Discovery and characterization of carbamothioylacrylamides as EP2 selective antagonists

Thota Ganesh[†, ‡, *], Jianxiong Jiang[†, ‡,*], Rangaiah Shashidharamurthy[#] and Ray Dingledine[†]

[†] Department of Pharmacology, School of Medicine, Emory University, Atlanta, Georgia 30322
[#]Department of Pharmaceutical Sciences, PCOM-SOP, GA Campus, Suwanee, Georgia 30024

‡T.G. and J.J. contributed equally to this work.

Corresponding Author

*E-mail: tganesh@emory.edu (T.G.); jjiang3@emory.edu (J.J.).

Methods

Cell Culture. The rat C6 glioma (C6G) cells stably expressing human DP1, EP2, EP4, or IP receptors (C6G-EP2) were created in the lab[1, 2] and grown in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen), supplemented with 10% (v/v) fetal bovine serum (FBS) (Invitrogen), 100 U/ml penicillin, 100 µg/ml streptomycin (Invitrogen), and 0.5 mg/ml G418. The C6G cell line stably expressing β2-adrenergic receptors (C6G-β2-AR) was a kind gift from Dr. Kenneth Minneman and was maintained in complete DMEM medium containing 250 µg/ml G418 and 200 µg/ml hygromycin B (Invitrogen).

Chemicals and Drugs. PGE_2, butaprost, Iloprost, BW245C and rolipram were purchased from Cayman Chemical. Isoproterenol and doxorubicin were purchased from Sigma-Aldrich.

Cell-Based cAMP Assay. Cytosol cAMP was measured with a cell-based homogeneous time-resolved fluorescence resonance energy transfer (TR-FRET) method (Cisbio Bioassays).[1, 2] The assay is based on generation of a strong FRET signal upon the interaction of two molecules: an anti-cAMP antibody coupled to a FRET donor (Cryptate),

and cAMP coupled to a FRET acceptor (d2). Endogenous cAMP produced by cells competes with labeled cAMP for binding to the cAMP antibody and thus reduces the FRET signal. Cells stably expressing human DP1, EP2, EP4, or IP receptors were seeded into 384-well plates in 30 µl complete medium (4,000 cells/well) and grown overnight. The medium was carefully withdrawn and 10 µl Hanks' Buffered Salt Solution (HBSS) (Hyclone) plus 20 µM rolipram was added into the wells to block phosphodiesterase. The cells were incubated at room temperature for 0.5-1 h, and then treated with vehicle or test compound for 10 min before addition of increasing concentrations of appropriate agonist: BW245C for DP1, PGE_2 for EP2 and EP4, or iloprost for IP. The cells were incubated at room temperature for 40 min, then lysed in 10 µl lysis buffer containing the FRET acceptor cAMP-d2 and 1 min later another 10 µl lysis buffer with anti-cAMP-Cryptate was added. After 60-90 min incubation at room temperature, the FRET signal was measured by an Envision 2103 Multilabel Plate Reader (PerkinElmer Life Sciences) with a laser excitation at 337 nm and dual emissions at 665 nm and 590 nm for d2 and Cryptate (50 µs delay), respectively. The FRET signal was expressed as: F665/F590 × 10^4.

Quantitative Real-Time PCR. P388D1 (mouse macrophage cell line) cells were incubated with 10 µM TG6-129 for 1 hour at 37^{o}C with RPMI-1640 supplemented with 10% (v/v) cosmic calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. Then 10 µM of selective EP2 receptor agonist 9-oxo-11α,16S-dihydroxy-17-cyclobutyl-prost-13E-en-1-oic acid commonly called butaprost (free acid) was added and incubation was continued for additional 2 hours under same conditions. Cells were harvested and total RNA was purified using RNeasy Mini Kit (Qiagen). cDNA synthesis was performed with 1 µg of total RNA, 2 µl Reverse Transcriptase (Invitrogen), and 2 µl random primers in a reaction volume of 20 µL at 42 °C for 50 min. The reaction was terminated by heating at 70 °C for 15 min. Quantitative real-time PCR (qRT-PCR) was performed by using 2 µl of 10× diluted cDNA, 0.8 µl 10 µM primers, and 10 µl iQ SYBR Green Supermix (Bio-Rad Laboratories) with a final volume of 20 µL in the iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories). PCR cycling conditions were as follows: 95 °C for 2 min followed by 40 cycles of 95 °C for 15 s and then 60 °C for 1 min. Melting-curve analysis

was used to verify single-species PCR product. Fluorescent data were acquired at the 60 °C step. Cycle thresholds for β-actin was used as internal control. Primers used for qRT-PCR were as follows: ***β-Actin*** (5'-AAG AGC TAT GAG CTG CCT GA-3' reverse primer 5'-TAC GGA TGT CAA CGT CAC AC-3'), ***IL 17*** (5'-TCC CTC TGT GAT CTG GGA AG-3' reverse primer 5'-AGC ATC TTC TCG ACC CTG AA-3'), ***IL- 23*** (5'-CCC AGC CTG AGT TCT AGT CA-3' reverse primer 5'-TGG TAG ATG TCT GGG CTG AT-3'), ***IL-12*** (5'-CAA TCA GGG CTT CGT AGG TA-3' reverse primer 5'-GGC CCT GGT TTC TTA TCA AT-3'), ***IL- 1β*** (5'- CCC AAC TGG TAC ATC AGC AC -3' reverse Primer 5'- TCT GCT CAT TCA CGA AAA GG -3'), ***COX-2*** (5'-TGA TCG AAG ACT ACG TGC AA-3' reverse primer 5'-GTG AGT CCA TGT TCC AGG AG-3'), ***TNF-α*** (5'-CCC ACT CTG ACC CCT TTA CT-3' reverse primer 5'-TTT GAG TCC TTG ATG GTG GT-3'), ***IL-6,*** 5'- CTA CCC CAA TTT CCA ATG CT -3', reverse primer 5'- ACC ACA GTG AGG AAT GTC CA -3' , ***EP2*** (5'-TTC TAC AGG CGC CAC TTA TC-3' reverse primer 5'-GAG GTT GAG AAC GCT GA-3').

Statistical Analysis. Data were plotted with Origin (OriginLab). Statistical analyses were performed using Prism (GraphPad Software) by one-way ANOVA with post hoc Bonferroni test. $P < 0.05$ was considered to be statistically significant. Data were normalized to control (vehicle) values and presented as mean ± SEM.

Synthesis of TG6-129 (compound 1)

Step-1. A solution of 5-ethyl-1,3,4-thiadiazol-2-amine (**B)** (320mg, 2.46 mmol) in pyridine (5mL) was added drop wise by 4-nitrobenzene-1-sulfonyl chloride (**A**) (600mg, 2,71 mmol, 1.1eq) at room temperature and the resulting reaction mixture was stirred for 3hrs. Pyridine was removed under vacuum and the crude reaction was dissolved in water. The precipitate formed was filtered and dried to get the product **C** (95% yield). ^{1}H NMR (400 MHz, $CDCl_3$). δ 8.21 (d, J = 9.2 Hz, 2H), 7.98 (d, J = 8.8 Hz, 2H), 2.74 (q, J = 7.6 Hz, 2H), 1.23 (t, J = 7.6 Hz, 3H).

Step-2. A solution of **C** (500 mg, 1.58 mmol) in ethyl acetate (10 mL) was added by tin-chloride dihydrate (6eq) and the resulting reaction mixture was refluxed for 36 hrs. Then reaction mixture was cooled and diluted with ethyl acetate (100 mL) and water (25 mL). Layers were separated, and organics were washed with saturated sodium bicarbonate

solution, dried and concentrated to provide crude product which on silica gel chromatography, eluting with 0-2% methanol in dichloromethane provided product **D**[3] (315mg, 70% yield). ^{1}H NMR (400 MHz; $CDCl_3$). δ 7.49 (d, *J* = 7.6 Hz, 2H), 6.54 (d, *J* = 7.6 Hz, 2H), 2.69 (q, *J* = 7.6 Hz, 2H), 1.19 (t, *J* = 7.6 Hz, 3H).

Step-3. A solution of ammonium thiocyanate (47 mg, 0.62 mmol) in acetone (1.5 mL) was brought to reflux for 15 minutes. Then 4-fluorocinnamyl chloride **E** (138 mg, 1.2 eq) in acetone (1.5 mL) was added and refluxed for 15 minutes. The amine **D** (215 mg, 1.2 eq) in acetone (1.5 mL) was added and the resulting reaction mixture was refluxed for 1 hr. The reaction mixture was cooled and the white precipitate was filtered and recrystallised from methanol to get product (300 mg, 81% yield). ^{1}H NMR (400 MHz, DMSO-d_6). δ 12.81 (s, NH), 11.66 (s, NH), 7.87 (d, *J* = 8.8 Hz, 2H), 7.78 (d, *J* = 8.8 Hz, 2H), 7.75 (d, *J* = 15.6 Hz, 1H), 7.68 (dd, *J* = 8.8, 5.6 Hz, 2H), 7.30 (t, *J* = 8.8 Hz, 2H), 6.97 (d, J = 15.6 Hz, 1H), 2.80 (q, *J* = 7.2 Hz, 2H), 1.18 (t, *J* = 7.6 Hz, 3H). ^{19}F NMR. -109.5. LC-MS > 95% pure at λ 254, MS (m/z) calculated for $C_{20}H_{18}FN_5O_3S_3$: 491; Found 492 $[M+H]^+$.

All other compounds (**2-9)** were acquired from commercial sources and used for biotesting.

Supporting information references

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