Supporting Information

Development of [^{3}H]2-carboxy-4,6-dichloro-1*H*-indole-3-propionic acid ([^{3}H]PSB-12150) – a useful tool for studying GPR17

*Meryem Köse[a‡], Kirsten Ritter[a‡], Katharina Thiemke[a], Michel Gillard[b], Evi Kostenis[c], and Christa E. Müller[a]**

[a]PharmaCenter Bonn, Pharmaceutical Institute, Pharmaceutical Chemistry I, University of Bonn, Bonn, Germany.
[b]UCB Pharma S.A., CNS Research, Chemin du Foriest, B - 1420 Braine-l'Alleud, Belgium.
[c]PharmaCenter Bonn, Institute of Pharmaceutical Biology, Section of Molecular-, Cellular-, and Pharmacobiology, University of Bonn, Bonn, Germany.
‡These authors contributed equally.

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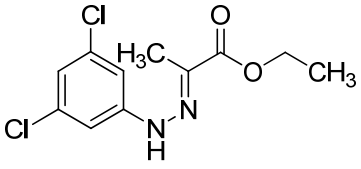
Experimental procedures

Materials and Methods

Chemicals, reagents and solvents were purchased from either Fluka, Riedel-de-Haen and Aldrich (products sold by Sigma-Aldrich, Steinheim, Germany), Alfa Aesar (Karlsruhe, Germany), VWR (Langenfeld, Germany), ABCR (Karlsruhe, Germany) or Acros (products sold by Fischer Scientific, Nidderau, Germany). Unless otherwise mentioned solvents were used without further purification. The reactions were monitored by thin layer chromatography (TLC) using silica gel 60 F_{254} (Merck, Darmstadt, Germany) pre-coated aluminum sheets. Purification via column chromatography was carried out using Merck silica gel 60 (0.06 mm to 0.20 mm) (Merck, Darmstadt, Germany). Purification via reverse phase HPLC was carried out on a Knauer-System (Knauer GmbH, Berlin) equipped with a Eurospher 100-column (250 x 20 mm, packed with RP-silica gel C-18, 10 µm (Knauer GmbH, Berlin)) and a Wellchrome K-2600 spectrometer detection unit. HPLC mobile phases consisted of methanol/water containing 1.5 % triethylamine from 20/80 to 100/0 in 40 min, flow rate 20 ml/min. After separation, the fractions were lyophilized with an Alpha 1-4 LSC freeze dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany). ^{1}H-NMR and ^{13}C-NMR spectra were measured on a Bruker Avance 500 NMR spectrometer using DMSO-d_6 or CD_3OD as solvent (^{1}H: 500 MHz, ^{13}C: 125 MHz). Chemical shifts are expressed in δ (ppm) values relative to the solvent peak used as internal reference. The respective chemical shifts are δ [ppm] DMSO-d_6: 2.49 and CD_3OD: 3.35 for ^{1}H spectra and δ [ppm] DMSO-d_6: 37.7 and CD_3OD: 49.3 for ^{13}C spectra. Abbreviations for signal coupling are as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. Coupling constants (*J*) are given in Hz. Melting points were obtained on a Büchi B-545 apparatus (BÜCHI Labortechnik GmbH, Essen, Germany) and are uncorrected.
CHO-K1 cells and 1321N1 astrocytoma cells were obtained from the European Collection of Cell Cultures (ECACC). The 1321N1 astrocytoma-hGPR17 and CHO-FITR-hGPR17 cell lines were generated as described previously.[1] Tissue culture media and reagents were purchased from Invitrogen (Karlsruhe, Germany). FCS was from Sigma-Aldrich (Munich, Germany). CysLT1 antagonists (pranlukast, montelukast) were obtained from Cayman Chemicals (Ann Arbor, MI, USA). All other laboratory reagents were obtained from Sigma-Aldrich unless specified otherwise.

Synthetic procedures

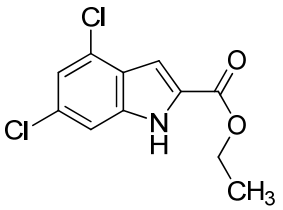


Ethyl 2-(2-(3,5-dichlorophenyl)hydrazono)propanoate (4)

To a suspension of 3,5-dichloroaniline (5.00 g, 30.9 mmol, 1.0 eq) in water (30 mL) concentrated hydrochloric acid (20 mL) was added. The suspension was cooled to 0 °C. A solution of sodium nitrite (2.34 g, 34.0 mmol, 1.1 eq) in water (11 mL) was added dropwise and the mixture was stirred for 20 min (solution 1). In a separate vessel, a solution of potassium hydroxide (15.5 g, 276.2 mmol, 8.9 eq) in water (30 mL) was added dropwise at 0 °C to a solution of ethyl 2-

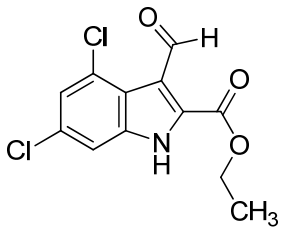
methylacetoacetate (6.6 g, 45.8 mmol, 1.5 eq) in ethanol (25 ml) (solution 2). Solution 1 was cooled to 0 °C and ice was added followed by solution 2 in one portion. The resulting mixture was warmed to 40 °C for 15 min. The reaction mixture was then allowed to cool and extracted three times with diethyl ether. The combined extracts were dried ($MgSO_4$) and after filtration, the solvent was evaporated. The crude residue was purified by column chromatography (petroleum ether/ethyl acetate, 9:1) to yield the pure product as beige-colored solid (6.34 g, 23.0 mmol, 75 % (both *E*- and *Z*-diastereomers)). m.p. 127 °C. R_f (petroleum ether/ethyl acetate, 9:1) 0.75 and 0.48. δ_H (500 MHz, DMSO-d_6) 1.26 (t, ^3J = 7.1 Hz, 3H, CH_2-$\underline{CH_3}$), 2.05 (s, 3H, CH_3), 4.20 (q, ^3J = 7.1 Hz, 2H, $\underline{CH_2}$-CH_3), 7.01 (pseudo-t, ^4J = 1.9 Hz, 1H, H-4), 7.21 (d, ^4J = 1.9 Hz, 2H, H-2 and H-6), 10.06 (s, 1H, NH). δ_C (125 MHz, DMSO-d_6) 12.26 (CH_3), 14.33 (CH_2-$\underline{C}H_3$), 61.00 ($\underline{C}H_2$-CH_3), 112.10 (C-2 and C-6), 119.67 (C-4), 134.72 (C-3 and C-5), 135.25 (C-N), 146.92 (C=N), 164.59 (C=O).

Ethyl-4,6-dichloro-1*H*-indole-2-carboxylate (5)



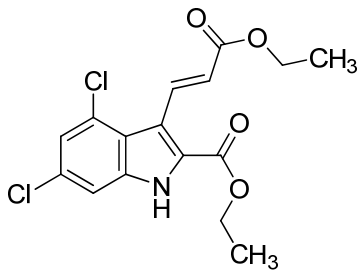
A mixture of ethyl 2-(2-(3,5-dichlorophenyl)hydrazono)propanoate (**4**, 750 mg, 2,73 mmol, 1.0 eq), polyphosphoric acid (5.0 g) and toluene (10 mL) was stirred for 20 min at 45 °C. The solvent was subsequently evaporated and ice was added to the residue. The resulting precipitate was filtered off and purified by column chromatography (petroleum ether/ethyl acetate, 9:1) to yield the pure product as a white solid (685 mg, 2.65 mmol, 97 %). m.p. 185 °C (lit. 185 – 187 °C)[2]. R_f (dichloromethane, 100 %) 0.69. δ_H (500 MHz, DMSO-d_6) 1.34 (t, ^3J = 7.1 Hz, 3H, CH_2-$\underline{CH_3}$), 4.35 (q, ^3J = 7.1 Hz, 2H, $\underline{CH_2}$-CH_3), 7.10 (d, ^4J = 0.9 Hz, 1H, H-7), 7.26 (d, ^4J = 1.7 Hz, 1H, H-3); 7.44 (dd, ^4J = 0.9 Hz, ^4J = 1.7 Hz, 1H, H-5), 12.38 (s, 1H, NH). δ_C (125 MHz, DMSO-d_6) 14.31 (CH_2-$\underline{CH_3}$), 61.08 ($\underline{CH_2}$-CH_3), 105.45 (C-3), 111.50 (C-7), 120.16 (C-5), 124.50 (C-2), 127.00 (C-3a), 129.20 (C-6), 129.36 (C-4), 137.72 (C-7a), 160.68 (C=O).

Ethyl 4,6-dichloro-3-formyl-1*H*-indole-2-carboxylate (6)



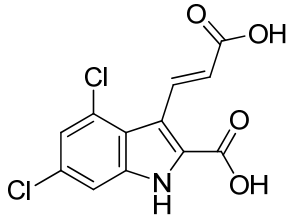
To a stirred suspension of 4,6-dichloro-1*H*-indole-2-carboxylic acid ethyl ester (**5**, 150 mg, 0.58 mmol, 1.0 eq) in anhydrous dichloromethane (10 mL) and *N*,*N*-dimethylformamide (68 µL, 0.87 mmol, 1.5 eq) at room temperature freshly distilled phosphorus oxychloride (80 µL, 0.87 mmol, 1.5 eq) was added. The mixture was then heated at reflux for 2.5 h, and was then allowed to cool down to room temperature. The resulting precipitate was filtered off and washed sparingly with water. The solid was then stirred in 1M NaOAc solution (10 mL) at room temperature for 1 h, filtered off, washed with water and dried under high vacuum to yield the product as an white solid (157 mg, 0.55 mmol, 95 %). R_f (dichloromethane, 100 %) 0.29. δ_H (500 MHz, DMSO-d_6) 1.37 (t, ^3J = 7.1 Hz, 3H, CH_2-$\underline{CH_3}$), 4.43 (q, ^3J = 7.1 Hz, 2H, $\underline{CH_2}$-CH_3), 7.41 (d, ^4J = 1.8 Hz, 1H, H-7), 7.57 (d, ^4J = 1.8 Hz, 1H, H-5), 10.62 (s, 1H, CHO), 13.19 (s, 1H, NH). δ_C (125 MHz, DMSO-d_6) 14.14 (CH_2-$\underline{C}H_3$), 62.32 ($\underline{C}H_2$-CH_3), 111.96 (C-7), 119.10 (C-3), 121.32 (C_{arom}), 124.07 (C-5), 127.54 (C_{arom}), 130.01 (C_{arom}), 134.24 (C-3a), 137.62 (C-7a), 160.00 (C=O), 186.04 (CHO).

Ethyl 4,6-dichloro-3-(3-ethoxy-3-oxoprop-1-enyl)-1*H*-indole-2-carboxylate (7)



Ethyl 4,6-dichloro-3-formyl-1*H*-indole-2-carboxylate (**6**, 100 mg, 0.39 mmol, 1.0 eq), ethyl (triphenylphosphoranylidene)acetate (173 mg, 0.50 mmol, 1.3 eq) and toluene (6.6 ml) were mixed in an atmosphere of argon in a previously dried, oven-heated flask. The mixture was heated at reflux overnight. Then the solvent was evaporated, and the crude product was applied to a silica gel column eluting it with petroleum ether/ethyl acetate (85:15). The product was obtained as a yellow solid (67 mg, with some impurities) and was used for the next step without further purification.

3-(2-carboxyvinyl)-4,6-dichloro-1*H*-indole-2-carboxylic acid (8)



Ethyl 4,6-dichloro-3-(ethoxy-3-oxoprop-1-enyl)-*1H*-indole-2-carboxylate (**7**, 66.8 mg, 0.19 mmol, 1.0 eq) was dissolved in tetrahydrofuran (10 mL) and water (10 mL) and lithium hydroxide monohydrate (23.6 mg, 0.56 mmol, 10 eq) was added. The reaction mixture was stirred at room temperature overnight. On the next day, another 28 mg of lithium hydroxide monohydrate was added and the mixture was stirred at room temperature for an additional night, then diluted with water and acidified with 6 M aq. hydrochloric acid. The crude product was extracted twice with ethyl acetate. The combined organic layers were dried ($MgSO_4$), filtered, and the solvent was evaporated. The crude product was suspended in methanol/water (10 mL, 20:80, containing 1.5 % triethylamine), applied to a preparative reversed phase HPLC column (C-18) and eluted with a gradient of 20 –100 % methanol. Lyophilisation yielded the product as a light yellow solid (42 mg, 47 % yield, *E*- and *Z*-diastereomeric ratio 1:4, containing 1.66 eq. of triethylamine). m.p. 84 °C. R_f (dichloromethane/ethanol, 1:1) 0.10. δ_H (500 MHz, CD_3OD) 1.30 (t, ^3J = 7.3 Hz, 15H, CH_2-$\underline{CH_3}$ (triethylamine)), 3.17 (q, ^3J = 7.3 Hz, 10H, $\underline{CH_2}$-CH_3 (triethylamine)), 6.17 (d, ^3J = 12.1 Hz, 1H, H-9$_{Z\text{-isomer}}$), 6.70 (d, ^3J = 16.0 Hz, 0.25H, H-9$_{E\text{-isomer}}$), 7.05 (d, ^3J = 1.7 Hz, 1H, H-7$_{Z\text{-isomer}}$), 7.15 (d, ^3J = 1.8 Hz, 0.25H, H-7$_{E\text{-isomer}}$), 7.40 (d, ^3J = 1.7 Hz, 1H, H-5$_{Z\text{-isomer}}$), 7.44 (d, ^3J = 1.7 Hz, 0.25H, H-5$_{E\text{-isomer}}$), 7.47 (d, ^3J = 12.2 Hz, 1H, H-8$_{Z\text{-isomer}}$), 8.66 (d, ^3J = 16.0 Hz, 1H, H-8$_{E\text{-isomer}}$), (3 signals (NH, 2 x CO_2H) not detectable). δ_C (125 MHz, DMSO-d_6) 9.45 (CH_2-$\underline{C}H_3$ (triethylamine)), 47.91 ($\underline{C}H_2$-CH_3(triethylamine)), 111.93 (C-7), 112.19 (C-9$_{E\text{-isomer}}$), 114.73 (C-9$_{Z\text{-isomer}}$), 122.14 (C-5), 123.24 (C-3), 124.80 (C-2), 125.91 (C-3a), 129.53 (C-6), 129.90 (C-4), 134.46 (C-8$_{E\text{-isomer}}$), 136.42 (C-8$_{Z\text{-isomer}}$), 138.21 (C-7), 168.77 (CO_2H), 172.16 (CO_2H).

Cell culture

CHO-K1 cells were cultivated in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) supplemented with 10% (v/v) FCS, glutamine 2 mM, 100 U/ml penicillin and 100 µg/ml streptomycin. 1321N1 cells and 1321N1 cells stably expressing human GPR17 (1321N1-hGPR17) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) FCS, 100 U/ml penicillin and 100 µg/ml streptomycin. Flp-In™ T-REx CHO cells stably expressing hGPR17 (CHO-FITR-hGPR17) were cultured in DMEM/F-12

containing 10% FCS, 100 U/mL penicillin / 0.1 mg/mL streptomycin, 30 µg/mL blasticidine and 300 µg/mL hygromycin B. Expression of GPR17 was induced by treatment with 1 µg/mL doxycycline for 14-20 h.

Membrane preparations

Cells were cultured in 150 cm^2 dishes. After reaching confluence the cells were washed with 10 mL of ice-cold PBS and the dishes were frozen at -80°C. Membranes were prepared by adding 1 mL of ice-cold 25 mM TRIS buffer, pH 7.4 containing 0.32 M sucrose, 1 mM EDTA, and protease inhibitors (1:100 dilution of the protease inhibitor Cocktail, Sigma 8340) and scratching the cells off the previously frozen cell culture dishes. The collected cell suspension was homogenized with an Ultra Turrax and was subsequently centrifuged at 1,000 x g for 10 min at 4°C. The supernatant was carefully removed before being centrifuged at 48,000 x g for 30 min at 4°C. The obtained pellet was resuspended in 50 mM TRIS buffer, pH 7.4 and washed and centrifuged once again under the same conditions. The final crude membrane pellet was resuspended at a protein concentration of 2-3 mg/mL in a 50 mM TRIS buffer (pH 7.4), homogenized in a glass/teflon homogenizer and stored at -80°C in 1-mL aliquots until further use. Protein concentrations were determined according to the method of Lowry.

Competition experiments with [^{3}H]PSB-12150

Binding affinities of unlabelled compounds for GPR17 receptors were determined by competition experiments with [^{3}H]PSB-12150. Membranes expressing GPR17 receptors (50 µg protein per vial) were incubated for 60 min at 25°C in 1 mL of a 50 mM Tris-HCl buffer (pH 7.4) containing 10 mM $MgCl_2$, 25 nM [^{3}H]PSB-12150 (17 Ci/mmol) and increasing concentrations of test compound. Nonspecific binding (NSB) was determined in the presence of 100 µM of unlabeled PSB-12150 (**1**, MDL29,951). Membrane-bound and free radioligand were separated by rapid filtration through GF/B glass fiber filters (Whatman, Dassel, Germany). Filters were washed three times, 2 ml each, with freshly prepared ice-cold 50 mM Tris-HCl buffer, pH 7.4. The entire filtration procedure did not exceed 10 seconds per sample. The filters were immediately transferred to mini vials. Scintillation cocktail (Ultima Gold, Canberra Packard, 2.5 ml) was added and after an incubation of 9 h filter-bound radioactivity was measured by liquid scintillation counting (Tri-Carb® 2810 TR, PerkinElmer, Inc., USA) at an efficiency of 54 %. Data were analyzed using Graph Pad Prism Version 6.0 (San Diego, CA, USA). For the calculation of K_i values the Cheng-Prusoff equation and a K_D value of 1190 nM was used.

Real Time PCR

The mRNA extraction was performed using the PerfectPure RNA cultured cell kit (5 PRIME, Hamburg, Germany) according to the manufacturer's instructions. Residual genomic DNA was removed by incubation with DNase I, Amplification Grade (Karlsruhe, Germany). The reverse transcription was performed using the QuantiTect Reverse Transcription kit (Qiagen, Venlo, Netherlands) as per manufacturer´s instructions. Amplifications were done using the Real Master Mix SYBR Rox kit (5 PRIME, Hamburg, Germany). 100 ng cDNA was mixed to the

RealMasterMix buffer containing the intercalant dye, Syber Green, and specific primers (5 pmol/μL) for the gene of interest. As positive control, the quantification of a housekeeping gene (β-actin) was performed in parallel. As negative control, a sample without template was used; as control for genomic DNA contamination, a sample with non-retro-transcribed mRNA was included instead of template cDNA. All reactions were run in duplicate on a CFX96 Touch™ Real-Time PCR Detection System (Biorad, Müchen, Germany).

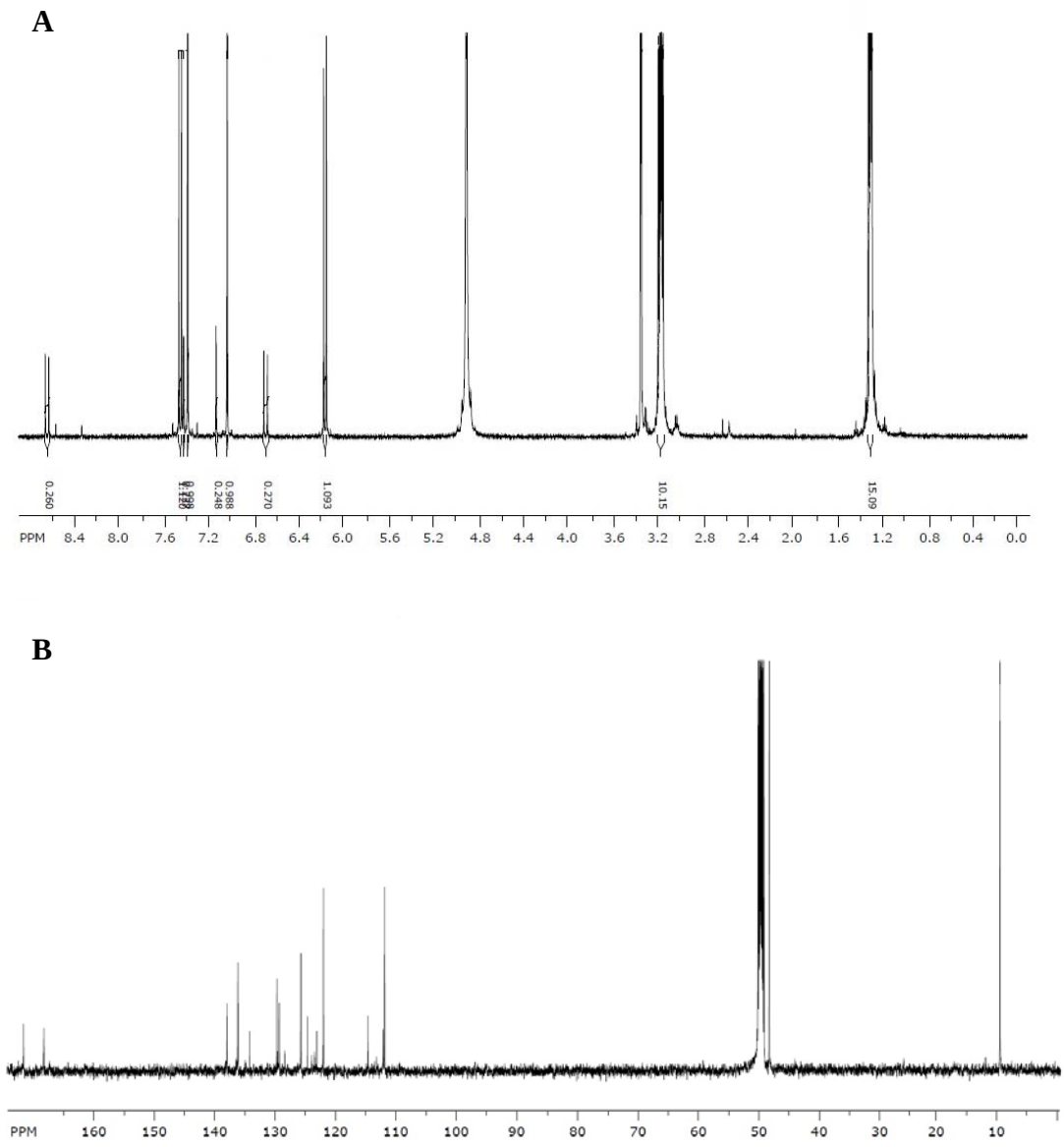


Figure S1. NMR spectra of the precursor **8** in CD_3OD. **(A)** ^{1}H- NMR spectrum of the precursor **8** in CD_3OD. (500 MHz, Bruker Avance 500 NMR spectrometer; the spectrum was referenced to the residual solvent signal with resonance at δ_H = 3.35 ppm). (**B**) ^{13}C- NMR spectrum of the precursor **8** in CD_3OD. (125 MHz, Bruker Avance 500 NMR spectrometer; the spectrum was referenced to the residual solvent signal with resonance at δ_C = 49.3 ppm).

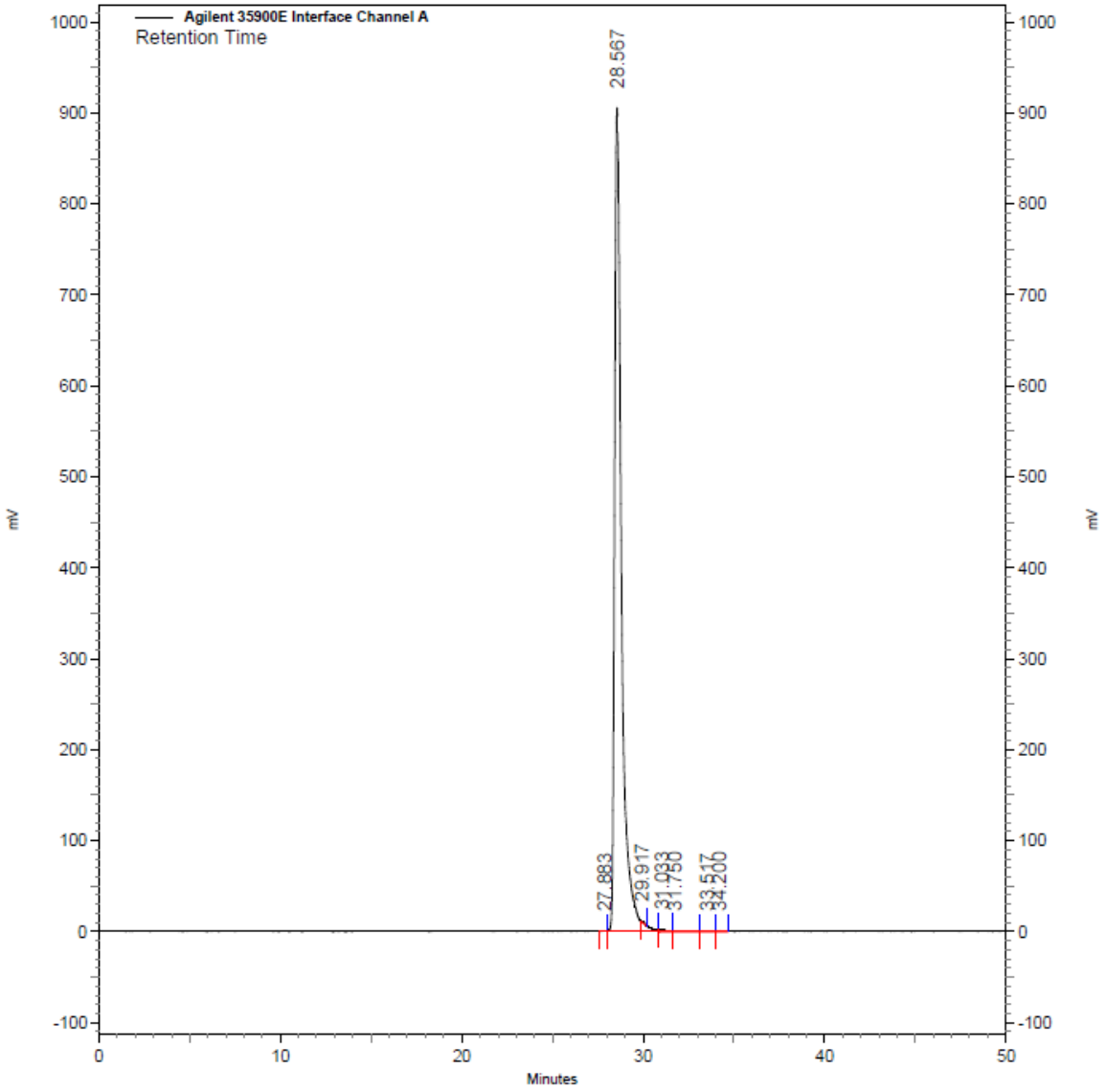


Figure S2. Radiochemical purity of [^{3}H]PSB-12150 determined by high performance liquid chromatography. HPLC chromatogram of [^{3}H]PSB-12150 and its purity determined by HPLC-DAD at 254 nm (99.4%). Column: Luna C18(2), 50x4.6mm, 3µM; solvent A: 2 mM ammonium acetate in water; solvent B: 2 mM ammonium acetate in methanol; gradient: 0 min (10%), 15 min (10%), 30 min (100%), 45 min (100%), 46 min (10%) 50 min (10%), temperature: 22°C; flow rate: 0.25 mL/min.

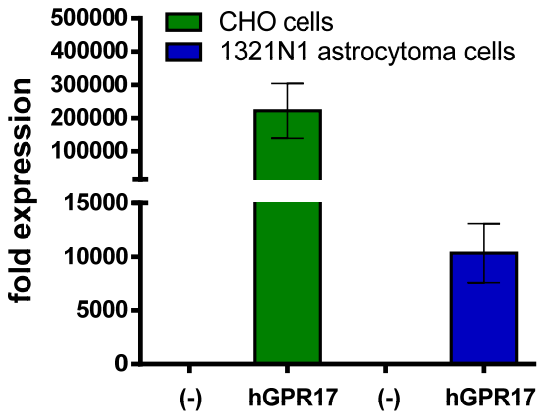


Figure S3. mRNA expression profile of GPR17 in CHO and 1321N1 astrocytoma cells. Real-time PCR was performed to measure mRNA expression using specific primers for GPR17 and the reference gene β-actin. The data were normalized to the cycle threshold value of the internal housekeeping gene β-actin and the relative mRNA level in the non-transfected 1321N1 astrocytoma cells were used as calibrator. The results are presented as mean fold in expression ± SEM of 8 independent experiments representing 4-8 biological replicates.
Primer sequences used for amplifications were as follows: human GPR17: forward: 5´-AACCGCTCCGTCTACGTG-3´, reverse: 5'-AGGAGGTGATGCGGTTTG-3'; human β-actin: forward: 5'-GGCCTCGCTGTCCACCTTCC-3',
reverse: 5'-TGTCACCTTCACCGTTCCAGTTTTT-3'.

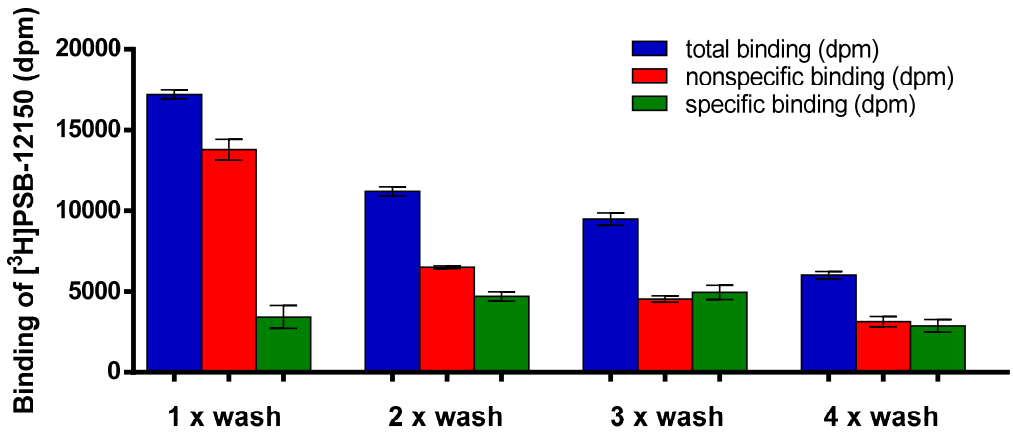


Figure S4. Recovery of radioligand binding in dependence of the GF/B filter washing procedure

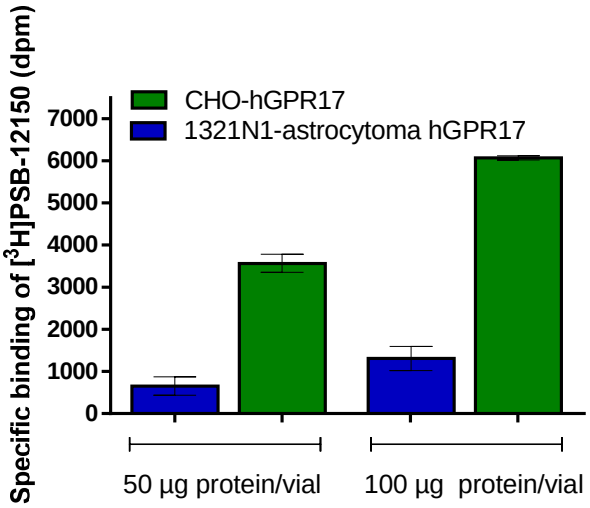


Figure S5. Specific binding of [^{3}H]PSB-12150 to CHO-hGPR17 and 13121N1 astrocytoma-hGPR17 membrane preparations. The experiments were performed at 25°C (60 min) using 25 nM [^{3}H]PSB-12150 in 50 mM TRIS buffer, pH 7.4, 10 mM $MgCl_2$. Data shown are mean values ± SEM of 2 independent experiments, each performed in duplicates.

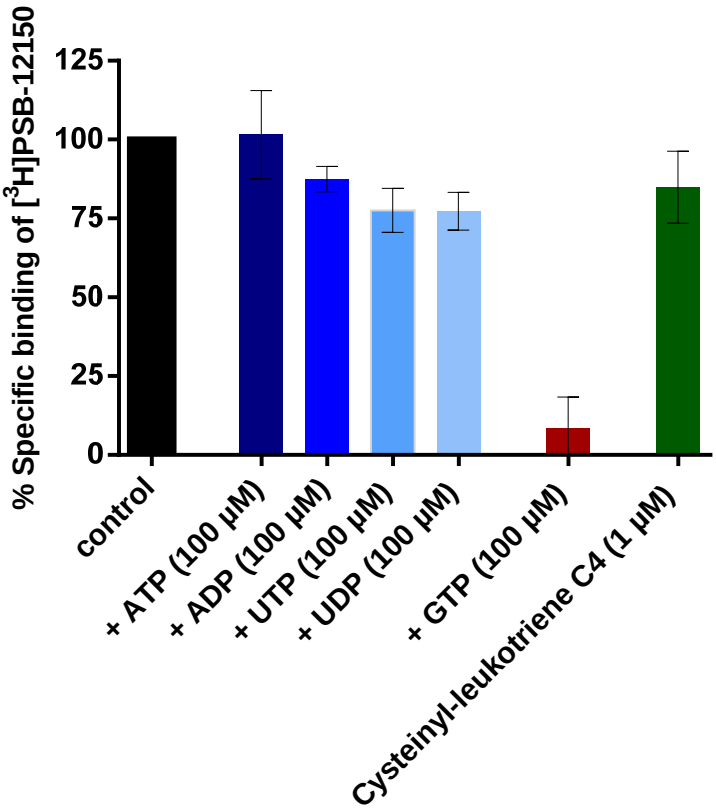


Figure S6. Specific binding of [^{3}H]PSB-12150 to CHO-hGPR17 membrane preparations in the absence and presence of nucleotides and cysteinyl-leukotriene C4. The experiments were

performed at 25°C (60 min) using 25 nM [^{3}H]PSB-12150 in 50 mM TRIS buffer, pH 7.4, 10 mM $MgCl_2$. Data shown are mean values ± SEM of 2 independent experiments, each performed in duplicates.

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