SUPPORTING INFORMATION

A novel and potent brain penetrant inhibitor of extracellular vesicle release

Running Title: Brain penetrant inhibitor of extracellular vesicle release

Camilo Rojas[1,2*], Michal Sala[8], Ajit G. Thomas[1], Amrita Datta Chaudhuri[3], Seung-Wan Yoo[3], Zhigang Li[3], Ranjeet Dash[1], Rana Rais [1,3], Norman Haughey[3]*, Radim Nencka[9]*, Barbara Slusher[1,3,4,5,6,7*]

[1]Johns Hopkins Drug Discovery, Departments of [2]Molecular and Comparative Pathobiology, [3]Neurology, [4]Psychiatry and Behavioral Sciences, [5]Neuroscience, [6]Medicine, [7]Oncology, Johns Hopkins School of Medicine, Baltimore, Maryland 21205, U.S.A., [8]Institute of Organic Chemistry and Biochemistry, Prague, Czech Republic

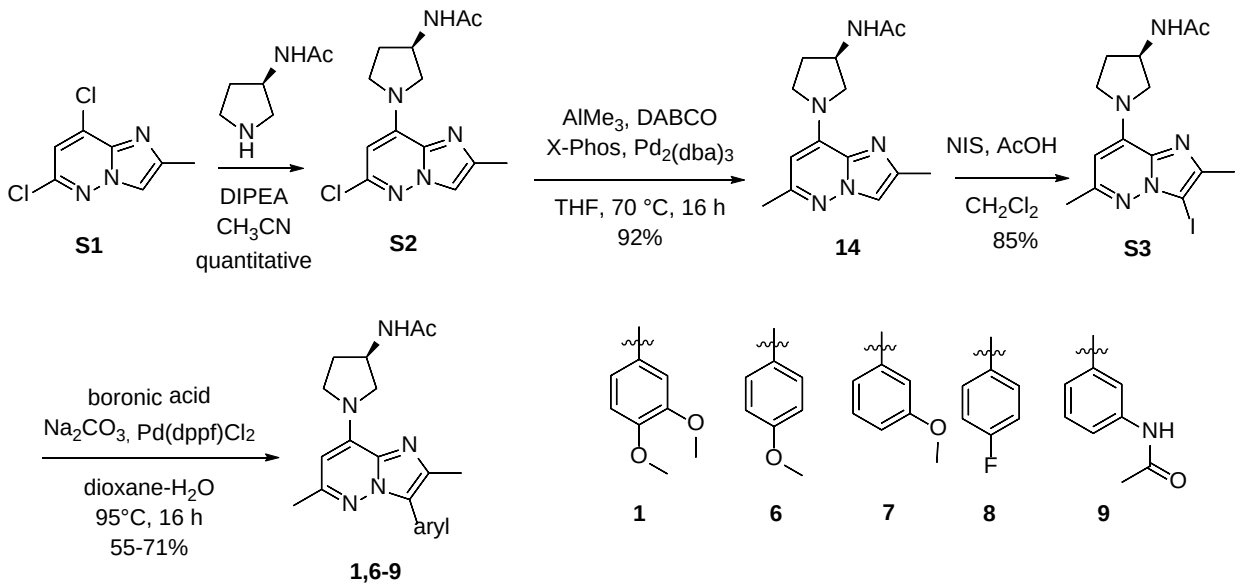
Supporting Information includes:

* Corresponding Authors
crojas2@jhmi.edu
nhaughey@jhmi.edu
radim.nencka@uochb.cas.cz
bslusher@jhmi.edu

Synthesis and authentication of compounds

Synthesis of compounds **1**, **6**, **7**, **8**, **9** (imidazo[1,2-*b*] pyridazine central core with modification on the southern part)

The synthetic strategy is outlined in Scheme 1. Dichloroderivative **S1** (Mejdrova et al., 2015) was used as a starting material. Halogen atom in position 8 was replaced by (*R*)-N-(pyrrolidin-3-yl) acetamide to give derivative **S2**. Second chlorine atom in position 6 was then converted to a methyl by treatment with trimethylaluminum-DABCO complex to afford compound **14**, which was in the next step iodinated by NIS in dichloromethane. Compound **S3** served as a starting material for synthesis of majority of the final compounds. Traditional Suzuki coupling with appropriate boronic acids was used to introduce aryl substituent to the position 3 to obtain final compounds **1**, **6**-**9**.

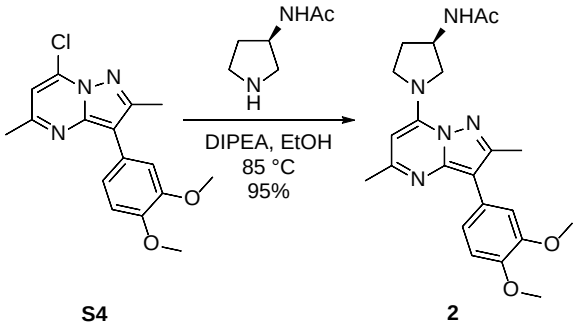


Scheme 1.

Compounds with modified central cores (**2**-**5**).

Synthesis of compound **2**.

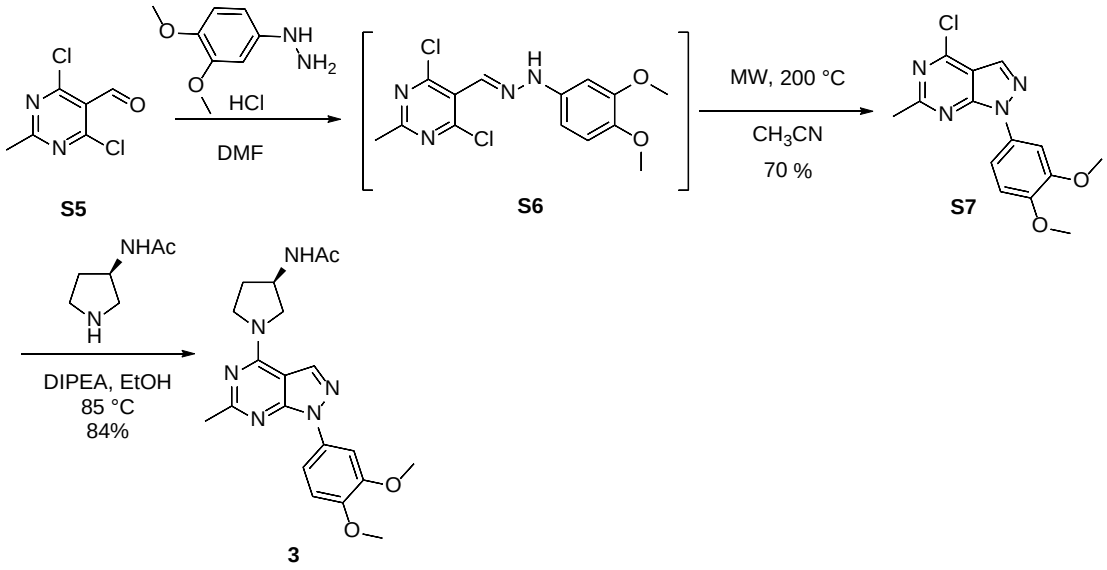
Nucleophilic aromatic substitution of the chlorine atom in compound **S4** (Mejdrova et al., 2015) with pyrazolo[1,5-*a*] pyrimidine central core was performed using (*R*)-N-(pyrrolidin-3-yl) acetamide in acetonitrile and lead to desired derivative **2** (Scheme **2**) in high yield (95%).



S4 **2**

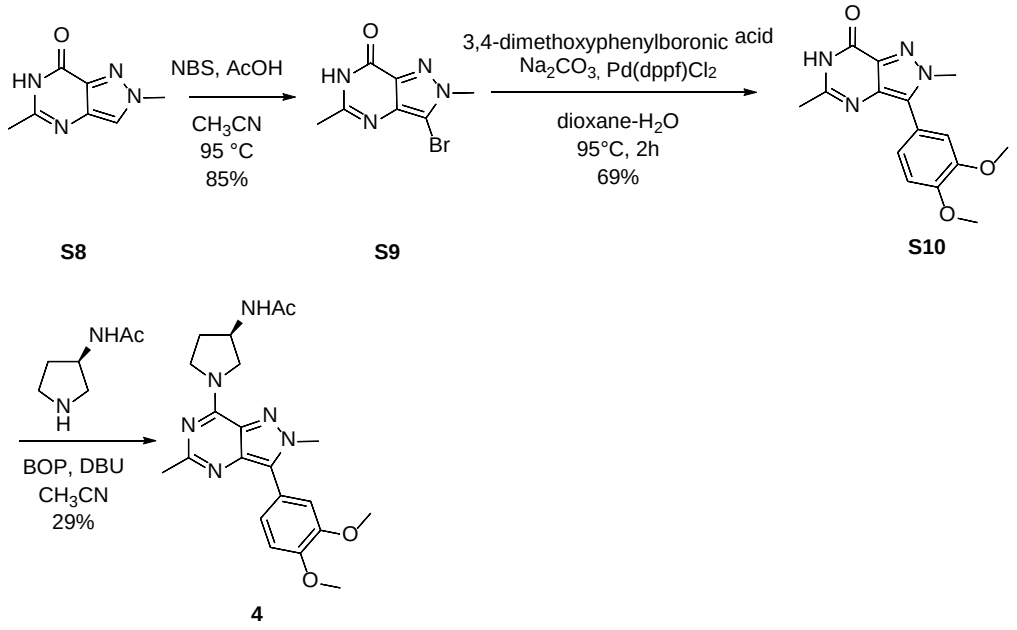
Scheme 2.

Synthesis of compound **3** - Synthesis of compound **3** with a pyrazolo[3,4-*d*] pyrimidine core started from a known aldehyde **S5** (Banno, Tanaka & Sasaki, 2012) which was treated with (3,4-dimethoxyphenyl) hydrazine hydrochloride in dimethylformamide (Scheme **3**). Obtained intermediate **S6** was precipitated from the reaction mixture and without further purification immediately used in the ring closure reaction by heating its solution in acetonitrile in microwave reactor at 200 °C (Babu, Morrill, Almstead & Moon, 2013). Obtained chloro derivative **S7** was then converted *via* nucleophilic aromatic substitution to the final compound **3**.



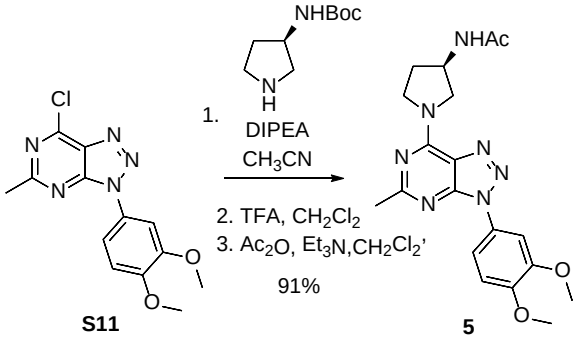
Scheme 3.

Synthesis of compound **4** - Compound **4** was prepared in three steps starting from oxo compound **S8,** (Squarcialupi et al., 2016) which was initially brominated with NBS, then the bromine was exchanged with 3,4-dimethoxyphenyl group under Suzuki cross-coupling conditions. Finally, pyrrolidine substituent was introduced by BOP-mediated reaction with (*R*)-N-(pyrrolidin-3-yl) acetamide.



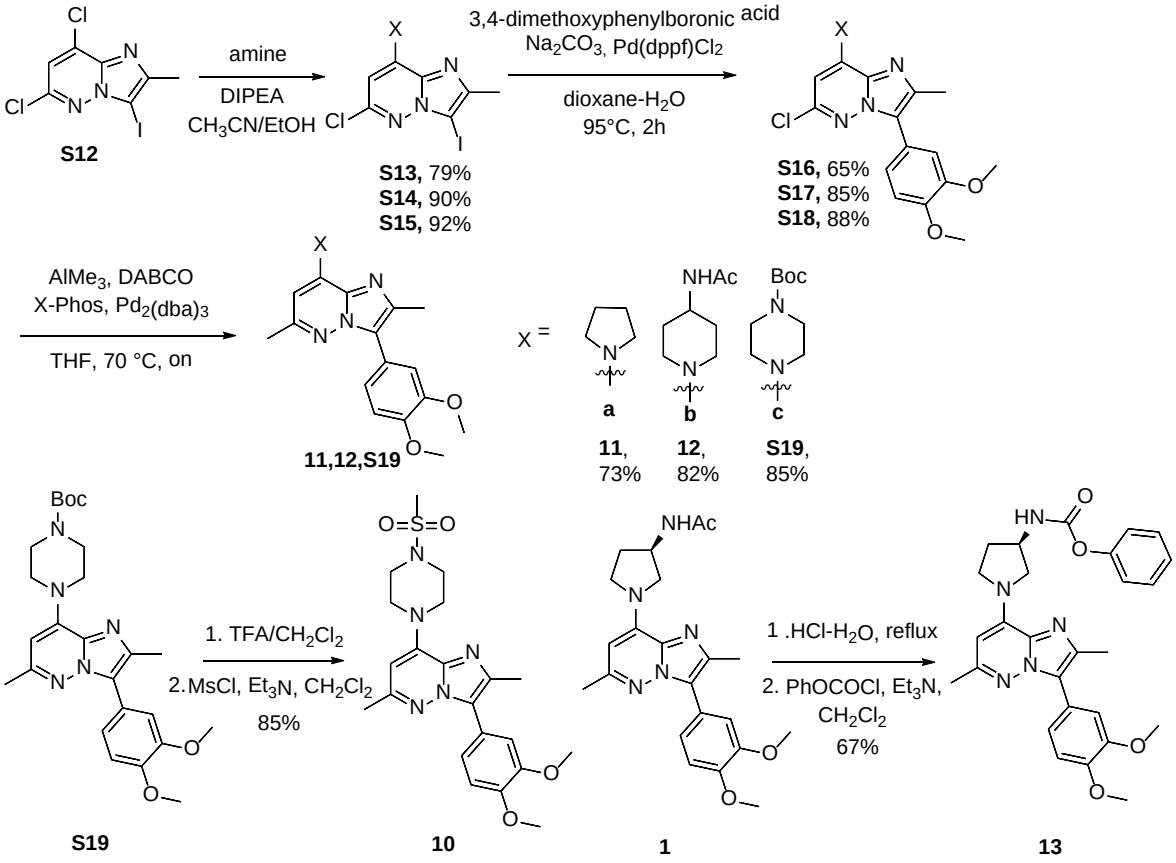
Scheme 4.

Synthesis of compound **5** - Compound **5** was synthesized by nucleophilic aromatic substitution of the chlorine atom in compound **S11** (Sala et al., 2016) with *tert*-butyl (3*R*)-pyrrolidin-3-ylcarbamate followed by acidic cleavage of the Boc-protecting group and immediate acetylation of the free amino function.



Scheme 5.

Compounds with modified northern part (**10**, **11**, **12**, **13**) - A slightly different strategy was used for preparation of compounds **10**-**13** with modified northern part (Scheme **6**). Firstly, chlorine atom at position 8 in a known compound **S12** (Mejdrova et al., 2015) was replaced with an appropriate amine (a - pyrrolidine, b - N-(piperidin-4-yl) acetamide, c - *tert*-butyl piperazine-1-carboxylate). Obtained derivatives **S13-S15** were then subjected to Suzuki cross coupling reaction with 3,4-dimethoxyphenylboronic acid (method A) and desired products **S16-S18** were isolated in high yields (65-88%). Second chlorine atom in position 6 was then exchanged for a methyl group by methylation reaction using trimethylaluminium-DABCO complex as a methylation agent. Final compounds **11** (73%) and **12** (82%) were obtained in high yields. Intermediate **S19** (85%) served as a starting material for preparation of the final compound **10**. Boc-protecting group was cleaved-off under acidic conditions (trifluoroacetic acid in dichlormethane) and free substrate was immediately mesylated with methane sulfonyl chloride in dichloromethane and triethylamine. Similar strategy was used to prepare compound **13**, the acetyl group in derivative **1** is cleaved-off under acidic conditions (aq. HCl, reflux) and the carbamate group is immediately installed by reaction of the free amino group with phenyl chloroformate.



S12

S13, 79%
S14, 90%
S15, 92%

S16, 65%
S17, 85%
S18, 88%

11,12,S19

11, 73%
12, 82%
S19, 85%

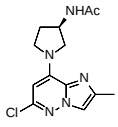
S19 **10** **1** **13**

Scheme 6.

AUTHENTICATION

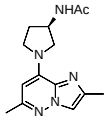
NMR spectra (δ, ppm; J, Hz) were measured on a Bruker Avance II-400 instrument (400.0 MHz for ^{1}H and 101 MHz for ^{13}C) in hexadeuterated dimethyl sulfoxide or $CDCl_3$ and referenced to the solvent signal (δ 2.50 and 39.70, respectively, 7.26 and 77.16). Mass spectra were measured on a LTQ Orbitrap XL (Thermo Fisher Scientific) using electrospray ionization (ESI). Column chromatography was performed on Silica gel 60 (Fluka) and thin-layer chromatography (TLC) on Silica gel 60 F254 foils (Merck). Solvents were evaporated at 2 kPa and bath temperature 30–60 °C; the compounds were dried at 13 Pa and 50 °C. For all the tested compounds satisfactory elemental analysis was obtained supporting > 95% purity. Optical rotation was measured on polarimeter Autopol IV (Rudolph Research Analytical) at 589 nm wavelength in chloroform or DMSO. UPLC samples were measured on Waters UPLC H-Class Core System, (column Waters Acquity UPLC BEH C18 1.7 µm, 2.1x100 mm), Waters Acquity UPLC PDA detector, Mass spectrometer Waters SQD2 and MassLynx Mass Spectrometry Software.

(*R*)-*N*-(1-(6-chloro-2-methylimidazo[1,2-*b*] pyridazin-8-yl) pyrrolidin-3-yl) acetamide (**S2**)



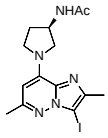
Mixture of starting material **S1** (600 mg, 2.97 mmol), (*R*)-*N*-(pyrrolidin-3-yl) acetamide (457 mg, 3.56 mmol), DIPEA (0.79 mL, 4.54 mmol) in acetonitrile (10 mL) was heated at 85 °C for 16 hours, then cooled down and evaporated. Residue was purified by column chromatography on silica gel (100 g, ethyl acetate → ethyl acetate-ethanol 7:1) to yield 864 mg (quantitative). Sample for analysis was obtained by crystallization from methanol. ^{1}H NMR (400 MHz, d6-DMSO) δ 1.81 (s, 3H), 1.83 – 1.96 (m, 1H), 2.07 – 2.23 (m, 1H), 2.30 (d, *J* = 0.9 Hz, 3H), 3.30 – 3.80 (br s, 1H),3.85 – 4.48 (br s + m, 3H), 5.91 (s, 1H), 7.75 (d, *J* = 0.9 Hz, 1H), 8.17 (d, *J* = 6.5 Hz, 1H). ^{13}C NMR (101 MHz, d6-DMSO) δ 14.5, 22.7, 92.7, 114.8, 132.0, 139.7, 142.0, 146.5, 169.4 (peaks on pyrrolidine ring were not detected). HRMS calcd for $C_{13}H_{17}ClN_5O$ m/z: 294.1116 $(M+H)^+$, found 294.1117.

(*R*)-*N*-(1-(2,6-dimethylimidazo[1,2-b] pyridazin-8-yl) pyrrolidin-3-yl) acetamide (**1A**)



To a solution of DABCO (448 mg, 4 mmol) in 15 mL freshly distilled THF, $AlMe_3$ (2M in hexanes, 4 mL, 8 mmol) was added dropwise and the mixture was stirred at r.t. for 30 minutes under argon atmosphere. A solution of **S2** (1.4 g, 4.77 mmol), $Pd_2(dba)_3$ (224 mg, 0.27 mmol) and X-Phos (234 mg, 0.49 mmol) in 80 mL freshly THF was subsequently added to the solution and the reaction mixture was stirred at 75 °C overnight under argon atmosphere. The mixture was cooled to 0 °C, quenched with sat. NH_4Cl (16 mL), diluted with acetone and ethyl acetate and filtered through Celite. The celite pad was thoroughly washed with acetone and ethyl acetate. The filtrate was evaporated and the residue was purified by silica gel column chromatography (200 g, chloroform-ethanol 10:1) yielding compound **14** (1.42 g, 92%) as an off-white solid. Analytical sample was obtained by crystallization from ethyl acetate. ^{1}H NMR (400 MHz, d6-DMSO) δ 1.81 (s, 3H), 1.84 – 1.93 (m, 1H), 2.08 – 2.19 (m, 1H), 2.27 (s, 3H), 2.28 (d, *J* = 0.8 Hz, 3H), 3.77 (br s, 3H), 4.03 (br s, 1H), 4.27 – 4.37 (m, 1H), 5.71 (s, 1H), 7.62 (d, *J* = 0.8 Hz, 1H), 8.16 (d, *J* = 6.6 Hz, 1H). ^{13}C NMR (101 MHz, d6-DMSO) δ 14.6, 21.6, 22.7, 30.5, 47.6, 48.8, 55.2, 93.6, 113.8, 132.6, 138.7, 141.0, 151.6, 169.4. HRMS calcd for $C_{14}H_{20}N_5O$ m/z: 274.1662 $(M+H)^+$, found 274.1663.

(*R*)-*N*-(1-(3-iodo-2,6-dimethylimidazo[1,2-*b*] pyridazin-8-yl) pyrrolidin-3-yl)acetamide (**S3**)

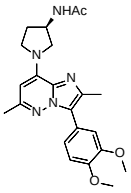


A solution of compound **14** (1.22 g, 4.5 mmol) in dichloromethane (50 mL) with acetic acid (0.19 mL) was cooled down to 0 °C then N-iodosuccinimide (1.1 g, 4.89 mmol) was added in one portion and the reaction mixture was stirred overnight (0 °C → r.t.). Reaction mixture was diluted with ethyl acetate (700 mL) and washed with sat. aq. $NaHCO_3$ (200 mL) and sat. aq. $Na_2S_2O_3$ (200 mL). Organic phase was dried over Na_2SO_4 and evaporated. The residue was purified by silica gel column chromatography (200 g, chloroform-ethyl acetate 20:1 → 15:1) which furnished compound **S3** (1.53 g, 85%) as an off-white solid. Recrystallization from hot acetone yielded an analytically pure sample. ^{1}H NMR (400 MHz, d6-DMSO) δ 1.81 (s, 3H), 1.83 – 1.93 (m, 1H), 2.09 – 2.19 (m, 1H), 2.31 (s, 3H), 2.34 (s, 3H), 3.77 (br s, 3H),4.01 (br s, 1H), 4.27 – 4.38 (m, 1H),5.84 (s, 1H), 8.16 (d, *J* = 6.6 Hz, 1H). ^{13}C NMR (101 MHz, d6-DMSO) δ 15.1, 21.5, 22.8, 30.5, 48.8, 55.3, 70.9, 94.7, 135.2, 140.8, 142.6, 152.2, 169.4 (one CH_2 peak was not detected). HRMS calcd for C_{14} $H_{19}IN_5O$ m/z: 400.0629 $(M+H)^+$, found 400.0630.

General procedure for Suzuki coupling with compound **S3** (method A)

Suspension of starting material **S3** (1 mmol), appropriate boronic acid (1.5 mmol), sodium carbonate (2 mmol) in dioxane-water (10 mL, 4:1) was three times purged argon. Then $Pd(dppf)_2Cl_2$ (0.1 mmol) was added and again flask was purged with argon. Reaction mixture was then heated to 95 °C overnight, cooled down and diluted with ethyl acetate or chloroform (300 mL). The suspension was dried over Na_2SO_4 and evaporated. Final compound was isolated by column chromatography and then crystallized.

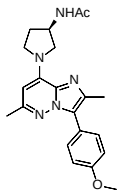
(*R*)-N-(1-(3-(3,4-dimethoxyphenyl)-2,6-dimethylimidazo[1,2-*b*] pyridazin-8-yl) pyrrolidin-3-yl) acetamide (**1**)



Prepared by method A from 3,4-dimethoxyphenylboronic acid in 64 %. Chromatography: $CHCl_3$-ethanol 15:1, crystallization from methanol. $[\alpha]_D^{20}$ = +6.5 (c 0.245, $CHCl_3$). ^{1}H NMR (400 MHz, d6-DMSO) δ 1.84 (s, 3H), 1.90 – 1.98 (m, 1H), 2.16 – 2.24 (m, 1H,), 2.30 (s, 3H), 2.41 (s, 3H), 3.76 – 3.86 (m, 8H), 3.93 (m, 1H), 4.12 (m, 1H), 4.35 – 4.41 (m, 1H, H-3), 5.78 (s, 1H, H-7'), 7.07 (d, *J* = 8.3 Hz, 1H), 7.19 (dd, *J* = 2.0, *J* = 8.3 Hz, 1H), 7.32 (d, *J* = 2.0 Hz, 1H), 7.94 (d, *J* = 6.1 Hz, 1H). ^{13}C NMR (101 MHz, d6-DMSO) δ 14.6, 21.2, 22.3, 30.3, 47.4, 48.5, 54.8, 55.7, 55.8, 93.6, 112.3, 113.9, 121.9, 122.4,

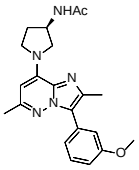
123.6, 131.7, 136.3, 141.0, 148.4, 148.5, 151.0, 169.0. HRMS calcd for $C_{22}H_{27}N_5O_3$ m/z: 410.2187 $(M+H)^+$, found 410.2186.

(*R*)-*N*-(1-(3-(4-methoxyphenyl)-2,6-dimethylimidazo[1,2-*b*] pyridazin-8-yl) pyrrolidin-3-yl) acetamide (**6**)



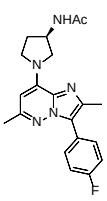
Prepared by method A from 4-methoxybenzeneboronic acid in 64 %. Chromatography: $CHCl_3$-ethanol 15:1, crystallization from ethyl acetate. $[\alpha]_D^{20}$ = +2.5 (c 0.258, $CHCl_3$). ^{1}H NMR (400 MHz, d6-DMSO) δ 1.82 (s, 3H), 1.86 – 1.95 (m, 1H), 2.10 – 2.22 (m, 1H), 2.27 (s, 1H), 2.36 (s, 1H), 3.63 – 3.97 (br s , 3H), 3.81 (s, 3H), 4.06 (br s, 1H), 4.35 (m, 1H), 5.79 (s, 1H), 7.06 (d, *J* = 8.4 Hz, 1H), 7.56 (d, *J* = 8.4 Hz, 1H), 8.17 (d, *J* = 6.6 Hz, 1H). ^{13}C NMR (101 MHz, d6-DMSO) δ 14.8, 21.7, 22.8, 30.5, 47.7, 48.8, 55.2, 55.3, 93.9, 113.9, 122.0, 123.8, 130.7, 131.9, 136.4, 141.1, 151.4, 158.6, 169.4. HRMS calcd for $C_{21}H_{26}N_5O_2$ m/z: 380.2081 $(M+H)^+$, found 380.3083.

(*R*)-*N*-(1-(3-(3-methoxyphenyl)-2,6-dimethylimidazo[1,2-*b*] pyridazin-8-yl)pyrrolidin-3-yl)acetamide (**7**)



Prepared by method A from 3-methoxybenzeneboronic acid in 71 %. Chromatography: $CHCl_3$-ethanol 15:1, crystallization from acetone. $[\alpha]_D^{20}$ = +3.6 (c 0.276, $CHCl_3$). ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.82 (s, 3H), 1.85 – 1.95 (m, 1H), 2.11 – 2.22 (m, 1H), 2.40 (s, 3H), 3.80 (s, 3H),3.61 – 3.97 (br s and s, 6H), 3.98 – 4.20 (br s, 1H), 4.30 – 4.39 (m, 1H), 5.82 (s, 1H), 6.94 (ddd, *J* = 8.2, 2.6, 1.0 Hz, 1H), 7.29 – 7.17 (m, 2H), 7.40 (t, *J* = 8.0 Hz, 1H), 8.17 (d, *J* = 6.6 Hz, 1H). ^{13}C NMR (101 MHz, DMSO) δ 15.0, 21.7, 22.8, 30.4, 39.1, 39.3, 39.5, 39.7, 39.9, 40.1, 40.3, 47.7, 48.8, 55.2, 94.2, 112.8, 115.0, 121.6, 123.7, 129.4, 131.0, 132.2, 137.2, 141.2, 151.6, 159.1, 169.4.

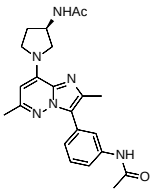
(*R*)-*N*-(1-(3-(4-fluorophenyl)-2,6-dimethylimidazo[1,2-*b*] pyridazin-8-yl)pyrrolidin-3-yl)acetamide (**8**)



Prepared by method A from 4-fluorobenzeneboronic acid in 55% yield. Chromatography: $CHCl_3$-ethanol 15:1, crystallization from acetone. $[\alpha]_D^{20}$ = +9.3 (c 0.259, $CHCl_3$). ^{1}H NMR (400 MHz, d6-DMSO) δ 1.82 (s, 1H), 1.86 – 1.96 (m, 1H), 2.11 – 2.23 (m, 1H), 2.28 (s, 1H), 2.37 (s, 1H), 4.31 – 4.39 (m, 1H), 5.82 (s, 1H), 7.39

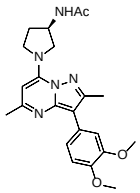
– 7.25 (m, 1H), 7.77 – 7.62 (m, 1H), 8.17 (d, *J* = 6.6 Hz, 1H). ^{13}C NMR (101 MHz, d6-DMSO) δ 14.8, 21.7, 22.8, 30.5, 47.7, 48.8, 55.3, 94.2, 115.3 (d, *J* = 21.4 Hz), 123.00, 126.1 (d, *J* = 3.2 Hz), 131.4 (d, *J* = 8.1 Hz), 132.2, 137.0, 141.1, 151.6, 161.4 (d, *J* = 244.7 Hz), 169.4. HRMS calcd for $C_{20}H_{23}N_5OF$ m/z: 368.1881 $(M+H)^+$, found 368.1882.

(*R*)-*N*-(1-(3-(3-acetamidophenyl)-2,6-dimethylimidazo[1,2-*b*]pyridazin-8-yl)pyrrolidin-3-yl)acetamide (**9**)



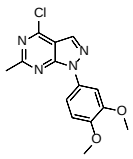
Prepared by method A from 3-*N*-acetylbenzeneboronic acid in 71 %. Chromatography: $CHCl_3$-ethanol 10:1 → 7:1, crystallization from acetone. $[\alpha]_D^{20}$ = +15.4 (c 0.234, $CHCl_3$). ^{1}H NMR (401 MHz, DMSO-d_6) δ 1.82 (s, 3H), 1.85 – 1.97 (m, 1H), 2.06 (s, 3H), 2.10 – 2.22 (m, 1H), 2.28 (s, 3H), 2.38 (s, 3H), 3.41 – 4.28 (2 x br s, 4H), 4.27 – 4.41 (m, 1H), 5.82 (s, 1H), 7.28 – 7.45 (m, 2H), 7.62 (dt, *J* = 8.0, 1.7 Hz, 1H), 7.83 (t, *J* = 1.9 Hz, 1H), 8.17 (d, *J* = 6.6 Hz, 1H), 10.02 (s, 1H). ^{13}C NMR (101 MHz, DMSO) δ 15.0, 21.7, 22.8, 24.2, 30.4, 47.8, 48.8, 55.3, 94.2, 118.1, 119.9, 123.9, 124.1, 128.6, 130.1, 132.2, 137.0, 139.4, 141.1, 151.6, 168.6, 169.4. HRMS calcd for $C_{21}H_{26}N_5O_2$ m/z: 380.2081 $(M+H)^+$, found 380.2082.

(*R*)-*N*-(1-(3-(3,4-dimethoxyphenyl)-2,5-dimethylpyrazolo[1,5-a] pyrimidin-7-yl) pyrrolidin-3-yl) acetamide (**2**)



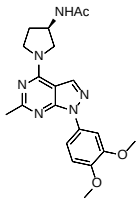
Chloro derivative **S4** (100 mg, 0.32 mmol), (*R*)-*N*-(pyrrolidin-3-yl) acetamide (51 mg, 0.4 mmol), DIPEA (86 µL, 0.5 mmol) in acetonitrile (4 mL) was heated at 85 °C (bath) for 16 hours, then cooled down and evaporated. Residue was purified by column chromatography on silica gel (50 g, ethyl acetate → ethyl acetate- ethanol 10:1) to yield 104 mg (75%). Solids were then crystalized from ethyl acetate. $[\alpha]_D^{20}$ = +8.5 (c 0.234, $CHCl_3$). ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.82 (s, 3H), 1.86 – 1.96 (m, 1H), 2.09 – 2.20 (m, 1H), 2.35 (s, 3H), 2.47 (s, 3H), 3.78 (s, 3H), 3.79 (s, 3H), 3.83 – 399 (m, 3H), 4.09 – 4.17 (m, 1H), 4.30 – 4.38 (m, 1H), 5.85 (s, 1H), 7.00 (d, *J* = 8.5 Hz, 1H), 7.21 (dd, *J* = 8.3, 2.0 Hz, 1H), 7.40 (d, *J* = 2.0 Hz, 1H), 8.18 (d, *J* = 6.6 Hz, 1H). ^{13}C NMR (101 MHz, DMSO) δ 14.8, 22.7, 24.7, 30.3, 48.5, 48.6, 55.7, 55.8, 55.9, 89.3, 104.5, 112.2, 112.7, 120.6, 126.3, 146.6, 146.7, 148.0, 148.6, 149.5, 158.6, 169.4. HRMS calcd for $C_{22}H_{27}N_5O_3$ m/z: 410.2187 $(M+H)^+$, found 410.2185.

4-chloro-1-(3,4-dimethoxyphenyl)-6-methyl-1*H*-pyrazolo[3,4-*d*] pyrimidine (**S7**)



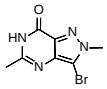
To a solution of aldehyde **S5** (500 mg, 2.6 mmol) in DMF (9 mL) was added (3,4-dimethoxyphenyl) hydrazine hydrochloride (511 mg, 2.48 mmol) at r.t. and reaction mixture was stirred for 90 min. Reaction was quenched by adding ice (10 g) and solution of sodium bicarbonate (330 mg) in water (5 mL). Then was added water and precipitated solid was filtered off and washed with water. Solids were dried in vacuum oved at 40 °C overnight and then used without further purification. Solids were suspended in acetonitrile (4 mL) and this reaction mixture was heated in microwave reactor for 20 min at 200 °C. Reaction mixture was poured to satd. sodium bicarbonate (100 mL) and water phase was extracted with ethyl acetate (2 x 200 mL). Combined organic fractions were dried over sodium sulfate and evaporated. Residue was chromatographed on silica gel (100 g, toluene-ethyl acetate 5:1) to obtain 400 mg (64%) of the product as yellowish solid (product was contaminated with inseparable impurity). UPLC-MS: t = 4.65 (M+H, 305.2/307.2). ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.73 (s, 3H), 3.83 (s, 4H), 3.84 (s, 3H), 7.15 (dd, *J* = 8.2, 0.9 Hz, 1H), 7.72 – 7.44 (m, 2H), 8.59 (s, 1H). ^{13}C NMR (101 MHz, DMSO) δ 25.9, 55.9, 56.0, 106.3, 112.0, 112.4, 113.9, 131.3, 133.4, 148.1, 149.1, 153.3, 153.6, 165.6.

(*R*)-N-(1-(1-(3,4-dimethoxyphenyl)-6-methyl-1*H*-pyrazolo[3,4-*d*] pyrimidin-4-yl)pyrrolidin-3-yl)acetamide (**3**)



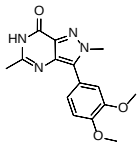
Chloroderivative **S7** (100 mg, 0.33 mmol), (*R*)-*N*-(pyrrolidin-3-yl) acetamide (51 mg, 0.4 mmol), DIPEA (86 µL, 0.5 mmol) in acetonitrile (4 mL) was heated at 85 °C (bath) for 16 hours, then cooled down and evaporated. Residue was purified by column chromatography on silica gel (75 g, ethyl acetate → ethyl acetate – acetone - Ethanol-H_2O 20:3:0.6:0.4) to yield 110 mg (84%). Solids were then crystalized from ethyl acetate. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.02 (s, 3H), 2.10 – 2.44 (br s, 2H), 2.58 (s, 3H), 3.71 (br s, 1H), 3.79 – 3.89 (m, 1H), 3.92 (s, 3H), 3.97 (s, 3H), 4.66 (br s, 1H), 5.96 (d, *J* = 7.1 Hz, 1H), 6.97 (d, *J* = 8.5 Hz, 1H), 7.83 – 7.51 (m, 2H), 7.93 (s, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 23.4, 26.7, 56.2, 56.3, 100.5, 106.2, 111.3, 114.0, 132.8, 133.3, 147.6, 149.3, 154.8, 155.3, 170.2 (peaks on pyrrolidine ring were not detected). HRMS calcd for $C_{20}H_{25}N_6O_3$ m/z: 397.1983 $(M+H)^+$, found 397.1979.

3-bromo-2,5-dimethyl-2,6-dihydro-7*H*-pyrazolo[4,3-*d*] pyrimidin-7-one (**S9**)



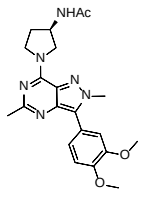
Starting material **S8** (600 mg, 3.65 mmol) was combined with NBS (783 mg, 4.4 mmol) in acetonitrile (20 mL) and acetic acid (60 µL) at r.t.. Reaction mixture was then heated in an oil bath (95 °C) for 4 h. After cooling down, precipitated solid was filtered off and washed with acetonitrile, ether and dried on air. It was obtained 754 mg (85%) of the product. UPLC-MS: t = 2.85 (M+H, 243.3/245.0). ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.29 (s, 3H), 4.04 (s, 3H), 11.93 (s, 1H). ^{13}C NMR (101 MHz, DMSO) δ 21.3, 39.3, 107.9, 134.6, 136.9, 153.3, 156.6.

3-(3,4-dimethoxyphenyl)-2,5-dimethyl-2,6-dihydro-7*H*-pyrazolo[4,3-*d*] pyrimidin-7-one (**S10**)



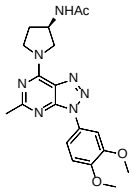
Prepared by method A from 3,4-dimethoxyphenylboronic acid in 69 %. Chromatography: ethyl acetate → ethyl acetate – acetone – ethanol - H_2O 20:3:0.6:0.4). UPLC-MS: t = 3.28 (M+H, 301.2). ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.26 (s, 3H), 3.81 (s, 3H), 3.83 (s, 3H), 4.05 (s, 3H), 7.13 (d, *J* = 8.4 Hz, 1H), 7.18 (dd, *J* = 8.2, 2.0 Hz, 1H), 7.25 (d, *J* = 2.0 Hz, 1H), 11.78 (s, 1H). ^{13}C NMR (101 MHz, DMSO) δ 21.4, 39.3*, 55.8, 55.9, 112.0, 113.3, 120.3, 122.6, 133.8, 135.3, 135.8, 148.8, 149.5, 152.0, 157.1.

(*R*)-N-(1-(3-(3,4-dimethoxyphenyl)-2,5-dimethyl-2*H*-pyrazolo[4,3-*d*] pyrimidin-7-yl)pyrrolidin-3-yl)acetamide (**4**)



Starting material **S10** (299 mg, 1 mmol) was suspended in acetonitrile (10 mL) and sequentially was added (*R*)-*N*-(pyrrolidin-3-yl) acetamide (192 mg, 1.5 mmol), BOP reagent (575 mg, 1.3 mmol) and then DBU (0.23 mL, 1.5 mmol) dropwise at r.t.. Reaction mixture was heated to 65 °C (oil bath) for 4 h and then cooled down and evaporated. Residue was chromatographed on silica gel column (200 g, ethyl acetate → ethyl acetate-acetone-ethanol-H_2O 20:3:0.6:0.4). Fractions containing the product were evaporated and chromatographed again (100 g, $CHCl_3$-ethanol 7:1). Obtained solid was twice crystalized (1. ethyl acetate, 2. acetone). It was obtained 120 mg (29%) of the product. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.72 (br s, 1H), 2.00 (s, 3H), 2.27 (s, 1H), 2.50 (s, 3H), 3.80 – 4.35 (2 x br s, 4H), 3.94 (s, 3H), 4.11 (s, 3H), 4.64 (br s, 1H), 6.09 (br s, 1H), 7.00 (d, *J* = 8.3 Hz, 1H), 7.13 (dd, *J* = 8.2, 2.0 Hz, 1H), 7.19 (d, *J* = 2.0 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 23.5, 26.6, 39.5, 56.2, 56.3, 111.7, 113.2, 121.3, 122.7, 133.8, 149.4, 149.8, 152.9, 161.9, 170.1 (peaks on pyrrolidine ring were not detected). HRMS calcd for $C_{21}H_{27}N_6O_3$ m/z: 411.2139 $(M+H)^+$, found 411.2135.

N-{(3*R*)-1-[3-(3,4-dimethoxyphenyl)-3*H*-[1,2,3] triazolo[4,5-*d*]pyrimidin-7-yl]pyrrolidin-3-yl}acetamide (**5**)

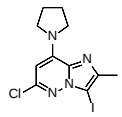


To a mixture of 7-chloro-3-(3,4-dimethoxyphenyl)-5-methyl-3*H*-[1,2,3] triazolo[4,5-*d*]pyrimidine **S11** (961 mg, 3.14 mmol), DIPEA (0.855 mL, 4.91 mmol) in acetonitrile (15 mL) was added *tert*-butyl (3*R*)-pyrrolidin-3-ylcarbamate (651 mg, 3.45 mmol) at r.t. and reaction mixture was heated at 85 °C for 2 hours, cooled down and evaporated. The residue was chromatographed on silica gel column (100 g, toluene-ethyl acetate 1:1). Fractions containing product were evaporated and dissolved in a mixture of trifluoroacetic acid and dichloromethane (44 mL, 1:10, v/v). Reaction mixture was stirred at r.t. for 16 hours, evaporated, two times co-evaporated with acetonitrile (2 x 40 mL) and redissolved in acetonitrile. To this solution was sequentially added Et_3N (1.96 mL, 14 mmol), catalytic amount of DMAP and acetic anhydride (0.49 mL, 5.2 mmol). Reaction mixture was stirred at r.t. for 2 hours and then methanol (1 mL) was added and mixture was evaporated. Residue was purified by column chromatography (200 g, toluene-acetone 1:2) to obtain 1.1 g (91 %) of the product. Obtained solid was recrystallized from ethyl acetate. $[\alpha]_D^{20}$ = +2.3 (c 0.258, $CHCl_3$). NMR spectrum showed signals of two rotamers. ^{1}H NMR (400 MHz, d6-DMSO) δ 1.83 (2 x s, 3H), 1.87 – 1.98 and 2.00 – 2.10 (2 x m, 1H), 2.12 – 2.24 and 2.24 – 2.37 (2 x m, 1H), 2.50* (s, 3H, covered by DMSO, observed in HSQC), 3.62 – 3.70 (m, 0.5H), 3.73 – 3.89 (m and 2 x s, 7.5H), 4.01 – 4.09 (m, 0.5H), 4.18 – 4.24 (m, 1H), 4.24 – 4.31 (m, 0.5H), 4.32 – 4.39 (m, 0.5H), 4.43 – 4.50 (m, 0.5H), 7.15 – 7.25 (m, 1H), 7.59 – 7.69 (m, 2H), 8.22 and 8.26 (2 x d, *J* = 6.5 Hz, 1H). ^{13}C NMR (101 MHz, d6-DMSO) δ 22.8, 26.4, 26.5, 29.5 and 31.2, 45.7 and 47.6, 47.7 and 49.4, 52.9 and 54.5, 56.00, 106.2 and 106.3, 112.1, 114.1 and 114.2, 124.1 and 124.2, 129.1 and 129.2, 148.9 (2x), 149.3, 149.8 and 149.9, 152.2 and 152.3, 166.4 (2x), 169.6 (2x). HRMS calcd for $C_{19}H_{27}N_7O_3$ m/z: 398.1935 $(M+H)^+$, found 398.1900.

General method B for preparation of the intermediates **S13, S14, S15**.

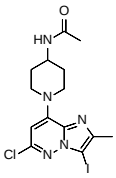
Compound **S12** (500 mg, 1.53 mmol), appropriate amine (2 mmol), DIPEA (3 mmol for amines) was dissolved in acetonitrile (10 mL). Reaction mixture was heated at 85 °C for 16 hours and cooled down. Products were separated by column chromatography or directly as precipitated solids were filtered-off and washed with acetonitrile.

6-chloro-3-iodo-2-methyl-8-(pyrrolidin-1-yl) imidazo[1,2-*b*] pyridazine (**S13**)



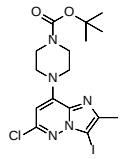
Yield: 79%. Chromatography: toluene: ethyl acetate 20:1. UPLC-MS: t = 5.28 (M+H, 363.1/365.1).

N-(1-(6-chloro-3-iodo-2-methylimidazo[1,2-*b*] pyridazin-8-yl) piperidin-4-yl) acetamide (**S14**)



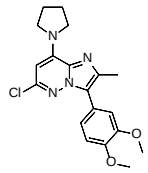
Yield: 90%, precipitated solid was filtered-off and used without further purification. UPLC-MS: t = 4.26 (M+H, 434.2/436.1).

tert-butyl 4-(6-chloro-3-iodo-2-methylimidazo[1,2-*b*] pyridazin-8-yl) piperazine-1-carboxylate (**S15**)



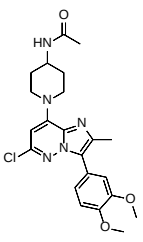
Yield: 92%. Chromatography: toluene: ethyl acetate 10:1. UPLC-MS: t = 5.46 (M+H, 478.2/480.2).

6-chloro-3-(3,4-dimethoxyphenyl)-2-methyl-8-(pyrrolidin-1-yl) imidazo[1,2-*b*] pyridazine (**S16**)



Prepared by method A from 3,4-dimethoxybenzeneboronic acid. Yield: 65%. Chromatography: $CHCl_3$:acetone 40:1. UPLC-MS: t = 5.03 (M+H, 373.2/375.2). ^{1}H NMR (400 MHz, d6-DMSO): δ 1.95 (4H, m), 2.32 (s, 3H), 3.41 (br s, 2H), 4.15 (br s, 2H), 5.97 (s, 1H). ^{13}C NMR (101 MHz, d6-DMSO) δ 15.1, 72.4, 93.6, 134.7, 141.8, 143.5, 147.1 (CH_2 peaks were not detected).

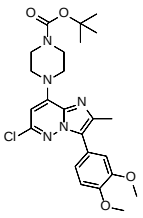
N-(1-(6-chloro-3-(3,4-dimethoxyphenyl)-2-methylimidazo[1,2-*b*] pyridazin-8-yl)piperidin-4-yl) acetamide (**S17**)



Prepared by method A from 3,4-dimethoxybenzeneboronic acid. Yield: 85%. Chromatography: $CHCl_3$:ethanol 20:1. UPLC-MS: t = 4.14 (M+H, 444.3/446.3). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.48 – 1.68 (m, 1H), 2.00 (s, 3H), 2.05 – 2.23 (m, 2H), 3.13 – 3.36 (m, 2H), 3.92 (s, 3H),3.94 (s, 3H),4.07 – 4.17 (m, 1H), 4.90 (d, *J* = 13.5 Hz, 2H), 5.39 (d, *J* = 8.0 Hz, 1H), 6.09 (s, 1H), 6.99 (d, *J* = 8.3 Hz, 1H), 7.15 – 7.21

(m, 1H), 7.23 (d, *J* = 2.0 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 15.1, 23.7, 32.1, 46.8, 47.4, 56.1, 56.1, 77.5, 96.7, 111.3, 112.9, 121.5, 122.5, 125.5, 132.1, 137.9, 144.4, 147.1, 148.9, 149.0, 169.6.

tert-butyl 4-(6-chloro-3-(3,4-dimethoxyphenyl)-2-methylimidazo[1,2-*b*] pyridazin-8-yl) piperazine-1-carboxylate (**S18**)

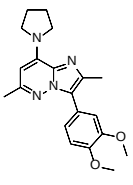


Prepared by method A from 3,4-dimethoxybenzeneboronic acid. Yield: 88%. Chromatography: $CHCl_3$: acetone 20:1. UPLC-MS: t = 5.30 (M+H, 484.3/490.4). ^{1}H NMR (400 MHz, $CDCl_3$): 1.43 (s, 9H), 2.40 (s, 3H), 3.51 (t, *J* = 5.2 Hz, 4H), 3.78 (s, 3H), 3.82 (s, 3H), 4.09 (t, *J* = 5.0 Hz, 4H), 6.41 (s, 1H),7.10 (d, *J* = 8.4 Hz, 1H), 7.15 (dd, *J* = 8.3, 1.9 Hz, 1H), 7.18 (d, *J* = 1.9 Hz, 1H). ^{13}C NMR (101 MHz, DMSO) δ 14.7, 28.2, 47.2, 55.7, 55.8, 79.4, 95.6, 111.9, 113.3, 120.8, 122.3, 124.8, 131.2, 137.1, 143.7, 146.6, 148.6, 148.9, 154.0.

General procedure for methylation of position 6 (method C)

To a solution of DABCO (98 mg, 0.88 mmol) in 3 mL freshly distilled THF, $AlMe_3$ (2M in hexanes, 0.89 mL, 1.78 mmol) was added dropwise and the mixture was stirred at r.t. for 30 minutes under argon atmosphere. A solution of chloro derivative **S16**/**S17**/**S18** (1 mmol), $Pd_2(dba)_3$ (50 mg, 0.06 mmol) and X-Phos (57 mg, 0.12 mmol) in 15 mL freshly THF was subsequently added to the solution and the reaction mixture was stirred at 75 °C overnight under argon atmosphere. The mixture was cooled to 0 °C and quenched with sat. NH_4Cl (4 mL), diluted with acetone and ethyl acetate and filtered through Celite. The Celite pad was thoroughly washed with acetone and ethyl acetate. The filtrate was evaporated and the residue was purified by silica gel column chromatography (120 g). Solids were then crystalized from appropriate solvent.

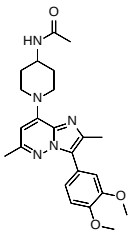
3-(3,4-dimethoxyphenyl)-2,6-dimethyl-8-(pyrrolidin-1-yl) imidazo[1,2-*b*]pyridazine (**11**)



Yield: 73%. Chromatography: $CHCl_3$-acetone 30:1. Crystallization: ethyl acetate. ^{1}H NMR (400 MHz, d6-DMSO) δ 1.91– 2.01 (m, 4H), 2.39 (s, 3H), 2.28 (s, 3H), 3.78 (s, 3H), 3.80 (br s, 4H), 3.81 (s, 3H), 5.76 (s, 1H), 7.29 (d, *J* = 2.0 Hz, 1H), 7.17 (dd, *J* = 8.3, 2.0 Hz, 1H), 7.07 (d, *J* = 8.4 Hz, 1H). ^{13}C NMR (101 MHz, d6-DMSO) δ 15.0,

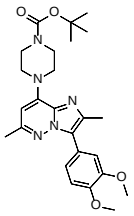
21.7, 25.1, 49.6, 55.7, 55.7, 93.7, 111.8, 113.3, 122.0, 122.3, 123.8, 132.0, 136.4, 141.1, 148.2, 151.3. HRMS calcd for $C_{20}H_{25}N_4O_2$ m/z: 353.1972 $(M+H)^+$, found 353.1972.

N-(1-(3-(3,4-dimethoxyphenyl)-2,6-dimethylimidazo[1,2-*b*] pyridazin-8-yl) piperidin-4-yl) acetamide (**12**)



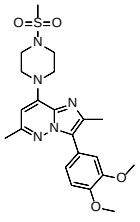
Yield: 82%. Chromatography: $CHCl_3$-ethanol 15:1. Crystallization: acetone. ^{1}H NMR (400 MHz, d6-DMSO) δ 1.39 – 1.54 (m, 2H), 1.79 – 1.89 (m, 3H), 1.81 (s, 3H), 3.23 (ddd, *J* = 13.8, 11.5, 2.6 Hz, 2H), 4.77 (d, *J* = 13.8 Hz, 2H), 6.24 (s, 1H), 7.07 (d, *J* = 8.4 Hz, 1H), 7.16 (dd, *J* = 8.4, 2.0 Hz, 1H), 7.26 (d, *J* = 2.0 Hz, 1H), 7.83 (d, *J* = 8.4 Hz, 1H). ^{13}C NMR (101 MHz, d6-DMSO) δ 15.2, 22.0, 23.3, 31.6, 47.0, 56.0, 97.4, 112.1, 113.7, 122.3, 122.4, 124.2, 132.4, 136.5, 143.3, 148.7, 148.6, 151.7, 168.8. HRMS calcd for $C_{23}H_{29}N_5O_3Na$ m/z: 446.2163 $(M + Na)^+$, found 446.2161.

tert-butyl 4-(3-(3,4-dimethoxyphenyl)-2,6-dimethylimidazo[1,2-*b*]pyridazin-8-yl)piperazine-1-carboxylate (**S19**)



Yield: 85%. Chromatography: $CHCl_3$-acetone 10:1. Crystallization: ethyl acetate. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.50 (s, 9H), 2.41 (s, 3H), 2.51 (s, 3H), 3.63 – 3.70 (m, 4H), 3.79 – 4.03 (br m, 2 x s 10H), 5.96 (s, 1H), 6.99 (d, *J* = 8.4 Hz, 1H), 7.22 (dd, *J* = 8.3, 2.0 Hz, 1H), 7.30 (d, *J* = 2.0 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 15.0, 22.3, 28.6, 47.9, 56.1, 80.2, 97.8, 111.2, 113.1, 122.4, 122.5, 124.7, 132.8, 137.5, 144.1, 148.7, 148.8, 151.6, 154.9. HRMS calcd for $C_{25}H_{34}N_5O_4$ m/z: 468.2605 $(M+H)^+$, found 468.2599.

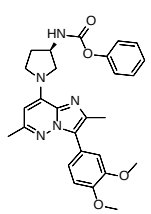
3-(3,4-dimethoxyphenyl)-2,6-dimethyl-8-(4-(methyl sulfonyl) piperazin-1-yl) imidazo[1,2-*b*] pyridazine (**10**)



Compound **S19** (178 mg, 0.38 mmol) was dissolved in dichloromethane (7 mL) and trifluoroacetic acid was added (1 mL) and reaction mixture was stirred for 16 h, evaporated and residue was co-evaporated with acetonitrile (3 x 10 mL). Oily intermediate was dissolved in acetonitrile (15 mL) and Et_3N (0.27 mL, 1.91 mmol) was added followed by addition of mesyl chloride (45 µL, 0.57 mmol). Reaction mixture was stirred for 1 h at r.t. and evaporated. Residue was chromatographed on silica gel column (100 g, $CHCl_3$-ethanol 30:1) and residue was crystalized from ethyl acetate. It was

obtained 144 mg (85%) of the product. ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.35 (s, 3H), 2.41 (s, 3H), 2.93 (s, 3H), 3.29 (t, *J* = 5.1 Hz, 5H), 3.78 (s, 3H), 3.82 (s, 3H), 4.07 (t, *J* = 4.9 Hz, 4H), 6.31 (s, 1H), 7.08 (d, *J* = 8.5 Hz, 1H), 7.16 (dd, *J* = 8.3, 2.0 Hz, 1H), 7.25 (d, *J* = 2.0 Hz, 1H). ^{13}C NMR (101 MHz, DMSO) δ 14.8, 21.7, 34.0, 45.2, 47.0, 55.7, 55.7, 97.9, 111.8, 113.4, 121.8, 122.1, 124.0, 131.9, 136.5, 142.8, 148.5, 148.5, 151.5.

phenyl (*R*)-(1-(3-(3,4-dimethoxyphenyl)-2,6-dimethylimidazo[1,2-*b*] pyridazin-8-yl)pyrrolidin-3-yl)carbamate (**13**)



Acetamide **1** (165 mg, 0.4 mmol) was heated to reflux overnight in a mixture of conc. HCl (3 mL) and water (3 mL). Reaction mixture was evaporated to dryness, co-evaporated with ethanol (2 x 15 mL) and dried overnight on vacuum. Intermediate was dissolved in dichloromethane (10 mL) and triethylamine (0.3 ml, 2 mmol) and catalytic amount of DMAP was added. Reaction mixture was cooled to 0 °C and then was slowly added phenyl chloroformate (76 µL, 0.6 mmol). Reaction mixture was stirred at r.t. for 16 h and then evaporated. Residue was chromatographed on silica gel column (100 g, toluene: ethyl acetate 3:1) and residue was crystalized from ethyl acetate. It was obtained 131 mg (67%) of the product. $[\alpha]_D^{20}$ = +11.5 (c 0.234, $CHCl_3$). ^{1}H NMR (400 MHz, d6-DMSO) δ 2.00 – 2.09 (m, 1H), 2.19 – 2.28 (m, 1H), 2.30 (s, 3H), 2.40 (s, 3H), 3.79 (s, 3H), 3.81 (s, 3H), 3.92 (br s, 3H), 4.15 (br s, 1H), 4.23 – 4.31 (m, 1H), 5.82 (s, 1H), 7.07 (d, *J* = 8.4 Hz, 1H), 7.10 – 7.25 (m, 4H), 7.28 (d, *J* = 2.0 Hz, 1H), 7.34 – 7.43 (m, 2H), 8.19 (d, *J* = 6.5 Hz, 1H). ^{13}C NMR (101 MHz, d6-DMSO) δ 14.8, 21.5, 30.2, 50.5, 55.1*, 55.2*, 55.6, 93.8, 111.6, 113.1, 121.1, 121.8, 122.0, 123.7, 125.0, 129.3, 131.7, 136.4, 141.0, 148.1, 148.2, 150.9, 151.2, 154.5. HRMS calcd for $C_{27}H_{30}N_5O_4$ m/z: 488.2292 $(M+H)^+$, found 488.2267.

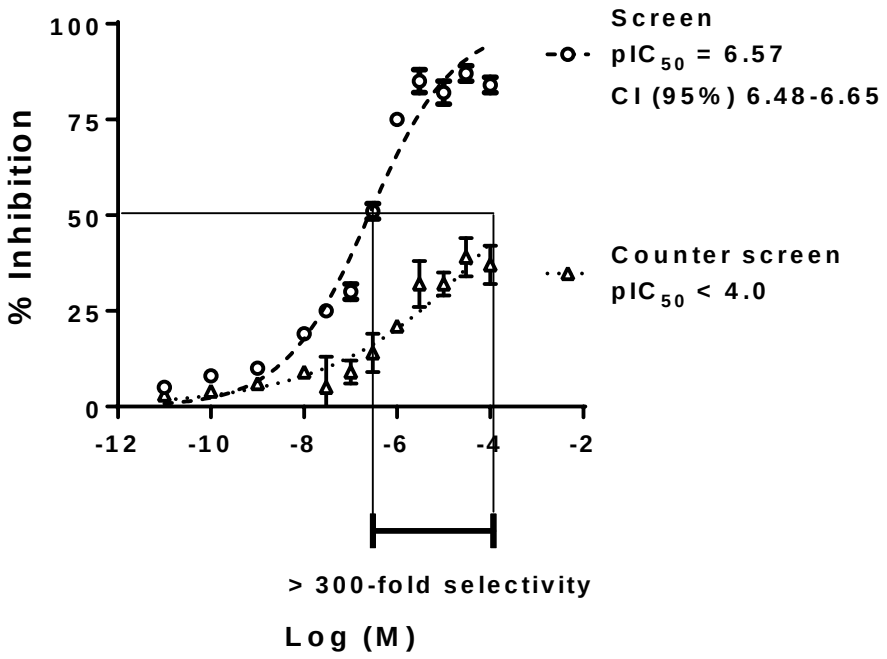


Fig 1S – Selectivity of **PDDC** for nSMase2 vs. coupling enzymes in fluorescence Amplex Red assay - pIC_{50} curves were generated in duplicate twelve-point dose response curves using the Amplex red Fluorescence-based assay. Lysate of cells expressing human recombinant nSMase2 (1.9 µg protein/50 µL) was incubated with sphingomyelin (20 µM) and detecting reagents (alkaline phosphatase 4U/mL, choline oxidase 0.1 U/mL, horse radish peroxidase 0.1 U/mL, and Amplex Red 50 µM) in the presence of different concentrations of **PDDC** for 3h (total reaction volume 50 µL). Percent inhibition vs. inhibitor concentration was plotted in GraphPad Prism. Data analysis and non-linear least squares curve fitting were carried out with GraphPad Prism 7. Reagents in the counter screen to establish selectivity against coupling enzymes (alkaline phosphatase, choline oxidase, and horse radish peroxidase) were the same as for the pIC_{50} determination as for inhibition of nSMase2 except that nSMase2 was not present and phosphorylcholine (2 µM) was used as substrate for alkaline phosphatase. CI (95%): 95% asymptotic confidence interval; lower bound – upper bound

Table 1S

Evaluation of PDDC at 10 µM in Eurofin's SafetyScreen 44					
Binding Assay	Catalog Ref	% Inhibition of Control Specific Binding	% of Control Specific Binding (n = 2)	Reference Compound	IC50 Ref (M)
A2A (h) (agonist radioligand)	0004	5	95.2	NECA	3.0E-08
alpha 1A (h) (antagonist radioligand)	2338	84	16.2	WB 4101	3.9E-10
alpha 2A (h) (antagonist radioligand)	0013	18	82.3	yohimbine	4.5E-09
beta 1 (h) (agonist radioligand)	0018	-6	105.6	atenolol	5.5E-07
beta 2 (h) (antagonist radioligand)	0020	0	100.1	ICI 118551	5.1E-10
BZD (central) (agonist radioligand)	0028	-25	124.9	diazepam	1.0E-08
CB1 (h) (agonist radioligand)	0036	32	67.6	CP 55940	2.0E-09
CB2 (h) (agonist radioligand)	0037	47	52.8	WIN 55212-2	1.6E-09
CCK1 (CCKA) (h) (agonist radioligand)	0039	-3	102.8	CCK-8s	5.9E-11
D1 (h) (antagonist radioligand)	0044	25	74.5	SCH 23390	4.2E-10
D2S (h) (agonist radioligand)	1322	4	95.9	7-OH-DPAT	4.4E-09
ETA (h) (agonist radioligand)	0054	4	95.7	endothelin-1	7.3E-11
NMDA (antagonist radioligand)	0066	12	87.6	CGS 19755	3.9E-07
H1 (h) (antagonist radioligand)	0870	15	84.7	pyrilamine	1.7E-09
H2 (h) (antagonist radioligand)	1208	-33	132.7	cimetidine	6.3E-07
MAO-A (antagonist radioligand)	0443	17	83.4	clorgyline	2.2E-09
M1 (h) (antagonist radioligand)	0091	16	84.2	pirenzepine	3.1E-08
M2 (h) (antagonist radioligand)	0093	4	96.3	methoctramine	2.7E-08
M3 (h) (antagonist radioligand)	0095	-15	115.2	4-DAMP	1.4E-09
N neuronal alpha 4beta 2 (h) (agonist radioligand)	3029	-11	111.0	nicotine	4.3E-09
delta (DOP) (h) (agonist radioligand)	0114	17	82.7	DPDPE	4.6E-09
kappa (KOP) (agonist radioligand)	1971	20	80.4	U 50488	7.7E-10
mu (MOP) (h) (agonist radioligand)	0118	11	88.7	DAMGO	1.3E-09
5-HT1A (h) (agonist radioligand)	0131	14	86.4	8-OH-DPAT	1.3E-09
5-HT1B (antagonist radioligand)	0132	42	58.3	serotonin	2.4E-08
5-HT2A (h) (agonist radioligand)	0471	40	60.1	(±)DOI	3.9E-10
5-HT2B (h) (agonist radioligand)	1333	44	56.3	(±)DOI	3.7E-09
5-HT3 (h) (antagonist radioligand)	0411	5	95.1	MDL 72222	1.3E-08
GR (h) (agonist radioligand)	0469	23	76.7	dexamethasone	2.5E-09
AR (h) (agonist radioligand)	0933	0	99.8	testosterone	3.0E-09
V1a (h) (agonist radioligand)	0159	22	78.2	[d(CH2)51,Tyr(Me)2]-	2.5E-09
Ca2+ channel (L, dihydropyridine site) (antagonist radioligand)	0161	67	33.4	nitrendipine	1.6E-10
Potassium Channel hERG (human)- [3H] Dofetilide	4094	19	81.5	Terfenadine	6.5E-08
KV channel (antagonist radioligand)	0166	-4	103.6	alpha -dendrotoxin	1.1E-10
Na+ channel (site 2) (antagonist radioligand)	0169	69	30.7	veratridine	6.5E-06
norepinephrine transporter (h) (antagonist radioligand)	0355	31	69.1	protriptyline	2.4E-09
dopamine transporter (h) (antagonist radioligand)	0052	79	20.7	BTCP	1.2E-08
5-HT transporter (h) (antagonist radioligand)	0439	41	59.4	imipramine	5.7E-09
Enzymatic Assays					
COX1(h)	4173	14	85.7	Diclofenac	1.0E-08
COX2(h)	4186	-22	122.2	NS398	8.6E-08
PDE3A (h)	4072	-17	117.4	milrinone	6.3E-07
PDE4D2 (h)	4077	-9	108.8	Ro 20-1724	8.0E-08
Lck kinase (h)	2906	-8	108.2	staurosporine	8.0E-08
acetylcholinesterase (h)	0363	15	84.8	galanthamine	9.0E-07

	% Maximal LDH Release
M-PER	100 ± 0.19
Media	0.0 ± 0.02
+DMSO (0.3%)	-0.2 ± 0.02
plus PDDC (3 µM)	-0.2 ± 0.02
plus PDDC (30 µM)	0.4 ± 0.03

Table 2S – Effect of **PDDC** on astrocyte viability as measured by lactate dehydrogenase (LDH) activity released into cell media – Primary astrocytes were treated in parallel incubations with **PDDC** using the same conditions as those used for the measurement of EV release from astrocytes (2h incubation up to 30 µM). Media was collected and used as LDH source to follow disappearance of NADH (1 mM) absorbance at 340 nM in the presence of sodium pyruvate (20 mM). Maximal LDH activity was obtained by using **m**ammalian **p**rotein **e**xtraction **r**eagent (M-PER, ThermoScientific) and normalized to 100%

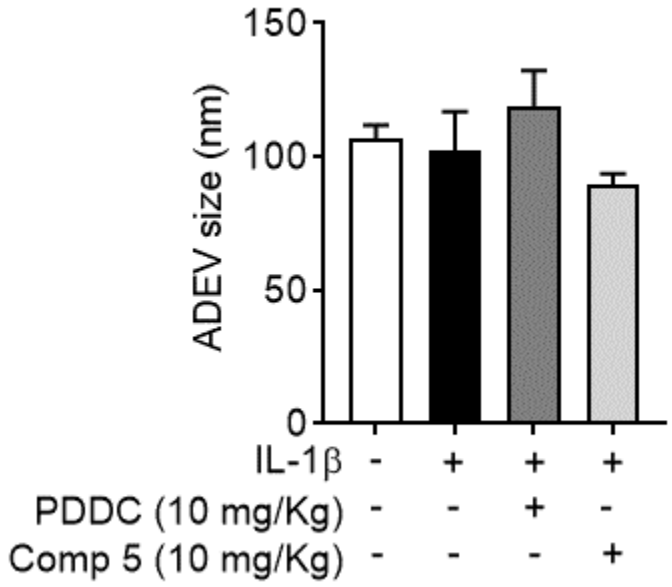


Fig 2S –Extracellular Vesicle (EV) size in an *in vivo* model of brain injury – EVs were isolated from plasma 2h following treatments with vehicle, IL-1β, IL-1β + **PDDC** (10 mg/kg IP) and IL-1β + compound **5** (10 mg/kg IP). Plasma was collected and centrifuged at 2700g for 15 min at 4°C. Supernatant was collected and size of EVs was measured using a Zetaview nanoparticle Tracker.

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