93-7.09 (m, 2H), 7.26-7.33 (m, 2H), 7.42-7.52 (m, 2H), 7.89-8.00 (m, 2H); ^{13}C NMR (DMSO-d_6): δ 21.0, 24.3, 26.0, 28.9, 44.0, 54.1, 106.8, 109.1, 116.6 (d, $^2J_{CF}$ = 23 Hz), 117.3, 118.9, 120.8, 126.5, 129.9 (d, $^3J_{CF}$ = 10 Hz), 134.4, 135.9, 137.0, 164.4 (d, $^1J_{CF}$ = 249 Hz), 170.4; IR ν_{max} 1726, 1591, 1493, 1466, 1334, 1166, 1155, 958, 839, 741 cm^{-1}; HRMS calcd for $C_{21}H_{21}FN_2O_4S$ $[M]^+$: 416.1206, found 416.1208. LC/MS ESI$^-$ R_t 4.40 min, *m/z* 415.0 [M - H], purity: >99% by UV-trace, >99% by MS-trace. Anal. ($C_{21}H_{21}FN_2O_4S$) C, H, N, S.

Purity of target compounds

Elemental analysis

Cmpd	Formula	Calc				Found			
		C	H	N	S	C	H	N	S
5	$C_{22}H_{23}FN_2O_4S \cdot ¼H_2O$	60.60	5.35	6.42	7.37	60.74	5.45	6.43	7.24
13	$C_{21}H_{21}FN_2O_4S$	60.56	5.08	6.73	7.70	60.23	5.08	6.74	7.61

LC/MS purity

Compound	R_t (min)	m/z	Purity by UV-trace	Purity by MS-trace
5	3.23	428.8 [M – H]	>95%	>95%
12	2.56	400.8 [M – H]	>97%	>97%
13	4.40	415.0 [M – H]	>99%	>99%

Biological assays

Generation/origin of the cDNA Constructs. The coding sequence of human CRTH2 (genbank accession no NM_004778) was amplified by PCR from a human hippocampus cDNA library and inserted into the pcDNA3.1(+) expression vector (invitrogen) via 5' Hind*III* and 3' EcoR*I*. To generate a CRTH2-Renilla luciferase (CRTH2-Rluc) fusion protein, the CRTH2 coding sequence without a STOP codon and Rluc were amplified, fused in frame by PCR and subcloned into the pcDNA3.1(+)Zeo expression vector. The thromboxane A2 (TXA2) receptor (gen bank accession no BC074749, version beta) has been cloned from a leukocyte cDNA library and inserted via 5' Hind*III* and 3' BamH*I* into pcDNA3.1(+). A TXA2-receptor-Rluc fusion protein was generated by PCR and inserted via 5' Hind*III* and 3' Xba*I* into pcDNA3.1(+). The prostaglandin D2 DP receptor (gen bank accession no NM_000953) was cloned from a bone marrow cDNA library and inserted via 5' Hind*III* and 3' BamH*I* into pcDNA3.1(+). Human β-arrestin2 (β-arr2) N-terminally tagged with GFP^2 (GFP^2-β-arr2) and Renilla luciferase were purchased from BioSignal Packard Inc, Montreal, Canada). The sequence identity of the constructs was verified by restriction endonuclease digests and sequencing in both directions on an ABI Prism (Applied Biosystems, Foster City, CA).

Cell Culture and Transfection. COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) 1885 supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 1000 µg/ml streptomycin, and kept at 37°C in a 10% CO_2 atmosphere. HEK293 cells were maintained in Minimum Essential medium (MEM) supplemented with 10% (v/v) heat inactivated fetal calf serum (HIFCS), 2mM Glutamax^{TM}-I, 1% non essential amino acids (NEAA), 1% sodium pyruvate and 10 µg/ml gentamicin. For transient transfections, cells were allowed to express the exogeneously added DNA for 48 hours before performing the binding or functional experiments. Transient transfections on COS7 cells were performed essentially as described by Heydorn et al.[2] using a calcium phosphate-DNA co-precipitation method with the addition of chloroquine. For functional inositolphosphate assays of CRTH2, COS7 cells were transiently cotransfected with the receptor cDNA and a promiscuous Gα protein[3] facilitating inositolphosphate production by the otherwise Gi/o-selective receptor. Inositolphosphate assays of the Gq/11-coupled TXA2 receptor were performed in the absence of any cotransfected Gα protein as was the case for cAMP assays performed in DP receptor expressing cells. To perform the binding and functional **B**ioluminescence **R**esonance **E**nergy **T**ransfer (BRET) assays, a HEK293 cell clone stably expressing βarr2-GFP^2 and CRTH2-Rluc was generated. BRET assays

on the TXA2 receptor were performed upon transient transfection of the TXA2-Rluc fusion protein into a HEK293 cell clone stably expressing βarr2-GFP^2.

Binding experiments. – *whole cell binding* - 24h after transfection COS-7 cells were seeded into 96well plates at a density of 30.000 cells/well. Competition binding experiments on whole cells were then performed about 18-24 h later using the appropriate radioligands (see below) in a binding buffer consisting of HBSS (GIBCO) and 10 mM HEPES (pH 7.5). The following radioligands were used: (i) 1.2 nM [^{3}H]PGD2 (NEN, 172 Ci/mmol) for CRTH2 expressing cells, (ii) 0,5 nM [^{3}H]PGD2 for DP receptor expressing cells, and (iii) 5 nM [^{3}H]SQ29548 (Perkin Elmer, 48,2 Ci/mmol) for TXA2 receptor expressing cells. Competing ligands were diluted in DMSO which was kept constant at 1% (v/v) of the final incubation volume. Total and nonspecific binding were determined in the absence and presence of 10 μM PGD2 for CRTH2 and DP expressing cells, and 50 μM U46619, respectively, for TXA2 expressing cells. Binding reactions were routinely conducted for 3 h at 4°C and terminated by 2 washes (100 μl each) with ice cold binding buffer. Radioactivity was determined by liquid scintillation counting in a TOPCOUNT (Packard) following over night incubation in Microscint 20. Stable HEK293 cells expressing CRTH2-Rluc were seeded at a density of 30.000 cells/well 18-24 h prior to the binding assay which was performed essentially as described for COS7 cells above. Fusion of Renilla luciferase to the C-terminus of CRTH2 resulted in a fusion protein that displayed virtually indistinguishable pharmacology from CRTH2wt. Determinations were made in duplicates.

BRET assay. Functional BRET assays were performed on HEK293 cells stably expressing human CRTH2-Rluc and GFP^2-β-arr2 or transiently expressing the TXA2-Rluc fusion protein together with GFP^2-β-arr2. Prior to their use in the BRET assay cells were detached and re-suspended in D-PBS with 1000 mg/l L-Glucose at a density of 2x10^6 cells/ml. DeepBlueC™ was diluted to 50 μM in D-PBS with 1000 mg/l L-Glucose (light sensitive). 100 μl of cells were transferred to wells in a 96-well microplate (white OptiPlate) and placed in the Mithras LB 940 instrument (BERTHOLD TECHNOLOGIES, Bad Wildbad, Germany). 12 μl/well agonist were then injected by injector 1 and 10 μl DeepBlueC™/well were injected simultaneously by injector 2. 5 seconds after the injections the light output from the well was measured sequentially at 400 nm and 515 nm, and the BRET signal was determined by calculating the ratio of the fluorescence emitted by GFP^2-β-arr2 (515 nM) over the light emitted by the receptor-Rluc (400 nM). Antagonists were added before placing the microplates into the Mithras LB 940 and allowed to preincubate for 15 minutes prior to the addition of agonist and DeepBlueC™. Compounds were dissolved in DMSO and the final DMSO concentration was kept constant at 1% in the assay.

Inositol phosphate accumulation assays. 24 h after transfection cells were seeded in 96-well tissue culture plates and loaded with 5 μCi *myo*-[2-^{3}H]-Inositol (16 Ci/mmol, TRK911, Amersham Biosciences). The next day cells were then washed twice in HBSS buffer (including $CaCl_2$ and $MgCl_2$, GIBCO cat. 14025-050) and stimulated with the respective agonists in HBSS buffer supplemented with 5 mM LiCl for 45 minutes at 37°C. The reactions were terminated by aspiration and addition of 10 mM ice-cold formic acid, and incubated for 30 minutes on ice. The lysate was applied to AG 1-X8 anion-exchange resin (Bio-Rad, Hercules, CA) and washed twice with buffer containing 60 mM sodium formate and 5 mM borax. The [^{3}H]-inositol-phosphate fraction was then eluted by adding 1 M ammonium formate and 100 mM formic acid solution and counted after addition of HiSafe3 scintillation fluid (PerkinElmer, Boston, MA).

cAMP accumulation assays. COS7 cells transiently expressing the DP receptor were metabolically labeled with 2 μCi of [^{3}H]adenine (Amersham, TRK311) in 6-well plates for 18-24 hours at 37°C. They were then washed twice with PBS and stimulated with increasing concentrations of PGD2 in HEPES-buffered saline supplemented with 1 mM isobutylmethylxanthine (IBMX) for 30 minutes at 37°C. The reaction was stopped by adding 5% (w/v) ice-cold trichloroacetic acid supplemented with 0.1 mM cAMP and 0.1 mM ATP. [^{3}H]cAMP was separated from the remaining nucleotides using anion exchange chromatography as described[4] and radioactivity was counted after addition of HiSafe3 scintillation fluid (PerkinElmer, Boston, MA).

Materials. White 96well Optiplates and DeepBlueC™ were obtained from Packard BioScience, Montreal, Canada. Tissue culture media and reagents were purchased from the GIBCO invitrogen corporation (Breda, Netherlands). PGD2 was from Cayman and [^{3}H]PGD2 from NEN. All other laboratory reagents were from Sigma (St. Louis, MO), unless explicitly specified.

Calculations and data analysis - IC_{50} and EC_{50} values were determined by nonlinear regression using the Prism 3.0 software (GraphPad Software, San Diego). Values of the dissociation and inhibition constants (K_d and K_i) were estimated from competition binding experiments using the equations $K_d = IC_{50} - L$ and $K_i = IC_{50}/(1 + L/K_d)$, where L is the concentration of radioactive ligand and K_d is its dissociation constant. Antagonistic potencies in functional assays are given as IC_{50} values that were obtained by competing with increasing concentrations of inhibitor compound for an agonist concentration required to elicit about 75-80% of the maximal agonist efficacy. IC_{50} values generated by this procedure are very closely matching the true affinity of antagonists determined by the analysis according to Arunlakshana & Schild.[5]

References:

1. Urrutia, A.; Rodríguez, J. G. Synthesis of Aspidospermidine Alkaloids from 1,2,3,4-tetrahydrocarbazole: Total Stereoselective Synthesis of (±)-18-Noraspidospermidine. *Tetrahedron* **1999**, *55*, 11095-11108.
2. Heydorn, A.; Ward, R. J.; Jorgensen, R.; Rosenkilde, M. M.; Frimurer, T. M.; Milligan, G., Kostenis, E. Identification of a novel site within G protein alpha subunits important for specificity of receptor-G protein interaction. *Mol. Pharmacol*. **2004**, 66, 250-259.
3. Kostenis, E. Is Galpha16 the optimal tool for fishing ligands of orphan G-protein-coupled receptors? *Trends Pharmacol. Sci*. **2001**, *22*, 560-564.
4. Holst, B.; Hastrup, H.; Raffetseder, U.; Martini, L.; Schwartz, T. W. Two active molecular phenotypes of the tachykinin NK1 receptor revealed by G-protein fusions and mutagenesis. *J. Biol. Chem*. **2001** *276*, 19793-19799.
5. Arunlakshana, O.; Schild, H. O. Some quantitative uses of drug antagonists. *Br J. Pharmacol.* **1959** *14*, 48-58.