Peripherally restricted highly potent, selective, aqueous-soluble EP2 antagonist with anti-inflammatory properties

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Supporting Information.

General: Chemical reagents for synthesis were purchased from TCI chemicals and Combi-blocks. Organic solvents for synthesis were purchased from ThermoFisher Scientific. Proton NMR spectra were recorded in DMSO-d_6 on a Varian Inova-400 (400 MHz). High-resolution mass spectrometry (HRMS) was performed on a Thermo LTQ-FTMS Ultra spectrometer. Thin layer chromatography was performed on pre-coated, aluminum-backed plates (silica gel 60 F_{254}, 0.25 mm thickness) from EM Science and was visualized by UV lamp. Column chromatography was performed with silica gel cartridges on a Teledyne-ISCO machine. Agilent 6120 Quadruple LC/MS was used to determine the purity of the products monitoring at 254 nm wavelength by using Eclipse XDB-C18 5 um 4.6 x 150 mm column at 1 ml/min flow rate with 0.1% AcOH in water and 0.1% AcOH in methanol as a mobile phase. Elemental analysis was performed by Atlantic Microlab Inc. (Norcross, GA). Chemicals: PGE_2, BW245C, iloprost, and rolipram were purchased from Cayman Chemical. EP2 agonist ONO-AE1-259-01 was obtained from ONO Pharmaceutical Co, Osaka, Japan.

Radioligand [H^3-PGE_2]-binding Assay. Binding assays for EP2, EP1 and EP3 receptors were performed at *Eurofins Cerep Panlabs (France)*. Cell membrane homogenates (20 μg protein) from HEK cells stably expressing human EP2 receptor were incubated with 3 nM [H^3-PGE_2] in the absence or

presence of the test compound in a buffer containing 10 mM Mes/KOH (pH 6.0), 10 mM $MgCl_2$, and 1 mM EDTA, at room temperature for 120 min. Nonspecific binding was determined in the presence of 10 μM PGE_2. Following incubation, the samples were filtered rapidly under vacuum through glass fiber filters (GF/B; Packard) presoaked with 0.3% polyethyleneimine (PEI) and rinsed several times with ice-cold 50 mM Tris·HCl, using a 96-sample cell harvester (UniFilter; Packard). The filters were dried and then counted for radioactivity in a scintillation counter (TopCount; Packard), using a scintillation mixture (MicroScint 0; Packard). The results were expressed as percent inhibition of the radioligand-specific binding. The dose–response curves were generated, and IC50 and Ki values were calculated with Origin (OriginLab).

Liver microsomal stability assay. This assay was performed at *Absorption Systems Inc*. Human liver microsomes and CD-1 mouse liver microsomes were obtained from XenoTech. The reaction mixture was prepared as described below. The test article was added into the reaction mixture to a final concentration of 1 μM, where microsome concentration is 0.5 mg/mL. An aliquot of the reaction mixture (without cofactor) was incubated in a shaking water bath at 37°C for 3 minutes. The control, testosterone, was run simultaneously with the test article in a separate reaction. The reaction was initiated by the addition of cofactor NADPH (1 mM) and the mixture was then incubated in a shaking water bath at 37°C. Aliquots (100 μL) were withdrawn at 0, 10, 20, 30 and 60 minutes for the test article and for testosterone, and they were immediately combined with 400 μL of ice-cold acetonitrile/*d*H2O (1:1) containing 0.1% formic acid and internal standard metoprolol to terminate the reaction. The samples were then mixed and centrifuged to precipitate microsomal proteins. All samples were assayed by LC-MS/MS using electrospray ionization. The peak area response ratio (PARR) to internal standard at 10, 20, 30 and 60 minutes was compared to the PARR at time 0 to determine the percent of test article remaining at each

timepoint. Half-life ($t_{1/2}$) (min) was derived from the equation 0.693/k, where k is gradient of line determined from plot of peak area ratio (compound peak area / internal standard peak area) against time.

CYP450 enzyme inhibition assays.[24] These assays were done at *Eurofins Cerep*. The test compound (10 µM) was pre-incubated with NADPH-generating system (1.3 mM NADP+ (β-Nicotinamide Adenine Dinucleotide Phosphate Sodium Salt Hydrate), 3.3 mM d-Glucose-6-Phosphate (G6P) and 0.4 U/mL Glucose-6-Phosphate Dehydrogenase (G6PDHase) in phosphate buffer (pH 7.4) for 5 min at 37 ºC. The reaction was initiated by adding a mixture of a CYP enzyme, a substrate (*SI* Table 1), and bovine serum albumin (BSA 0.4 mg/mL). The assay measured the appearance of a fluorescent metabolite of a non-fluorescent substrate for each CYP. After an appropriate incubation time the percent inhibition of enzyme activity by TG8-69 was calculated. Additional details of assay including the CYP enzymes, substrates concentrations, and metabolites are provided in *SI* Table 1 (below).

***Human-h*ERG potassium channel inhibition assay.** [25] This assay was done at *Eurofins Cerep*. Cell membrane homogenates from HEK-293 cells transfected with human hERG K^+ channel (about 40 µg protein) were incubated for 60 min at 22°C with 3 nM [^{3}H]-dofetilide in the absence or presence of the test compound (10 µM) in a buffer containing 50 mM Tris-HCl (pH 7.4), 10 mM KCl and 1 mM $MgCl_2$. Nonspecific binding was determined in the presence of 25 µM terfenadine. Following incubation, the samples were filtered rapidly under vacuum through glass fiber filters (GF/B, Packard) presoaked with 0.3% PEI and rinsed several times with ice-cold 50 mM Tris-HCl, 10 mM KCl and 1 mM $MgCl_2$ using a 96-sample cell harvester (Unifilter, Packard). The filters were dried then counted for radioactivity in a scintillation counter (Topcount, Packard) using a scintillation cocktail (Microscint 0, Packard). The results were expressed as percent inhibition of the radioligand specific binding. The standard reference compound is terfenadine, which was tested in each experiment at several concentrations to obtain a competition curve from which its IC_{50} is calculated.

In vivo pharmacokinetics. Pharmacokinetic studies were done at *Sai Life Sciences Limited (India)*. Twenty four male C57BL/6 mice were used in the study. Animals were divided into two groups, Group 1 consisting of nine animals and Group 2 consisting of 15 animals. Animals in Group 1 and Group 2 were administered TG8-69 by intravenous (5 mg/kg) and oral (10 mg/kg) routes respectively, in 5% NMP, 5% Solutol HS-15 & 90% normal saline. Blood samples (approximately 60 μL) were collected from retro orbital plexus under light isoflurane anesthesia such that the samples were obtained at 0.08, 0.25, 0.5, 1, 2, 4, 8, 12 and 24 hr (i.v.) and 0.25, 0.5, 1, 2, 4, 6, 8, 12 and 24 hr (p.o.). The blood samples were collected from sets of three mice (Group1) and from sets of five animals (Group 2) at each time point in micro centrifuge tubes containing K_2EDTA as anticoagulant. Plasma samples were separated by centrifugation of whole blood and stored below -70ºC until bioanalysis. Whole brain samples were homogenized using phosphate buffer saline (pH 7.4) and the total homogenate volume was thrice the brain weight. All samples were processed for analysis by protein precipitation using acetonitrile (ACN) and analyzed by LC/MS/MS (lower limit of quantification (LLOQ) was determined to be 1.23 ng/mL). Waters Xterra MS C18 column was used with flow rate 0.8 ml/min using the mobile phase 0.1 % formic acid in acetonitrile (pump A) and 0.1 % formic acid in water (pump B). Internal standard glipizide (ret. time 1.60 min) MRM transition *m/z* 444.3-319.0; TG8-69 (ret time 1.46 min) transition *m/z* 345.1-289.1 was used to quantify the peaks and the ratio was used for concentration analysis. Pharmacokinetic parameters were calculated using the non-compartmental analysis tool of Phoenix WinNonlin (Version 6.3). The overall pharmacokinetic parameters are summarized Table 2. Similarly, a brain to plasma ratio study was conducted in C57BL6 mice by dosing at 10mg/kg, IP dosing into male mice by the same vehicle as shown above. The concentrations in the plasma and brain were quantified by LC-MS/MS at 0.5, 2 and 6 hours to determine the B/P ratio, shown in Table 2.

Chemical Synthesis.

Compounds **1a**, **1b**, and **2a-2l** are commercially available from various vendors.

Typical procedure for the synthesis of compounds shown in Scheme 1. A solution of **1b** (1.15 mmol, 1.15 eq) and **2b** (1 mmol, 1 eq) were dissolved in *N*,*N*-dimethyl formamide (1 mL) and dichloromethane mixture (3 mL), then was added 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI) (1.5 mmol, 1.5 eq) and catalytic amount of *N*,*N*-dimethylaminopyridine (DMAP) (10 mg), and stirred at room temperature overnight. Reaction mixture was dissolved in ethyl acetate (50 mL), washed with 2N HCl followed by 2N $NaHCO_3$, water, and saturated NaCl solution (5 mL each). The organic solution was dried with Na_2SO_4 and concentrated. The crude product was purified by silica-gel column chromatography eluting with hexane and ethyl acetate (20-80%) mixture to obtain a pure product **3b**. The yield for each product is shown below. The yields are not optimized.

Characterization data

3a (TG8-15) see ref [21].

3b (TG8-185) *(N-(2-(2-methyl-1H-indol-1-yl)ethyl)-4-(2-methyl-2H-tetrazol-5-yl)benzamide)*. (75 % yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.56 – 7.46 (m, 3H), 7.32 (d, *J* = 8.1 Hz, 1H), 7.14 – 7.02 (m, 2H), 6.62 – 6.54 (m, 2H), 6.24 (s, 1H), 6.05 (t, *J* = 6.0 Hz, 1H), 4.31 (t, *J* = 6.0 Hz, 2H), 3.73 (q, *J* = 6.0 Hz, 2H), 3.49 – 3.42 (m, 3H), 2.38 (s, 3H); LCMS (ESI): > 98% purity at λ = 254 nm (ret. time 2.91 min); MS *m/z* 361.17 $[M+H]^+$.

3c (TG8-130) *(N-(2-(2-methyl-1H-indol-1-yl)ethyl)-4-(1H-pyrazol-4-yl)benzamide)*. (60 % yield). ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.92 (s, 1H), 8.61 (t, *J* = 5.6 Hz, 1H), 8.26 (s, 1H), 7.97 (s, 1H), 7.76-7.65 (m, 4H), 7.42- 7.37 (dd, *J* = 9.0, 4.2 Hz, 2H), 7.03 - 6.90 (m, 2H), 6.16 (s, 1H), 4.25 (t, *J* = 6.5 Hz, 2H), 3.61 – 3.40 (m, 2H), 2.36 (s, 3H); LCMS (ESI): > 98% purity at λ = 254 nm (ret. time 2.51 min); MS *m/z* 345.1 $[M+H]^+$.

3d (TG8-168) *(N-(2-(2-methyl-1H-indol-1-yl)ethyl)-4-(1H-pyrazol-3-yl)benzamide)*. (50 % yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.78 – 7.68 (m, 2H), 7.64 – 7.51 (m, 4H), 7.34 (d, *J* = 7.9 Hz, 1H), 7.16 – 7.05 (m, 2H), 6.64 (s, 1H), 6.28 (s, 2H), 4.36 (d, *J* = 5.6 Hz, 2H), 3.81 (q, *J* = 5.7 Hz, 2H), 2.39 (s, 3H); LCMS (ESI): > 98% purity at λ = 254 nm (ret. time 2.45 min); MS *m/z* 345.1 $[M+H]^+$.

3e (TG8-184) *(N-(2-(2-methyl-1H-indol-1-yl)ethyl)-4-(5-methyl-4H-1,2,4-triazol-3-yl)benzamide)*. (60 % yield).^{1}H NMR (400 MHz, DMSO-d_6): δ 8.73 (t, *J* = 5.7 Hz, 1H), 8.00 (d, *J* = 8.4 Hz, 2H), 7.83 (d, *J* = 8.4 Hz, 2H), 7.40 (dd, *J* = 12.4, 7.9 Hz, 2H), 7.04 – 6.98 (m, 1H), 6.95 – 6.90 (m, 1H), 6.17 (s, 1H), 4.26 (t, *J* = 6.7 Hz, 2H), 3.51 (q, *J* = 6.4 Hz, 2H), 2.37-2.36 (m, 6H); LCMS (ESI): >98% purity at λ = 254 nm (ret. time 2.51 min); MS *m/z* 360.1 [M+H]+.

3f (TG8-237) (*4-(1H-imidazol-4-yl)-N-(2-(2-methyl-1H-indol-1-yl)ethyl)benzamide)*. (60 % yield). ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.61 (t, *J* = 5.6 Hz, 1H), 7.88 – 7.65 (m, 6H), 7.40 (dd, *J* = 14.2, 7.9 Hz, 2H), 7.01 (td, *J* = 8.4, 4.2 Hz, 1H), 6.93 (td, *J* = 8.4, 4.2 Hz, 1H), 6.16 (s, 1H), 4.25 (t, *J* = 6.7 Hz, 2H), 3.50 (q, *J* = 6.5 Hz, 2H), 2.36 (s, 3H); LCMS (ESI): > 98% purity at λ = 254 nm (ret. time 3.67 min); MS *m/z* 345.1 $[M+H]^+$.

3g (TG8-239) (*4-(1H-imidazol-2-yl)-N-(2-(2-methyl-1H-indol-1-yl)ethyl)benzamide*). (60 % yield). ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.69 (t, *J* = 5.7 Hz, 1H), 7.96 (d, *J* = 8.5 Hz, 2H), 7.82 (d, *J* = 8.5 Hz, 2H), 7.40 (dd, *J* = 12.4, 7.9 Hz, 2H), 7.27 (s, 1H), 7.04 - 6.99 (m, 2H), 6.96 – 6.89 (m, 1H), 6.17 (s, 1H), 4.26 (t, *J* = 6.6 Hz, 2H), 3.56 – 3.47 (m, 2H), 2.36 (s, 3H); LCMS (ESI): > 98% purity at λ = 254 nm (ret. time 3.55 min); MS *m/z* 345.1 $[M+H]^+$.

3h (TG8-186) *(N-(2-(2-methyl-1H-indol-1-yl)ethyl)-4-(pyrimidin-5-yl)benzamide)*. (70 % yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.20 (s, 1H), 8.91 (s, 2H), 7.74 – 7.70 (m, 2H), 7.60 – 7.55 (m, 2H), 7.52 (dd, *J* = 7.0, 1.3 Hz, 1H), 7.34 – 7.29 (m, 1H), 7.13 – 7.03 (m, 2H), 6.32 – 6.24 (m, 2H), 4.38 (t, *J* = 5.9

Hz, 2H), 3.82 (q, *J* = 6.0 Hz, 2H), 2.40 (d, *J* = 0.9 Hz, 3H); LCMS (ESI): > 98% purity at λ = 254 nm (ret. time 2.91 min); MS *m/z* 357.1 $[M+H]^+$.

3i (TG8-238) *(N-(2-(2-methyl-1H-indol-1-yl)ethyl)-4-(pyrimidin-2-yl)benzamide)*. (70 % yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.80 (d, *J* = 4.8 Hz, 2H), 8.50 – 8.38 (m, 2H), 7.76 – 7.65 (m, 2H), 7.52 (d, *J* = 7.5 Hz, 1H), 7.32 (d, *J* = 8.0 Hz, 1H), 7.22 – 7.18 (m, 1H), 7.09-7.04 (m, 2H), 6.34 – 6.15 (m, 2H), 4.37 (t, *J* = 5.9 Hz, 2H), 3.80 (q, *J* = 6.0 Hz, 2H), 2.39 (s, 3H); LCMS (ESI): > 98% purity at λ = 254 nm (ret. time 2.7 min); MS *m/z* 357.1 $[M+H]^+$.

3j (TG8-280) *(N-(2-(2-methyl-1H-indol-1-yl)ethyl)-4-(2-methylthiazol-4-yl)benzamide)*. (70 % yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.91 – 7.84 (m, 2H), 7.66 – 7.60 (m, 2H), 7.52 (d, *J* = 7.7 Hz, 1H), 7.38 (d, *J* = 2.1 Hz, 1H), 7.32 (d, *J* = 8.0 Hz, 1H), 7.14 – 7.03 (m, 2H), 6.25 (d, *J* = 0.7 Hz, 1H), 6.15 (s, 1H), 4.39 – 4.32 (m, 2H), 3.85 – 3.70 (m, 2H), 2.76 (d, *J* = 1.0 Hz, 3H), 2.39 (s, 3H); LCMS (ESI): > 98% purity at λ = 254 nm (ret. time 1.91 min); MS *m/z* 376.1 $[M+H]^+$.

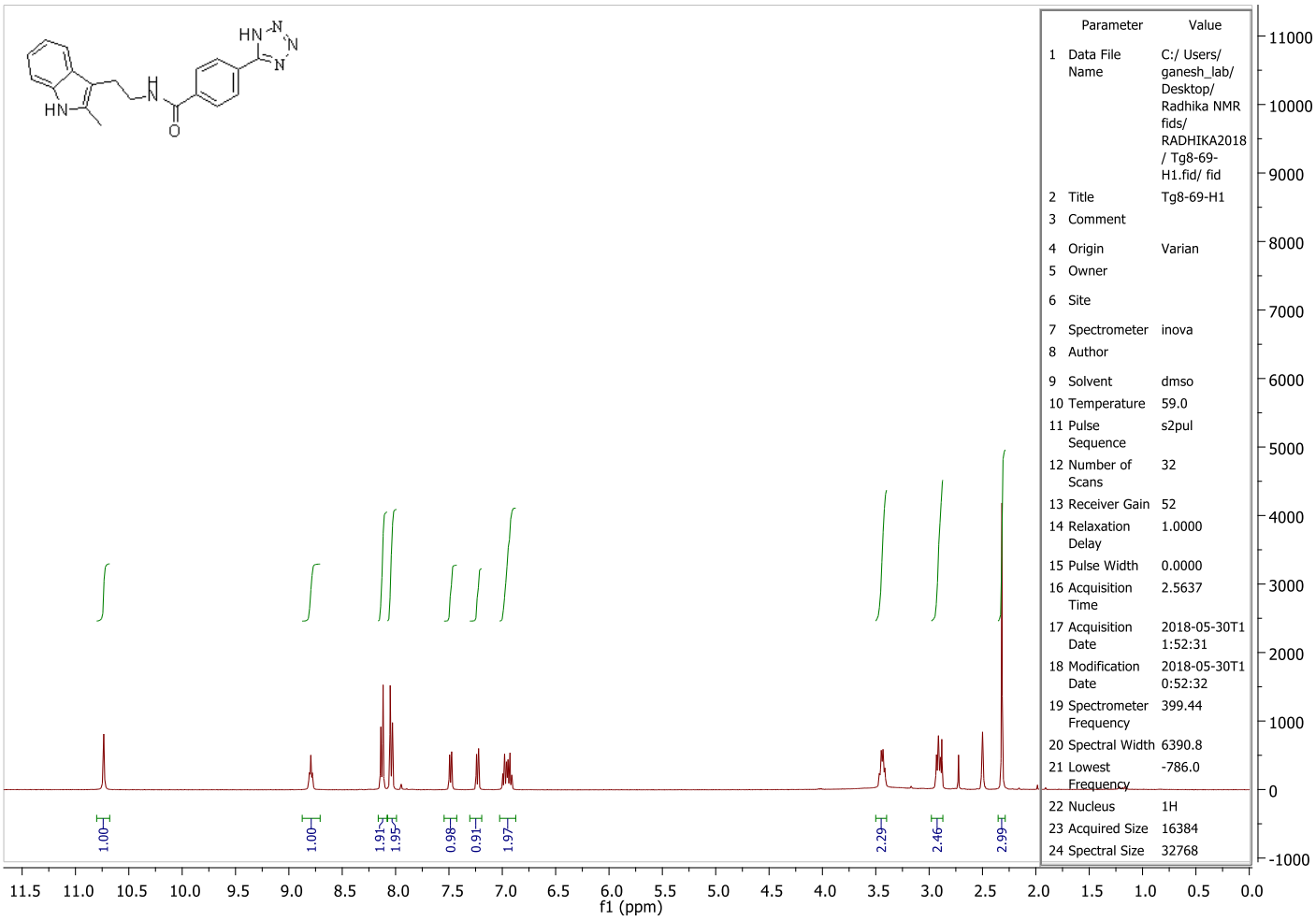
3k (TG8-258) (*4-(isoquinolin-6-yl)-N-(2-(2-methyl-1H-indol-1-yl)ethyl)benzamide)*. (60 % yield). ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.35 (s, 1H), 8.95 (t, *J* = 5.8 Hz, 1H), 8.55 (d, *J* = 5.7 Hz, 1H), 8.32 (s, 1H), 8.17 (d, *J* = 8.6 Hz, 1H), 7.96 (dd, *J* = 8.6, 1.5 Hz, 1H), 7.86 (d, *J* = 5.8 Hz, 1H), 7.46 - 7.35 (m, 2H), 7.01 (t, *J* = 7.1 Hz, 1H), 6.92 (t, *J* = 7.4 Hz, 1H), 6.17 (s, 1H), 4.30 (t, *J* = 6.6 Hz, 2H), 3.58 (q, *J* = 6.4 Hz, 2H), 2.38 (s, 3H); LCMS (ESI): > 98% purity at λ = 254 nm (ret. time 1.99 min); MS *m/z* 330 $[M+H]^+$.

3l (TG8-246) (*4-(2-methyl-1H-benzo[d]imidazole-6-yl)-N-(2-(2-methyl-1H-indol-1-yl)ethyl)benza mide)*. (70 % yield). ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.60 (t, *J* = 5.6 Hz, 1H), 7.92 (s, 1H), 7.61 (d, *J* = 8.0 Hz, 1H), 7.40 (dd, *J* = 18.9, 7.8 Hz, 3H), 7.07 – 6.98 (m, 1H), 6.98 – 6.86 (m, 1H), 6.16 (s, 1H), 4.26 (t, *J* = 6.7 Hz, 2H), 3.51 (q, *J* = 6.5 Hz, 2H), 2.47 (s, 3H), 2.36 (s, 3H). LCMS (ESI): >98% purity at λ = 254 nm (ret. time 3.62 min); MS *m/z* 330 $[M+H]^+$.

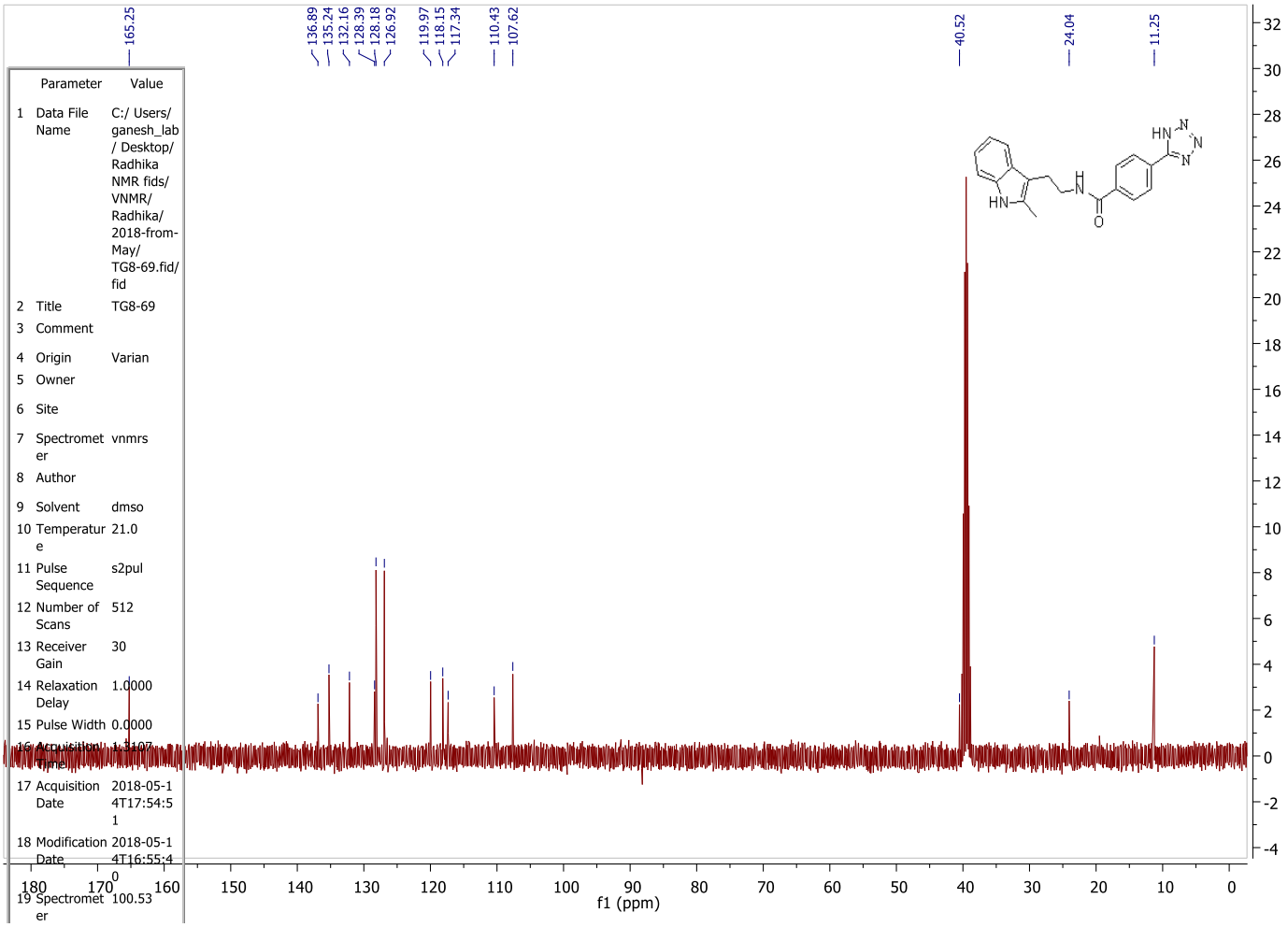
Synthesis of TG8-69. A solution of 2-(2-methyl-*1H*-indol-3-yl)ethan-1-amine (**1a**) (200 mg, 1.15 mmol) and 4-(1H-tetrazol-5-yl)benzoic acid (**2a**) (220 mg, 1 mmol) were dissolved in *N*,*N*-dimethylformamide (1 mL) and dichloromethane mixture (3 mL), then was added 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI) (300 mg, 1.5 mmol) and catalytic amount of *N*,*N*-dimethylaminopyridine (DMAP) (10 mg), and stirred at room temperature overnight. Reaction mixture was dissolved in ethyl acetate (50 mL), washed with 2N HCl followed by 2N $NaHCO_3$, water and saturated NaCl solution (5 mL each). The organic solution was dried with Na_2SO_4 and concentrated. The crude product was purified by silica-gel column chromatography eluting with hexane and ethyl acetate (20-80%) mixture to obtain a pure product (65% yield). ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.73 (s, 1H), 8.79 (t, *J* = 5.6 Hz, 1H), 8.13 (d, *J* = 8.4 Hz, 2H), 8.04 (d, *J* = 8.4 Hz, 2H), 7.48 (d, *J* = 7.5 Hz, 1H), 7.23 (d, *J* = 7.5 Hz, 1H), 6.95 (m, 2H), 3.44 (q, *J* = 6.9 Hz, 2H), 2.91 (t, *J* = 6.9 Hz, 2H), 2.32 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 165.25, 136.89, 135.24, 132.16, 128.39, 128.18, 126.92, 119.97, 118.15, 117.34, 110.43, 107.62, 40.52, 24.04, 11.25. LCMS (ESI): >97% purity at λ = 254 nm (ret. time 2.46 min); MS *m/z* 347 $[M + H]^+$. Elemental analysis calculated for $C_{19}H_{18}N_6O$; C, 65.88; H, 5.24; N, 24.26; Found: C, 65.21; H, 5.53; N, 23.69. HRMS Calculated for $C_{19}H_{19}ON_6$ (M+H), 347.16149; found 347.16230. Please see supporting information for NMR and MS spectra of TG8-69 and full characterization data for other compounds.

TG8-69 NMR spectra

^{1}H NMR



^{13}C NMR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136.89
135.24
132.16
128.39
128.18
126.92
119.97
118.15
117.34
110.43
107.62
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24.04
11.25
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3 Comment
4 Origin Varian
5 Owner
6 Site
7 Spectrometer vnmrs
8 Author
9 Solvent dmso
10 Temperature 21.0
11 Pulse Sequence s2pul
12 Number of Scans 512
13 Receiver Gain 30
14 Relaxation Delay 1.0000
15 Pulse Width 0.0000
16 Acquisition Time 1.3107
17 Acquisition Date 2018-05-14T17:54:51
18 Modification Date 2018-05-14T16:55:40
19 Spectrometer 100.53
HN
N
N
N
H
N
HN
O
f1 (ppm)

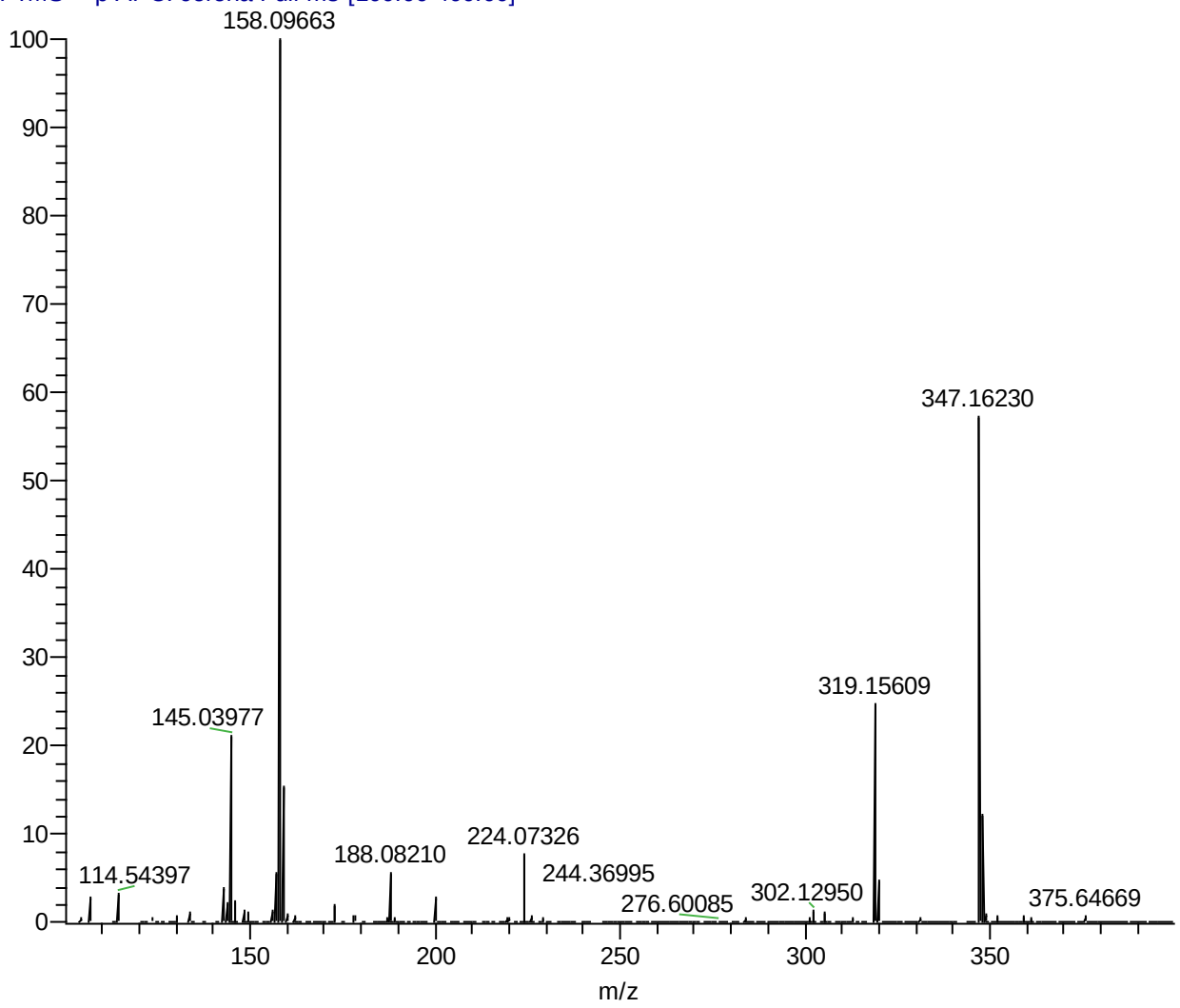
TG8-69 HRMS

Elemental composition search on mass 347.16230

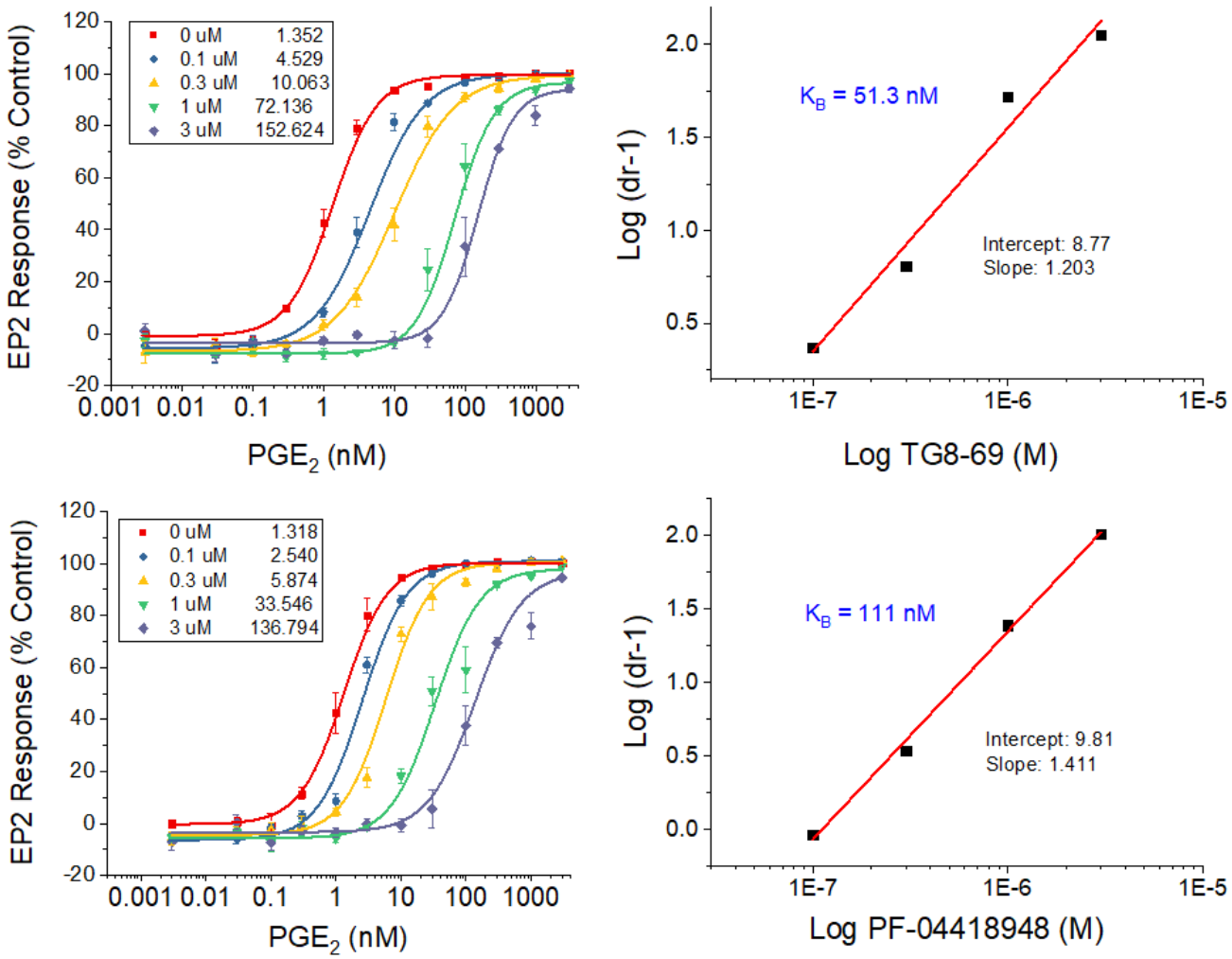
m/z= 342.16230-352.16230

m/z	Theo. Mass	Delta (mmu)	RDB equiv.	Composition
347.16230	347.16149	0.81	13.5	$C_{19}H_{19}ON_6$
	347.16334	-1.04	0.5	$C_7H_{23}O_8N_8$

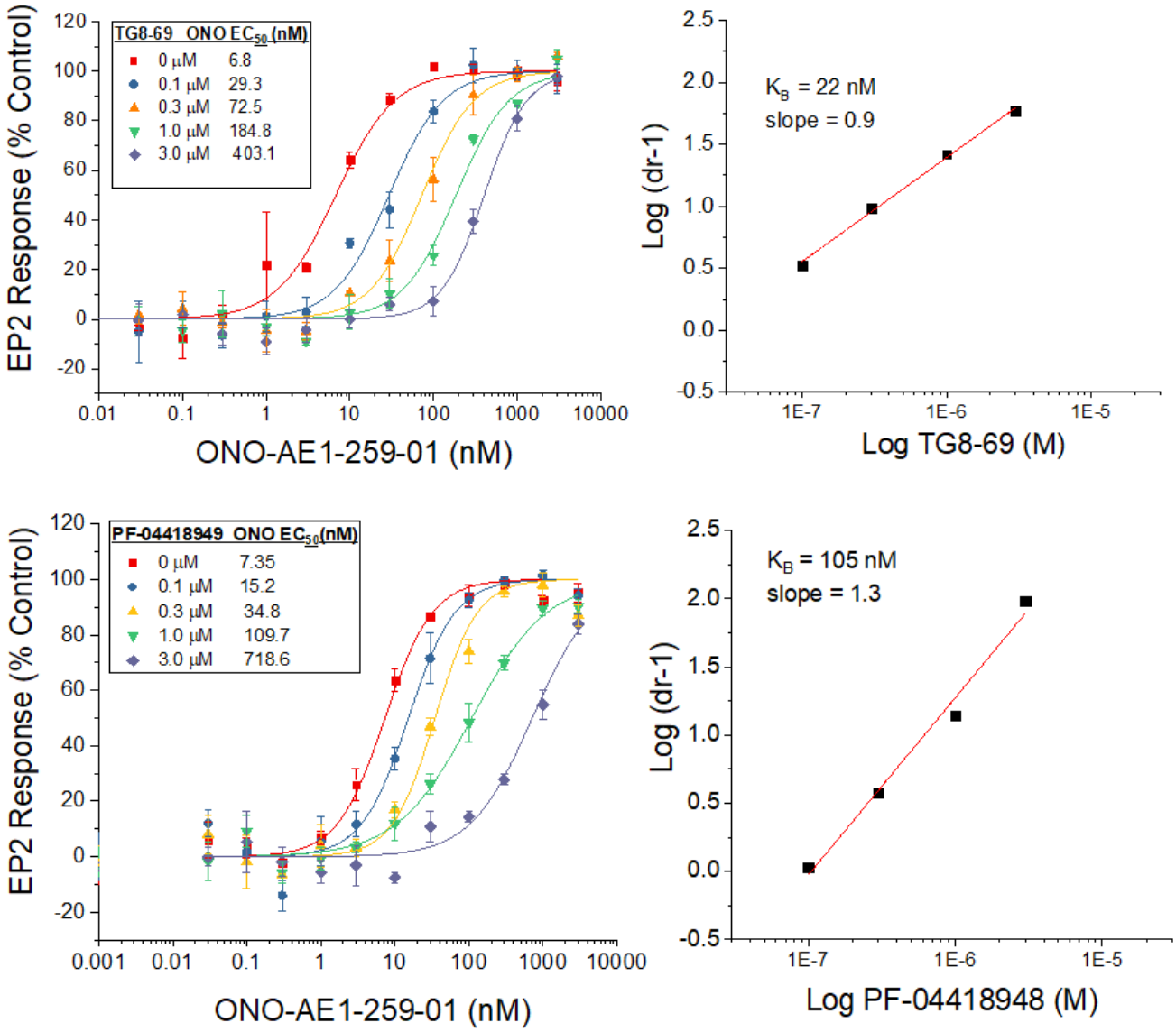
FT40543_180518095419 #37-42 RT: 0.54-0.62 AV: 6 NL: 1.56E7
T: FTMS + p APCI corona Full ms [100.00-400.00]



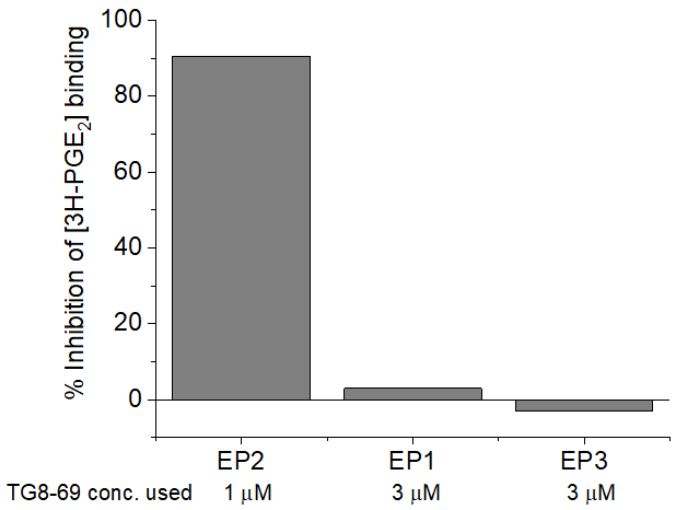
Additional Supporting Figures



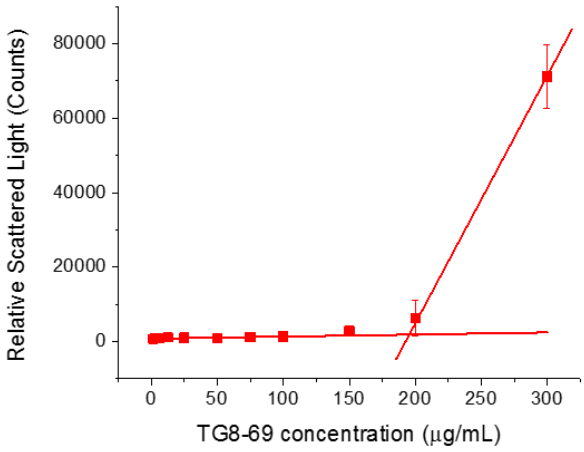
SI Figure 1. EP2 potency of TG8-69 and PF-04418948 with total 90 minutes of pre-incubation time. The Schild plots indicates that longer incubation time did not impact the potency of the compounds. The Schild K_Bs, 51.3 nM for TG8-69, 111 nM for Pfizer compound (PF-04418948) are within in range of K_Bs we found with total 50 minutes of pre incubation with these compounds (see main text and **Fig 1**).



SI Figure 2. EP2 potency of TG8-69 and PF-04418948 in BV2 cell line with human EP2 receptors. TG8-69 and the Pfizer compound displayed concentration response fold shifts to highly selective EP2 agonist ONO-AE1-259-01 EC_{50}. Schild analysis indicated that Pfizer compound (PF-04418948) is 5-times less potent than TG8-69 (n = 2).



SI, Figure 3. Inhibition of PGE_2 binding to the EP1-3 receptors by TG8-69. TG8-69 did not inhibit the binding of H^3-PGE_2 to EP1 and EP3 at 3 µM, but it inhibited H^3-PGE_2 binding to EP2 by 90.6% at 1 µM confirming it is selective to EP2 against EP1 and EP3 receptors.



SI, Figure 4: Aqueous solubility of TG8-69. TG8-69 was dissolved in DMSO at a stock concentration of 30 mg/mL, and then was serially diluted in DMSO for a concentration profile of 30 to 0.15 mg/mL. The solutions were transferred to a 96-well microplate and further diluted 100 × in PBS at pH 7.4 with the final DMSO concentration 1%. The plates were incubated for 90 min at room temperature and then analyzed by a BMG Labtech Nephelostar microplate nephelometer. Each concentration was assayed in a replicate of seven wells. This experiment was repeated two more times with replicates of seven wells.

SI Table 1: CYP450 enzymes inhibition assay parameters, substrates and metabolites used [a].

Fluorescent Assays	**CYP1A2**	**CYP 3A4**	**CYP 3A4**	**CYP 2B6**	**CYP 2C8**	**CYP 2C9**	**CYP 2C19**	**CYP 2D6**
CYP Enzyme Concentration (pmol/mL)	1.25	2.5	5	5	20	15	15	20
Substrate	CEC	BFC	BzRes	7-EFC	DBF	7-MFC	CEC	7-MFC
Substrate Concentration (µM)	5	50	1	1.5	0.5	75	10	50
Metabolite	CHC	HFC	Resorufin	HFC	Fluorescein	HFC	CHC	HFC
Reference	Furafyl-line	Ketoconazole	Ketoconazole	Ketoconazole	Quer-cetin	Sulfaph-enazole	Tranylc-ypro-mine	Quinidi-ne
Incubation time (min)	30	30	20	30	50	40	50	40
Buffer (PPB, pH7.4)	100 mM	100 mM	100 mM	100 mM	50 mM	50 mM	100 mM	100 mM
Glucose-6-Phoshpate Dehydrogenase	0.4 U/mL	0.4 U/mL	0.4 U/mL	0.4 U/mL	0.4 U/mL	0.4 U/mL	0.4 U/mL	0.4 U/mL
NADP	8.2µM	8.2µM	8.2µM	8.2µM	8.2µM	8.2µM	8.2µM	8.2µM
Excitation Filter (nm)	395/25	395/25	530/25	395/25	485/20	395/25	395/25	395/25
Emission Filter (nm)	460/40	528/20	590/10	528/20	528/20	528/20	460/40	528/20
Sensitivity (%)	50	75	75	75	65	75	50	75

[a]Test Concentration: 10 µM, Final volume: 200 µL/well

Abbreviations used for substrates: CEC: 3-Cyano-7-ethoxycoumarin; BFC: 7-Benzyloxy-4-(trifluoromethyl)- coumarin; BzRes: 7-Benzyloxyresorufin (Resorufin Benzyl Ether); 7-EFC: 7-Ethoxy-4-(trifluoromethyl)-coumarin; 7-MFC: 7-Methoxy-4-trifluoro-methylcoumarin; DBF: Dibenzylfluorescein;

Abbreviations used for metabolites: CHC: 3-Cyano-7-hydroxy-coumarin, Excitation max/ Emission max: 409/450; Resorufin: Excitation max/ Emission max: 571/585; HFC: 7-Hydroxy-4-trifluoro-methylcoumarin, Excitation max/ Emission max: 385/502; Fluorescein: Excitation max/ Emission max: 485/538.

SI Table 2: Real time PCR primers sequences

Genes	Forward Primer (sequence 5'-3')	Reverse Primer (sequence 5'-3')
GAPDH	TGTCCGTCGTGGATCTGAC	CCTGCTTCACCACCTTCTTG
COX-2	ACCAACGCTGCCACAACT	GGTTGGAACAGCAAGGATTT
IL1β	CAGGAAGGCAGTGTCACTCA	TCCCACGAGTCACAGAGGA
IL-6 (IL6)	AACTCCATCTGCCCTTCAGGAACA	AAGGCAGTGGCTGTCAACAACATC
hEP2	ACCCTTGGGTCTTTGCCATCCTTA	AGGTCAGCCTGTTTACTGGCATCT