Supporting Material

Structure Based Design of a Potent, Selective and Brain Penetrating PDE2 Inhibitor with Demonstrated Target Engagement.

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Contents.

Experimental Docking Procedure of Bay 60-7550: Docking was performed using the GOLD software (version 5.2.2). The protein structure was prepared by: firstly, the pdb was imported into MOE[1] (Molecular Operating Environment, version 2013.0801), structure preparation and protonate 3D tools were used to prepare the protein, Hydrogen atoms were added, charges then calculated, and the ligand removed. Docking used a 14 Å radius active site defined around Gln859 (alpha carbon). All waters were retained. The active site Gln859 can adopt two rotational conformations, both were used for docking. No placement restraints to enforce specific ligand-protein interactions were used. The GOLD slow, also known as most accurate, approach was used with the GoldScore fitness function and ten genetic algorithm runs per ligand. All other docking parameters were set to the defaults. Ionisation state of the ligand was checked by calculating their pK_a at pH 7.4 with the ACD[2] software. The molecule was considered in an unionised states. A single 3D conformation was used as input for docking. The structure was generated by minimising in MOE with the MMFF94x force field ensuring the correct diastereomer.

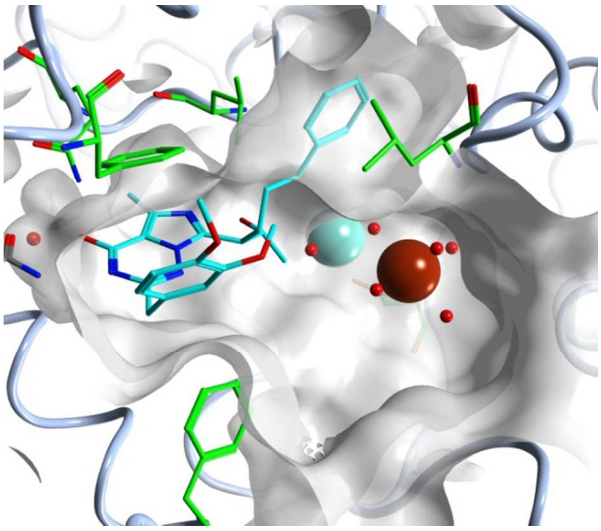


Figure S1. Docked pose of Bay 60-7550 showing access of the phenbutyl group to the channel above the active site.

Protocols for measuring inhibition of PDE's *in vitro*. Phosphodiesterases 1B1 and 11A4 were expressed in HEK cells from full-length human recombinant clones. Human recombinant phosphodiesterases 2A, 4D3, 5A3, 7A1, 9A1, 10A2, and rat PDE10A2 were expressed in Sf9 cells, using a recombinant baculovirus construct containing the full length sequence containing a 6xHis sequence following the start Met to allow metal affinity purification of the recombinant protein. Cells were harvested and the phosphodiesterase protein was purified by metal chelate chromatography on Ni-sepharose 6FF. PDE6AB, PDE3A and PDE8A were purchased as partially purified Sf9 cell lysates (Scottish Biomedical, UK). All enzymes were diluted in 50 mM Tris pH 7.8, 1.7 mM EGTA, 8.3 mM $MgCl_2$ except for PDE9A that was diluted in 50 mM Tris pH 7.8, 5 mM $MnCl_2$ and PDE1B was diluted in 50 mM Tris pH 7.8, 1.7 mM EGTA, 8.3 mM $MgCl_2$ complemented with 624 U/mL calmodulin and 800 µM $CaCl_2$. The affinity of the compounds for phophodiesterases (PDE) was measured by a scintillation proximity assay (SPA). PDE Yttrium Silicate SPA beads allow PDE activity to be measured by direct binding of the primary phosphate groups of non-cyclic AMP or GMP to the beads via a complex iron chelation mechanism. The amount of bound tritiated product ([^{3}H]-AMP or [^{3}H]-GMP) was measured by liquid scintillation counting in a TopCount (Packard). The compounds were dissolved and diluted in 100% DMSO in polystyrene plates to a concentration of 100-fold the final concentration in the assay. Rat PDE10A or human PDE2A enzyme solution (10 µl) was added to 20 µl of incubation buffer (50 mM Tris pH 7.8, 8.3 mM $MgCl_2$, 1.7 mM EGTA), 10 µl substrate solution consisting of a mixture of non-tritiated and tritiated substrate (60 nM cAMP, 0.008 µCi ^{3}H-cAMP for PDE10A, 10 µM cGMP, 0.01 µCi ^{3}H-cGMP for PDE2A), and 0.4 µl compound in 100% DMSO in a 384-well plate, and incubated for 60 or 40 minutes at room temperature for PDE10A and PDE2A respectively. After incubation, the reaction was stopped with 20 µl of stop solution, consisting of PDE SPA beads (17.8 mg beads/mL in 18 mM or 200 mM zinc sulfate for PDE10A or PDE2A respectively). After sedimentation of the beads for 30 minutes, the radioactivity was measured in a Perkin Elmer Topcount scintillation counter and results were expressed as counts per minute (cpm). To measure the low control, no enzyme was added to the reaction mixture. The same assay principle was applied for the measurement of the inhibition of other members of the PDE family, with appropriate modifications of enzyme concentration, incubation buffer, substrate solution, incubation time and stop solution. Data were calculated as the percentage of inhibition of total activity measured in the absence of test compound (% control). A best-fit curve was fitted by a minimum sum of squares method to the plot of % control *vs* compound concentration, from which an IC_{50} value (inhibitory concentration causing 50 % inhibition of hydrolysis) was obtained.

In vivo PDE2 occupancy method. Male Wistar rats were treated by systemic administration (sc or po) of vehicle or different dosages of selective PDE2A inhibitors (n = 3 per dose). After 15 min, every rat received a s.c. injection of the PDE10 inhibitor **MP-10** at the dose of 2.5 mg/kg followed 55 min later by an i.v. injection of the selective PDE2A radioligand [^{3}H]-**3**[3]. Dosing rats with MP-10, a potent and selective PDE10 inhibitor, prior to the radioligand injection allowed to increase the signal to noise ratio of [^{3}H]-**3** since its binding is increased when intracellular cGMP concentration raised. Rats were sacrificed 5 min after the tracer injection and their brains dissected and frozen. Then 20 µm thick coronal sections were cut using a cryostat, collected on glass slides and dried. Brain sections were loaded in a β-imager for 12 h. The specific binding was determined as the difference between [^{3}H]-**3** binding quantified in the striatum (a brain area showing a high density of PDE2A) and in the cerebellum (a brain area where PDE2A is virtually absent). Occupancy was calculated as the inhibition of specific [^{3}H]-**3** binding in drug-treated animals relative to vehicle-treated animals. For the determination of the ED_{50} (dose occupying 50% of PDE2A), the percentage of PDE2A occupancy was plotted against dosage, and the sigmoidal log dose-effect curve of the best fit was calculated by nonlinear regression analysis, using the GraphPad Prism program.

Protocol for brain tissue binding.[4] 1 in 10 diluted brain tissue homogenate is prepared by adding 9 mL PBS (pH=7.4) to 1 g of brain tissue. Brain tissue homogenate, containing test compound at 5 µM, is incubated at 37ºC in the Rapid Equilibrium Dialysis (RED) Device. The RED device consists of a Teflon 48-well base plate which contains disposable inserts. These inserts are bisected by a semi-permeable (MWCO= 8 kD) membrane, creating two chambers. Aliquots (300 µl) of spiked 1 in 10 diluted brain tissue homogenate are loaded in to one chamber and phosphate buffered saline (PBS pH=7.4, 500µl) is loaded into the other. The plate is then sealed and placed in a shaking incubator at 37 °C for 5 h. After 5 h, samples are removed and analyzed from both the buffer and brain tissue homogenate side to obtain free and bound concentrations. These concentrations are then used to calculate the percentage compound bound to brain tissue (%BTB). Recovery in the experiment is calculated by measuring the actual concentration of test compound remaining in the buffer and brain homogenate compartments at the end of the incubation and comparing this to the actual spiked brain homogenate concentration at the start of the experiment. A calibration curve with a concentration range of 1500 to 5 nM in DMSO is prepared. For purpose of matrix matching, 50 µl of this DMSO concentration range + 12.5 µl blank homogentae + 50 µl blank buffer and 300 µl MeCN is added in BA_curve plates. After shaking and centrifugation, these plates are ready for MS-injection. To check leakage from homogenate compartment in buffer compartment, 10 µl buffer and 200 µl Bradford reagent (20ml buffer+5ml Bradford stock) is added in an empty plate. Leakage is observed when the well coloured blue.

Materials:

1) 48-well Single-Use Red plate with inserts, 8K molecular weight cut-off, Thermo Scientific CatNo: 90006
2) Frozen rat or mouse brain homogenate is thawed and warmed just prior to use
3) Phosphate buffered saline (PBS), pH 7.4, Gibco life technologies CatNo: 10010-031)
4) 2 mM test compounds in DMSO
5) Bradford reagent (bio-Rad laboratories GmbH CatNo: 500-0006)

Methods and Procedures

Test compounds

Test compounds are received as neat compounds and diluted to 2 mM in DMSO.

The final concentration in brain tissue homogenate is 5 µM.

Assay procedure

Pre-Dialysis

RED inserts are in the RED base plate. Blank 1 in 10 diluted brain tissue homogenate and 2mM stock solutions are supplied to the robot. In addition, RED device and plates are placed on the robot deck (Tecan, Freedom EVO). The robot automatically spikes brain tissue homogenate at 5 µM (final DMSO concentration in the incubation is 1%), mixes and fills RED device with buffer and spiked homogenate. All BTB measurements are performed in triplicate. The RED device is sealed and is allowed to incubate for 5 h at 37 °C on a vortex shaker (500 rpm) in an air incubator.

Preparation standards

Calibration curves across an appropriate concentration range are prepared by the robot. These are used for quantitative analysis of the homogenate and buffer samples.

Post-Dialysis

After incubation, the RED device is automatically sampled with the robot by taking 12.5 µl homogenate and 50 µl buffer, which is transfered to a 96 well plate. After addition of matrix (12.5 µl homogenate or 50 µl buffer, 50 µl DMSO and 300 µl acetonitrile) the samples are ready for LC-MS/MS analysis.

LC-MS/MS Analysis

Samples are analyzed by LC-MS/MS in Multiple Reaction Monitoring (MRM) mode. One LC-MRM method is used for all tuned compounds, using optimal mass transitions and MS settings found with tuning. Absolute concentrations of test compound in buffer and homogenate samples is quantified as measured by the calibration curve.

Typical MS method on vantage:

-column: Waters Xbridge BEH C18xp 2.5µm 2.1*30mm

- Flow rate: 0.7mL/min
- column temperature: Room temperature
- solvent A: H_2O+0.1%FA
- solvent B: ACN+0.1%FA
- run time: 2.5 min (multiplexed)
- gradient profile:

0.00 min	5 %B	12sec
0.2min	95 %B	40sec
0.87 min	95 %B	40sec
1.53 min	5 %B	3sec
1.58 min	5% B	55sec

Typical MS method on 4000Qtrap:

-column: Acquity UPLC BEH C18 1.7µm 2.1*30mm

- Flow rate: 1.03mL/min
- column temperature: 60°C
- solvent A: H_2O+0.1%FA
- solvent B: ACN+0.1%FA
- run time: 1.7 min

- gradient profile:

0.00 min	5 %B
0.21min	95 %B
0.66 min	95 %B
1.11 min	5 %B
1.15 min	5% B

Calculations and Formulas

The formula used for determining the apparent unbound fraction ($f_{u,app}$) is as follows:

$$f_{u,app} = \frac{[A]_{buffer}}{4 \times [A]_{\text{homogenate}}}$$

where $[A]_{\text{homogenate}}$ is the concentration measured in the homogenate and $[A]_{\text{buffer}}$ the concentration measured in the buffer. Homogenate concentrations have to be multiplied by a factor 4 since four times the volume of buffer as homogenate is used for concentration determination. Since homogenates are diluted (in this case 10 times) the $f_{u,app}$ has to be corrected for the dilution factor in order to get the real unbound fraction in brain tissue ($f_{u,brain}$). This is accomplished with the following formula:

$$f_{u,brain} = \frac{f_{u\,app}}{D + f_{u\,app} - D \times f_{u\,app}}$$

Where D is the dilution factor. Subsequently, the percentage compound bound to brain tissue (%BTB) is determined as follows:

$$\%BTB = (1 - f_{u,brain}) \times 100\%$$

Protocol for plasma tissue binding.[5]

Assay Principle

Plasma, containing test compound at 5 µM, is incubated at 37ºC in the Rapid Equilibrium Dialysis (RED) Device. The RED device consists of a 48-well base plate which contains inserts. These inserts are bisected by a semi-permeable membrane, creating two chambers. Aliquots (300 µl) of spiked plasma are loaded into one chamber and phosphate buffered saline (PBS, 500µl) is loaded into the other. The plate is then sealed and placed in a shaking incubator at 37 °C for 4.5 h. After 4.5 h, samples are removed and analyzed from both the buffer and plasma side to obtain free and bound concentrations. These concentrations are then used to calculate the bound fraction of the drug (f_b). Recovery in the experiment is calculated by measuring the actual concentration of test compound remaining in the buffer and plasma compartments at the end of the incubation and comparing this to the actual spiked plasma concentration at the start of the experiment.

To check leakage from plasma compartment in buffer compartment, 10 µl buffer and 200 µl Bradford reagent (20ml buffer+5ml Bradford stock) is added in an empty plate. Leakage is observed when the well coloured blue. A calibration curve with a concentration range of 1250 to 5 nM in DMSO is prepared. For purpose of matrix matching, 50 µl of this DMSO concentration range + 10 µl blank plasma + 50 µl blank buffer and 300 µl MeCN is added in BA curve plates. After shaking and centrifugation, these plates are ready for MS-injection.

Materials

1) 48-well Single-Use Red plate with inserts, 8K molecular weight cut-off, Thermo Scientific CatNo: 90006

2) Frozen human, rat, dog, mouse or guinea pig plasma is thawed and warmed just prior to use (PH check at 37°C).
3) Phosphate buffered saline (PBS), pH 7.4, Gibco life technologies CatNo: 10010-031)
4) 2 mM test compounds in DMSO
5) Bradford reagent (bio-Rad laboratories GmbH CatNo: 500-0006)

Methods and Procedures

Test compounds

Test compounds are received as neat compounds and diluted to 2 mM in DMSO.

The final concentration in plasma is 5000nM.

Assay procedure

Pre-Dialysis

Blank plasma and 2mM stock solutions are supplied to the robot. In addition, RED device and plates are placed on the robot deck (Tecan, Freedom EVO) The robot automatically spikes plasma at 5µM (final DMSO concentration in the incubation is 1%), mixes and fills RED device with buffer and spiked plasma. All PPB measurements are performed in triplicate. The RED device is sealed and is allowed to incubate for 4.5 h at 37 °C on a vortex shaker (500 rpm) in an air incubator.

Preparation standards

Calibration curves across an appropriate concentration are prepared by the robot. These are used for quantitative analysis of the plasma and buffer samples.

Post-Dialysis

After incubation, the RED device is automatically sampled with the robot by taking 10 µl plasma and 50 µl buffer, which is transferred to a 96 well plate. After addition of matrix (10 µl plasma or 50 µl buffer, 50 µl DMSO and 300 µl acetonitrile) the samples are ready for LC-MS/MS analysis.

High free fraction protocol

For highly free compounds: 10µl buffer + 40µl blank buffer is added to get a final buffer volume of 50µl. The buffer concentration is multiplied by 5 in the calculations.

LC-MS/MS Analysis

Samples are analyzed by LC-MS/MS in Multiple Reaction Monitoring (MRM) mode. One LC-MRM method is used for all tuned compounds, using optimal mass transitions and MS settings found with tuning. Absolute concentrations of test compound in buffer and plasma samples is quantified as measured by the calibration curve.

Typical MS method on vantage:

-column: Waters Xbridge BEH C18xp 2.5µm 2.1*30mm
- Flow rate: 0.7mL/min
- column temperature: Room temperature
- solvent A: H_2O+0.1%FA
- solvent B: ACN+0.1%FA
- run time: 2.5 min (multiplexed)
- gradient profile:

0.00 min	5 %B	12sec
0.2min	95 %B	40sec
0.87 min	95 %B	40sec
1.53 min	5 %B	3sec
1.58 min	5% B	55sec

Typical MS method on 4000Qtrap:

-column: Acquity UPLC BEH C18 1.7μm 2.1*30mm

- Flow rate: 1.03mL/min
- column temperature: 60°C
- solvent A: H_2O+0.1%FA
- solvent B: ACN+0.1%FA
- run time: 1.7 min
- gradient profile:

0.00 min	5 %B
0.21min	95 %B
0.66 min	95 %B
1.11 min	5 %B
1.15 min	5% B

Calculations and Formulas

The formula used for determining the bound fraction (f_b) is as follows:

$$f_b = \frac{2 \times [plasma] - [buffer]}{2 \times [plasma]}$$

here [plasma] is the concentration measured in the plasma and [buffer] the concentration measured in the buffer. Plasma concentrations have to be multiplied by factor 2 since twice the volume of buffer as plasma is used for concentration determination. Subsequently the unbound fraction (f_u) is determined as follows:

$$f_u = 1 - f_b$$

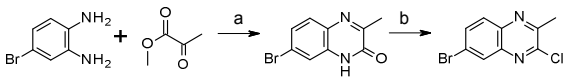
Synthesis procedures and characterization information

Analytical methods: All final compounds were characterized by ^{1}H NMR and LC/MS. ^{1}H Nuclear Magnetic Resonance spectra were recorded on a Bruker 300, 360 or 500 MHz spectrometer. For the ^{1}H spectra, all chemical shifts are reported in part per million (δ) units, and are relative to the residual signal at 7.26 and 2.50 ppm for $CDCl_3$ and DMSO, respectively.

All the LC/MS analyses were performed using an Agilent G1956A LC/MS quadrupole coupled to an Agilent 1100 series liquid chromatography (LC) system consisting of a binary pump with degasser, autosampler, thermostated column compartment and diode array detector. The mass spectrometer (MS) was operated with an atmospheric pressure electro-spray ionization (API-ES)

source in positive ion mode. The capillary voltage was set to 3000 V, the fragmentor voltage to 70 V and the quadrupole temperature was maintained at 100°C. The drying gas flow and temperature values were 12.0 L/min and 350 °C, respectively. Nitrogen was used as the nebuliser gas, at a pressure of 35 psig. Data acquisition was performed with Agilent Chemstation software.

Analyses were carried out on a YMC pack ODS-AQ C18 column (50 mm long x 4.6 mm I.D.; 3 µm particle size) at 35 °C, with a flow rate of 2.6 mL/min. A gradient elution was performed from 95% (Water + 0.1% Formic acid)/5% Acetonitrile to 5% (Water + 0.1% Formic acid)/95% Acetonitrile in 4.8 min; the resulting composition was held for 1.0 min; from 5% (Water + 0.1% formic acid)/95% Acetonitrile to 95% (Water + 0.1% formic acid)/5% Acetonitrile in 0.2 min. The standard injection volume was 2 µL. Acquisition ranges were set to 190-400 nm for the UV-PDA detector and 100-1400 m/z for the MS detector.

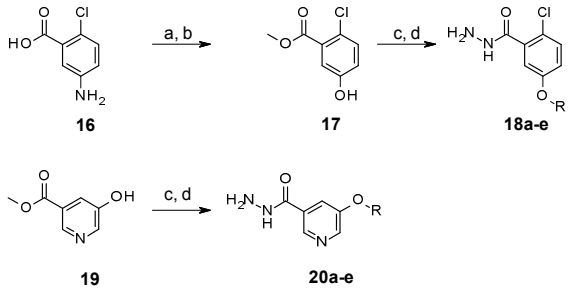


14 **15**

Scheme 1. (a) Toluene, reflux, water removal with a Dean Stark trap, 22 h, 25% (after selective recrystallization); (b) $POCl_3$, 1,2-dichloroethane, reflux, 16 h, 95%.

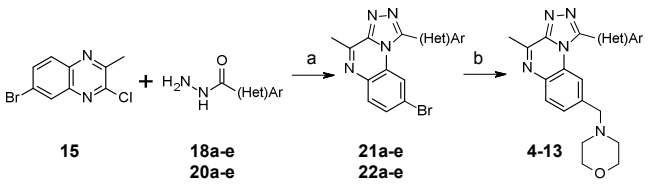
7-bromo-3-methylquinoxalin-2(1*H*)-one, 14. A slurry of 4-bromo-1,2-diaminobenzene (54.2 g, 0.290 mol) in toluene (0.75 L) was treated with methyl pyruvate (34.6 ml, 0.30 mol) and the ensuing reaction mixture was boiled under reflux for 22h while removing water and methanol by means of a Dean Stark apparatus. The reaction mixture was concentrated *in vacuo* and the resulting was diluted with i-PrOH (0.6 L), boiled for 30 min, allowed to come to r.t. stirring was continued for 2 days. The solid was collected by filtration washed with diethyl ether and dried to give a brown solid. This solid was diluted with methanol (0.3 L), boiled under reflux for 30 min, allowed to come to r.t. and stirring was continued for 16 h. The brownish solid was collected by filtration and dried to afford 7-bromo-3-methylquinoxalin-2(1*H*)-one (**4**) (18.5 g, 0.0774 mol, 25%). LC/MS (*m*/*z*): 95% pure, t_R = 0.71 min, $[M+1]^+$ 238.9. ^{1}H NMR (400 MHz, DMSO-d6) d ppm 2.38 (s, 3 H), 7.38 - 7.44 (m, 2 H), 7.62 (d, *J*=9.3 Hz, 1 H), 12.34 (br. s., 1 H).

6-bromo-3-chloro-2-methyl-quinoxaline, 15. To a mixture of 7-bromo-3-methylquinoxalin-2(1*H*)-one (**4**) (18.5 g, 0.0774 mol) in dichloroethane (250 mL) was added dropwise phosphorous oxychloride (50 mL, 0.54 mol) and the ensuing r.m. was boiled under reflux for 16h. The r.m. was cooled, concentrated *in vacuo* and the reside was diluted with dichloromethane (0.5 L) and poured onto ice water (0.5 L) and treated with aqueous ammonia until pH = 9. The aqueous layer was extracted with dichloromethane (0.2 L) and the combined organic layers were dried ($MgSO_4$), filtered and concentrated *in vacuo* to yield 6-bromo-3-chloro-2-methyl-quinoxaline **5** as a reddish solid (17.6 g, 0.0687 mol, 95%).: GC/MS (*m*/*z*): 100% pure, t_R = 6.08 min $[M^{+\cdot}]$ = 255. ^{1}H NMR (300 MHz, CHLOROFORM-d) d ppm 2.83 (s, 4 H), 7.80 - 7.93 (m, 2 H), 8.17 (d, *J*=1.9 Hz, 1 H)



Scheme 2. (a) $NaNO_2$, H_2SO_4 (1.7 %), 5°C, 1 h then hot water 3 h, reflux; (b) MeOH, $SOCl_2$, reflux, 2 h, 95-100%; (c) Alkyl iodide, Cs_2CO_3, acetonitrile, reflux or alcohol DIAD, Ph_3P in THF r.t. 50-100%; (d) Hydrazine hydrate EtOH reflux 2 h, 45-95%.

5-butoxypyridine-3-carbohydrazide, 20d. To a solution of 5-hydroxynicotinic acid methyl ester (10.1 g, 0.0657 mol) in THF (150 mL) was added subsequently 1-butanol (7.2 mL, 0.078 mol), triphenylphosphine (25.9 g, 0.0985 mol) and the diisopropyl azodicarboxylate (19.5 ml, 0.0990 mol) was added drop wise and the ensuing reaction mixture was boiled under reflux for 2h. The reaction mixture concentrated *in vacuo* and the crude concentrate was purified by silicagel chromatography using dichloromethane/methanol 99.5/0.5 as the eluent to afford methyl 5-butoxypyridine-3-carboxylate (13.3 g, 0.0635 mol) as an oil. A solution of methyl 5-butoxypyridine-3-carboxylate (13.3 g, 0.0635 mol) in ethanol (65 mL) was treated with aqueous hydrazine (6.4 mL, 0.127 mol) and boiled under reflux for 16 h and then concentrated *in vacuo*. The solid was triturated from hot hexane yielding the first crop of 5-butoxypyridine-3-carbohydrazide. The filtrate was concentrated *in vacuo* and the residue was purified by silicagel flash chromatography (120 g Redisep Flash column using a gradient of DCM/MeOH: 100/0 to 9/1) to afford a second crop of 5-butoxypyridine-3-carbohydrazide and both crops afforded 5-butoxypyridine-3-carbohydrazide as a solid (6.47g, 0.031 mol, 49%). LC/MS (*m*/*z*): 100% pure, t_R = 0.63 min, $[M+1]^+$ 211. ^{1}H NMR (360 MHz, DMSO-d6) δ ppm 0.93 (t, *J*=7.3 Hz, 3 H), 1.35 - 1.50 (m, 2 H), 1.64 - 1.79 (m, 2 H), 4.08 (t, *J*=6.4 Hz, 2 H), 4.57 (s, 2 H), 7.70 (dd, *J*=2.6, 1.8 Hz, 1 H), 8.38 (d, *J*=2.9 Hz, 1 H), 8.56 (d, *J*=1.5 Hz, 1 H), 9.94 (s, 1 H).



Scheme 3: (a) n-BuOH, 135-160°C 15-30 min; (b) (Morpholin-4-yl)methyl- trifluoroborate, $Pd(OAc)_2$ (5 mol%), 2-dicyclohexyl phosphino-2′,4′,6′-triisopropylbiphenyl (1 mol%), Cs_2CO_3, THF/H_2O, 110°C, 2.5 h, 40-66%.

8-bromo-1-(5-butoxypyridin-3-yl)-4-methyl[1,2,4] triazolo [4,3-*a*]quinoxaline, 22d . A heavy-walled tube was charged with a magnetic stirring bar, 5-butoxypyridine-3-carbohydrazide (6.75 g, 0.0323 mol), 6-bromo-3-chloro-2-methyl-quinoxaline (8.31g, 0.0323 mol) and n-butanol (130 mL) and this was placed in an oil bath of 160°C and stirred for 45 minutes. After cooling to room temperature, the reaction mixture was filtered and the filtrate was evaporated. Both filtrate and the filter cake were separately diluted with dichloromethane (200 mL), washed with saturated NaHCO3 (50 mL), dried ($MgSO_4$), filtered and concentrated *in vacuo*. The residue of the filtrate was purified by silicagel flash chromatography (120 g Redisep flash column using a gradient DCM/MeOH 100/0 to 9/1). The pure fractions were collected, evaporated and dried. All the solid fractions were gathered and the product was recrystallized from diethyl ether yielding 8-bromo-1-(5-butoxypyridin-3-yl)-4-methyl[1,2,4] triazolo[4,3-*a*]quinoxaline as an off white solid (6.89g, 0.0167 mol, 52%). LC/MS (*m*/*z*): 100% pure, t_R = 1.04 min, $[M+1]^+$ 412. ^{1}H NMR (300

MHz, DMSO-d_6) d ppm 0.93 (t, *J*=7.4 Hz, 3 H), 1.45 (sxt, *J*=7.5 Hz, 2 H), 1.75 (quin, *J*=6.3 Hz, 2 H), 2.92 (s, 3 H), 4.13 (t, *J*=6.3 Hz, 2 H), 7.48 (d, *J*=1.6 Hz, 1 H), 7.82 (dd, *J*=8.7, 1.8 Hz, 1 H), 7.91 (br. s., 1 H), 7.99 (d, *J*=8.7 Hz, 1 H), 8.55 (br. s, 1 H), 8.65 (d, *J*=2.6 Hz, 1 H).

1-(5-butoxypyridin-3-yl)-4-methyl-8-(morpholin-4-ylmethyl) [1,2,4]triazolo[4,3-*a*]quinoxaline hydrochloride, 12. A heavy-walled tube was charged with a magnetic stirring bar, 8-bromo-1-(5-butoxypyridin-3-yl)-4-methyl[1,2,4]triazolo[4,3-*a*]quinoxaline (6.89g, 0.0167 mol), (Morpholin-4-yl)methyltrifluoroborate (6.07 g, 0.0359 mol), Palladium acetate (0.19 g, 0.00084 mol, 5 mol%), 2-Dicyclohexylphosphino-2 ,4, 6-triisopropylbiphenyl (0.8 g, 0.00167 mol, 1 mol%), cesium carbonate (21.8 g, 0.0669 mol), THF (160 mL) and water (16 mL). The tube was capped, placed in an oil bath of 120°C with vigorous stirring for 2.5 h. The reaction mixture was cooled to room temperature and the THF was evaporated. Water (100 mL) and ethyl acetate (300 mL) were added and the layers were separated. The organic layer was washed with water (100 mL). the combined organic layers were extracted with ethyl acetate (200 mL). The combined organic layers were treated with brine (100 mL), dried ($MgSO_4$), filtered and concentrated *in vacuo* to give a brown solid. This was purified by silicagel flash chromatography (120 g Redisep flash column using a gradient DCM/MeOH 100/0 to 9/1) to afford white solid. This was dissolved in acetone (200 mL) and treated with 1M HCl in diethyl ether (30 mL, approximately 3 eq.) to afford 1-(5-butoxypyridin-3-yl)-4-methyl-8-(morpholin-4-ylmethyl)[1,2,4]triazolo [4,3-*a*]quinoxaline dihydrochloride as a white crystalline solid (5.58 g, 0.011 mol, 66%). LC/MS: t_R = 0.92 min, (m/z): $[M+1]^+$ 432. ^{1}H NMR (360 MHz, DMSO-d6) δ ppm 0.94 (t, *J*=7.5 Hz, 3 H), 1.34 - 1.54 (m, 2 H), 1.68 - 1.82 (m, 2 H), 2.95 (s, 5 H), 3.18 (d, *J* =12.1 Hz, 2 H), 3.73 - 3.96 (m, 4 H), 4.18 (t, *J* =6.4 Hz, 2 H), 4.35 (br. s., 2 H), 7.66 - 7.71 (m, 1 H), 7.99 (dd, *J* =8.4, 1.5 Hz, 1 H), 8.08 - 8.17 (m, 2 H), 8.76 (d, *J* =2.9 Hz, 1 H), 8.71 (d, *J* =1.8 Hz, 1 H), 12.02 (br. s., 1 H). Anal. calcd. for $C_{24}H_{28}N_6O_2 \cdot 2HCl$: C,57.03; H, 5.98: N, 16.63 found C,56.40; H, 5.97: N, 16.41. No clear m.p. was observed with DSC.

Syntheses of other compounds is analogous or is described in the previous paper.[3]

1-(2-chloro-5-methoxyphenyl)-4-methyl-8-(morpholin-4-ylmethyl)[1,2,4]triazolo[4,3-*a*]quinoxaline **4**: LC/MS (*m*/*z*): 94% t_R = 0.92 min, $[M+1]^+$ 424, mp 159.5°C, t_R = 1.57 min, ^{1}H NMR (300 MHz, DMSO-d6) δ ppm 2.23 (br. s., 4 H), 2.93 (s, 3 H), 3.40 - 3.56 (m, 6 H), 3.83 (s, 3 H), 7.32 (s, 1 H), 7.36 - 7.45 (m, 2 H), 7.52 (d, *J*=8.0 Hz, 1 H), 7.74 (d, *J*=9.8 Hz, 1 H), 7.98 (d, *J*=8.2 Hz, 1 H)

1-(2-chloro-5-ethoxyphenyl)-4-methyl-8-(morpholin-4-ylmethyl)[1,2,4]triazolo[4,3-*a*]quinoxaline hydrochloride **5**: LC/MS (*m*/*z*): 99% pure, t_R = 1.75 min, $[M+1]^+$ 438, mp 225.0°C , ^{1}H NMR (300 MHz, DMSO-d6) d ppm 1.36 (t, *J*=6.9 Hz, 3 H), 2.97 (s, 5 H), 3.20 (d, *J*=8.2 Hz, 2 H), 3.77 (br. s., 2 H), 3.91 (br. s., 2 H), 4.05 - 4.19 (m, 2 H), 4.31 (br. s., 2 H), 7.24 (s, 1 H), 7.34 - 7.44 (m, 2 H), 7.74 (d, *J*=8.7 Hz, 1 H), 7.97 (br. s., 1 H), 8.16 (d, *J*=8.2 Hz, 1 H), 11.36 (br. s., 1 H). Anal. calcd. for $C_{23}H_{24}ClN_5O_2 \cdot 2HCl$: C, 58.23; H, 5.31: N, 14.76 found C, 57.95; H, 5.16: N, 14.61.

1-(2-chloro-5-propoxyphenyl)-4-methyl-8-(morpholin-4-ylmethyl)[1,2,4]triazolo[4,3-a]quinoxaline monohydrochloride **6**: LC/MS (*m*/*z*): 99% pure, t_R = 1.96 min, $[M+1]^+$: 452. mp 250.5°C. ^{1}H NMR (300 MHz, DMSO-d6) δ ppm 1.36 (t, *J*=6.9 Hz, 3 H), 2.97 (s, 5 H), 3.20 (d, *J*=8.2 Hz, 2 H), 3.77 (br. s., 2 H), 3.91 (br. s., 2 H), 4.05 - 4.19 (m, 2 H), 4.31 (br. s., 2 H), 7.24 (s, 1 H), 7.34 - 7.44 (m, 2 H), 7.74 (d, *J*=8.7 Hz, 1 H), 7.97 (br. s., 1 H), 8.16 (d, *J*=8.2 Hz, 1 H), 11.36 (br. s., 1 H). Anal. calcd. for $C_{24}H_{26}ClN_5O_2 \cdot HCl$: C, 59.02; H, 5.39: N, 14.35 found C, 59.02; H, 5.57: N, 14.34.

1-[2-chloro-5-(propan-2-yloxy)phenyl]-4-methyl-8-(morpholin-4-ylmethyl)[1,2,4]triazolo[4,3-*a*]quinoxaline **7**: LC/MS (*m*/*z*): 99% pure, t_R = 1.98 min, $[M+1]^+$: 452. mp 247.6°C. ^{1}H NMR (300 MHz, DMSO-d6) δ ppm 1.29 (d, *J*=5.9 Hz, 3 H), 1.33 (d, *J*=5.9 Hz, 3 H),

2.97 (s, 5 H), 3.18 (br. s., 2 H), 3.79 (br. s., 2 H), 3.89 (br. s., 2 H), 4.30 (br. s., 2 H), 4.71 (dt, *J*=12.0, 5.9 Hz, 1 H), 7.26 (s, 1 H), 7.32 - 7.44 (m, 2 H), 7.72 (d, *J*=8.7 Hz, 1 H), 8.00 (br. s., 1 H), 8.15 (d, *J*=8.2 Hz, 1 H), 11.56 (br. s., 1 H)

1-(5-butoxy-2-chlorophenyl)-4-methyl-8-(morpholin-4-ylmethyl)[1,2,4]triazolo[4,3-*a*]quinoxaline monohydrochloride **8**: LC/MS (*m*/*z*): 98% pure, t_R = 2.19 min, $[M+1]^+$ 466. mp>300°C. ^{1}H NMR (300 MHz, DMSO-d6) δ ppm 0.93 (t, *J*=7.4 Hz, 3 H), 1.34 - 1.53 (m, 2 H), 1.64 - 1.83 (m, 2 H), 2.97 (s, 5 H), 3.17 (br. s., 2 H), 3.77 (br. s., 2 H), 3.92 (br. s., 2 H), 4.00 - 4.13 (m, 2 H), 4.31 (br. s., 2 H), 7.25 (s, 1 H), 7.32 - 7.46 (m, 2 H), 7.73 (d, *J*=8.8 Hz, 1 H), 7.96 (br. s., 1 H), 8.16 (d, *J*=8.1 Hz, 1 H), 11.31 (br. s., 1 H). Anal. calcd. for $C_{25}H_{28}ClN_5O_2$·HCl: C, 58.90; H, 5.81: N, 13.78 found C, 59.76; H, 5.82: N, 13.94.

1-(5-methoxypyridin-3-yl)-4-methyl-8-(morpholin-4-ylmethyl)[1,2,4]triazolo[4,3-*a*]quinoxaline **9**: : LC/MS (*m*/*z*): 100% pure, t_R = 1.29 min, $[M+1]^+$: 391 mp 136.3°C. ^{1}H NMR (500 MHz, CHLOROFORM-d) δ ppm 2.32 - 2.40 (m, 4 H), 3.08 (s, 3 H), 3.48 (s, 2 H), 3.63 (t, *J*=4.3 Hz, 4 H), 3.98 (s, 3 H), 7.53 (d, *J*=8.1 Hz, 1 H), 7.56 - 7.60 (m, 1 H), 7.66 (s, 1 H), 8.02 (d, *J*=8.4 Hz, 1 H), 8.57 (d, *J*=1.4 Hz, 1 H), 8.64 (d, *J*=2.6 Hz, 1 H)

4-methyl-8-(morpholin-4-ylmethyl)-1-(5-propoxypyridin-3-yl)[1,2,4]triazolo[4,3-*a*]quinoxaline monohydrochloride **10**: LC/MS (*m*/*z*): 96% pure, t_R = 1.54 min, $[M+1]^+$: 419, mp 213.8°C. ^{1}H NMR (300 MHz, DMSO-d6) δ ppm 0.90 - 1.12 (m, 6 H), 1.78 (sxt, *J*=6.9 Hz, 2 H), 2.95 (br. s., 4 H), 3.47 (br. s., 2 H), 3.52 - 3.81 (m, 3 H), 3.89 (br. s., 1 H), 4.09 (t, *J* =6.5 Hz, 2 H), 4.36 (br. s., 1 H), 7.56 (s, 1 H), 7.89 (br. s., 2 H), 8.15 (br. s., 1 H), 8.58 (br. s., 1 H), 8.66 (d, *J* =2.6 Hz, 1 H), 10.81 (br. s., 1 H). Anal. calcd. for $C_{25}H_{28}ClN_5O_2$·HCl: C, 58.88; H, 5.97: N, 17.72 found C, 60.72; H, 5.98: N, 18.47.

4-methyl-8-(morpholin-4-ylmethyl)-1-[5-(propan-2-yloxy)pyridin-3-yl][1,2,4]triazolo[4,3-*a*]quinoxaline **11**: LC/MS (*m*/*z*): 99% pure, t_R = 1.79 min, $[M+1]^+$: 419. ^{1}H NMR (500 MHz, CHLOROFORM-d) δ ppm 1.43 (d, *J*=6.1 Hz, 7 H), 2.32 - 2.42 (m, 4 H), 3.07 (s, 3 H), 3.48 (s, 2 H), 3.59 - 3.66 (m, 4 H), 4.69 (dt, *J*=12.1, 6.1 Hz, 1 H), 7.50 - 7.56 (m, 2 H), 7.66 (s, 1 H), 8.02 (d, *J*=8.1 Hz, 1 H), 8.52 (d, *J*=1.7 Hz, 1 H), 8.60 (d, *J*=2.6 Hz, 1 H).

1-[5-(2-methoxyethoxy)pyridin-3-yl]-4-methyl-8-(morpholin-4-ylmethyl)[1,2,4]triazolo[4,3-*a*]quinoxaline **13**: LC/MS (*m*/*z*): 97% pure, t_R = 1.39 min, $[M+1]^+$: 435. ^{1}H NMR (400 MHz, CHLOROFORM-d) δ ppm 2.36 (br. s., 4 H), 3.07 (s, 3 H), 3.39 - 3.52 (m, 5 H), 3.63 (br. s., 4 H), 3.82 (d, *J*=2.8 Hz, 2 H), 4.23 - 4.32 (m, 2 H), 7.53 (d, *J*=8.3 Hz, 1 H), 7.60 - 7.66 (m, 2 H), 8.01 (d, *J*=8.3 Hz, 1 H), 8.56 (s, 1 H), 8.67 (br. s., 1 H).

In vitro hPDE2 inhibitory activity of final compounds

Compound	***4***	***5***	***6***	***7***	***8***	***9***	***10***	***11***	***12***	***13***
pIC_{50}(hPDE2)	7.78	8.04	8.25	8.29	7.8	7.83	7.28	6.3	7.65	6.9
pIC_{50}(hPDE2)	7.95	8.09	8.06	8.35	7.8	7.96	7.47	6.61	7.72	6.92
pIC_{50}(hPDE2)	7.95	8.21	8.04	8.78	8.08	7.96	7.54	6.27	7.85	6.81
pIC_{50}(hPDE2)	8	8.51	8.4		8.18		7.59		7.99	7.05
pIC_{50}(hPDE2)	8.24				8.27		7.7		8.02	
pIC_{50}(hPDE2)					8.28				8.16	
pIC_{50}(hPDE2)					8.39				8.18	
pIC_{50}(hPDE2)									8.39	
pIC_{50}(hPDE2)										
Mean pIC_{50}	7.98	8.21	8.19	8.39	8.11	7.92	7.52	6.39	8.00	7.00
SD	0.17	0.21	0.17	0.04	0.23	0.08	0.16	0.19	0.25	0.19
n	5	4	4	3	7	3	5	3	8	4
Upper Limit pIC_{50}	8.13	8.42	8.35	8.6	8.29	8.00	7.65	6.61	8.17	7.17
Lower Limit pIC_{50}	7.84	8.01	8.02	8.26	7.94	7.83	7.38	6.18	7.82	6.83
IC_{50} (nM)	10.38	6.13	6.49	4.79	7.69	12.12	30.48	404.27	10.12	123.25
Upper Limit IC_{50}(nM)	14.49	9.86	9.54	5.48	11.47	14.73	41.78	660.20	15.05	148.71
Lower Limit IC_{50}(nM)	8.43	3.81	4.42	4.19	5.15	9.96	22.23	247.55	6.8	67.87

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