

Supporting information

Lead Optimization of Imidazopyrazines: A New Class of Antimalarial with Activity on *Plasmodium* Liver-stages

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Content

| | |
|--|---------|
| 1. General materials and method | (3) |
| 2. Synthesis of compound 2 | (4-5) |
| 3. Synthesis of compound 3 | (6-8) |
| 4. Synthesis of compound 20 | (9-10) |
| 5. Analytical data of analogues 10-19 | (11-12) |
| 6. X-ray structure of compound 20 (KDU691) | (13-14) |
| 7. Cytotoxicity data for compounds 3, 18, 20 ; and cytotoxicity assays | (15) |
| 8. Solubility assay | (16) |
| 9. Mouse microsome stability assay | (16) |
| 10. Methods for the <i>in vitro</i> assays (<i>P. falciparum</i> asexual blood-stage assay, <i>P. yoelii</i> liver schizont assay, and <i>P. cynomolgi</i> liver assay), <i>in vivo</i> pharmacokinetic studies, and the <i>in vivo</i> efficacy (causal prophylaxis <i>P. berghei</i> mouse model), see: <i>Nature</i> 2013 , 504, 248-253 and the Supplementary Information, and references therein. | |

General Materials and Methods

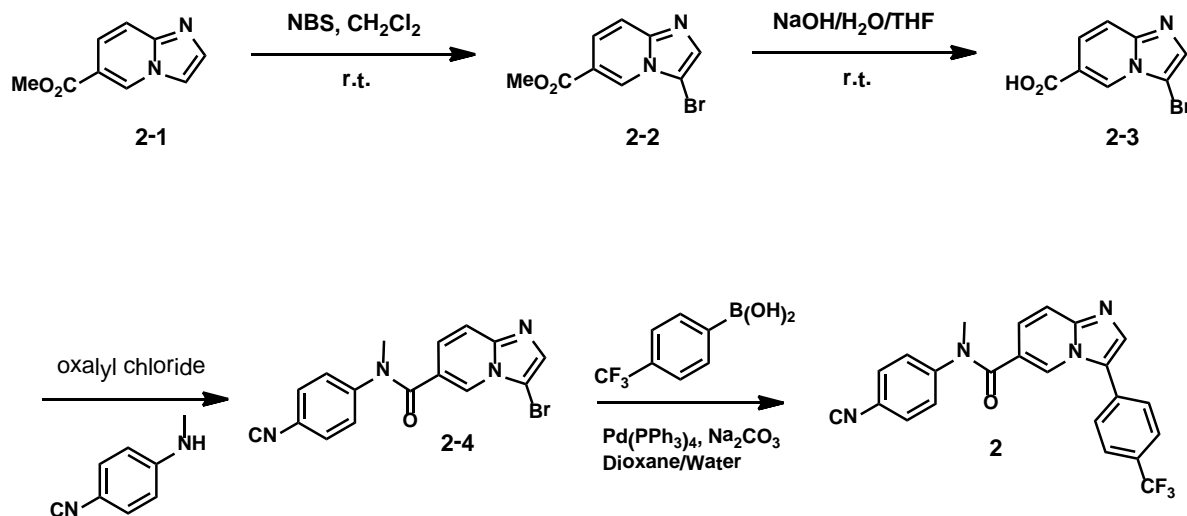
Unless otherwise noted, materials were obtained from commercial suppliers and were used without purification. Removal of solvent under reduced pressure refers to distillation using Büchi rotary evaporator attached to a vacuum pump (~3 mmHg). Products obtained as solids or high boiling oils were dried under vacuum (~1 mmHg). Purification of compounds by high pressure liquid chromatography was achieved using a Waters 2487 series with Ultra 120 5 μ m C18Q column with a linear gradient from 10% solvent A (acetonitrile with 0.035% trifluoroacetic acid) in solvent B (water with 0.05% trifluoroacetic acid) to 90% A in seven and half minutes, followed by two and half minute elution with 90% A.

¹H NMR spectra were recorded on Bruker XWIN-NMR (400 MHz). Proton resonances are reported in parts per million (ppm) downfield from tetramethylsilane (TMS). ¹H NMR data are reported as multiplicity (s singlet, d doublet, t triplet, q quartet, quint quintet, sept septet, dd doublet of doublets, dt doublet of triplet, bs broad singlet), number of protons and coupling constant in Hertz. For spectra obtained in CDCl₃, DMSO-*d*₆, CD₃OD, the residual protons (7.27, 2.50 and 3.31 ppm respectively) were used as the reference.

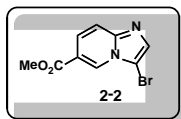
Analytical thin-layer chromatography (TLC) was performed on commercial silica plates (Merck 60-F 254, 0.25 mm thickness); compounds were visualized by UV light (254 nm). Flash chromatography was performed either by CombiFlash® (Separation system Sg. 100c, ISCO) or using silica gel (Merck Kieselgel 60, 230-400 mesh). Agilent 1100 series liquid chromatograph/mass selective detector (LC/MSD) was used to monitor the progress of reactions and check the purity of products using 254 nm and 220 nm wavelengths, and electrospray ionization (ESI) positive mode. Mass spectra were obtained in ESI positive mode. Elemental analyses were carried out by Midwest microlabs LLC, Indianapolis.

The synthesized compounds **2**, **3**, **10-20** are all with purity >95% based on HPLC analysis.

Synthesis of Compound 2



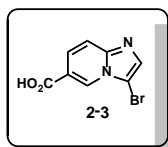
Methyl 3-bromoimidazo[1,2-a]pyridine-6-carboxylate (2-2). To a stirred solution of methyl H-



imidazo[1,2-a]pyridine-6-carboxylate (7.7 g, 43.75 mmol, 1.00 equiv.) in CH_2Cl_2 (150 mL) was added *N*-bromosuccinimide (11.7 g, 65.73 mmol, 1.50 equiv.). The resulting solution was allowed to react, with stirring, for 12 hours at room temperature. After a filtration, the resulting mixture was washed with aqueous

saturated $\text{Na}_2\text{S}_2\text{O}_3$ solution (2x100 mL), brine (2x100 mL), dried over Na_2SO_4 and concentrated under *vacuum*. The residue was applied onto a silica gel column with Petroleum Ether /EtOAc (2:1) to result in 8.2 g (74%) of **2-2** as a white solid. ^1H NMR (400 MHz, CDCl_3) δ ppm 8.89 (s, 1H), 7.79 (d, J = 7.2 Hz, 1H), 7.70 (s, 1H), 7.63 (d, J = 6.9 Hz, 1H), 3.99 (s, 3H); ^{13}C NMR (101 MHz, DMSO) δ 164.5, 145.7, 135.2, 127.7, 123.8, 117.3, 116.6, 96.8, 52.6; ESIMS: m/z $[\text{M}+\text{H}]^+$ 255.0.

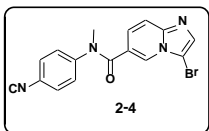
3-bromoimidazo[1,2-a]pyridine-6-carboxylic acid (2-3). 1N NaOH (4.0 mL) was added to a solution of **2-**



2 (500 mg, 1.96 mmol) in THF (2.0 mL). The reaction mixture was stirred at room temperature for 2 hours. The solvent was removed and the residue was diluted with water. The mixture was acidified using 1N HCl, resulting in a white precipitate that was collected by vacuum filtration (387 mg, 86%). ^1H NMR (400 MHz, DMSO) δ

8.77 (t, J = 1.3, 1H), 7.88 (s, 1H), 7.73 (t, J = 1.2, 2H); ^{13}C NMR (101 MHz, DMSO) δ 165.5, 145.7, 135.9, 127.5, 124.3, 117.7, 117.0, 96.6; HRMS: m/z calcd for $[\text{C}_8\text{H}_5\text{BrN}_2\text{O}_2 + \text{H}]^+$ 238.9462, found: 238.9463.

3-bromo-N-(4-cyanophenyl)-N-methylimidazo[1,2-a]pyridine-6-carboxamide (2-4). oxalyl chloride



(0.182 mL, 2.074 mmol) was added to a solution of 3-bromoimidazo[1,2-

a]pyridine-6-carboxylic acid (250 mg, 1.037 mmol), DCM (Volume: 1 mL, Ratio:

100) and DMF (Volume: 0.01 mL, Ratio: 1.00). The reaction mixture was stirred at

room temperature for 3 h. The solvent was removed and fresh DCM (1 mL) was

added, followed by a solution of 4-(methylamino)benzonitrile (206 mg, 1.556 mmol) in DCM (1 mL).

Et₃N (0.723 mL, 5.19 mmol) was added and the reaction was stirred at room temperature for 30

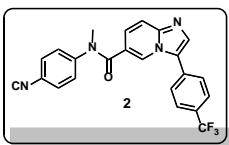
minutes. The solvent was removed and the crude material was purified by flash chromatography to give

2-4 (221 mg, 60%). ¹H NMR (400 MHz, DMSO) δ 8.32 (m, 1H), 7.79 (m, 3H), 7.52 (ddd, *J* = 1.6, 6.8, 11.0,

3H), 7.11 (dd, *J* = 1.7, 9.3, 1H), 3.46 (s, 3H); ¹³C NMR (101 MHz, DMSO) δ 166.2, 148.2, 144.5, 134.7,

133.3, 127.6, 126.4, 124.5, 122.0, 118.3, 116.4, 108.8, 96.0, 37.6; HRMS: *m/z* calcd for [C₁₆H₁₁BrN₄O + H]⁺ 355.0189, found: 355.0188.

N-(4-cyanophenyl)-N-methyl-3-(4-(trifluoromethyl)phenyl)imidazo[1,2-a]pyridine-6-carboxamide (2).



Pd(PPh₃)₄ (34 mg, 0.029 mmol) was added to a mixture of imidazopyridine **2-4**

(209 mg, 0.59 mmol), (4-(trifluoromethyl)phenyl)boronic acid (224 mg, 1.18

mmol), sodium carbonate (163 mg, 1.18 mmol) in dioxane/water (10 mL/10 mL).

The reaction was heated to 100 °C for 12 hours. After cooling to room

temperature, the solvent was removed. The residue was dissolved in ethyl acetate (100 mL) and filtered

through celite. The filtrate was concentrated and purified by silica gel chromatography, eluting with

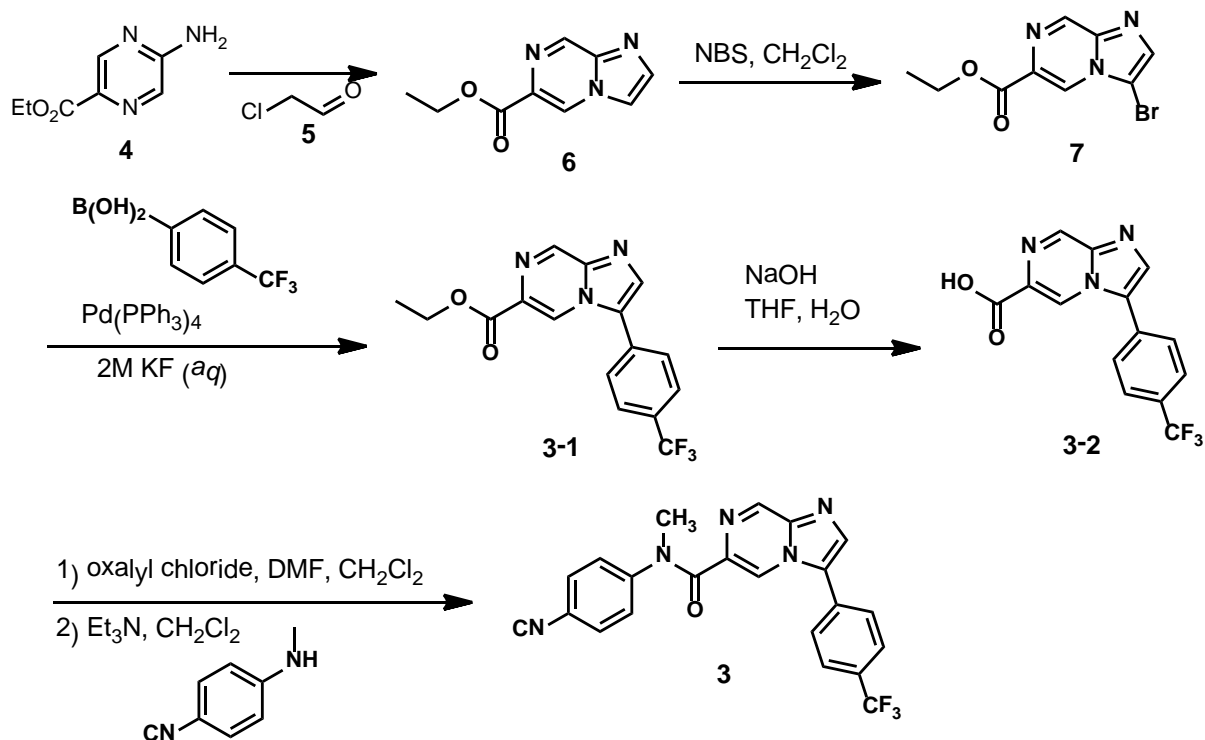
ethyl acetate and hexanes to afford compound **2** (47 mg, 19%). ¹H NMR (400 MHz, DMSO) δ 8.51 (m,

1H), 7.94 (s, 1H), 7.90 (d, *J* = 8.2, 2H), 7.85 (m, 2H), 7.62 (d, *J* = 8.1, 2H), 7.58 (dd, *J* = 0.8, 9.4, 1H), 7.54

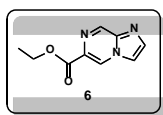
(m, 2H), 7.20 (dd, *J* = 1.6, 9.3, 1H), 3.44 (s, 3H). HRMS: *m/z* calcd for [C₂₃H₁₅F₃N₄O + H]⁺ 421.1271, found:

421.1263.

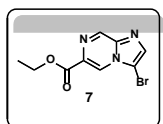
Synthesis of Compound 3



Ethyl pyrrolo[1,2-a]pyrazine-3-carboxylate (6). Chloroacetaldehyde (9.5 mL of a 50 wt% solution in water) was added to a mixture of aminopyrazine **4** (2.00 g, 12.0 mmol), sodium bicarbonate (2.00 g, 23.8 mmol) in ethanol (100 mL). The reaction mixture was heated to 90 °C for 16 hours. After cooling to room temperature, the reaction mixture was concentrated and purified by flash chromatography (silica, 0-10% methanol/chloroform) to give pure **6** as a tan colored solid (1.6 g, 70% yield). ¹H NMR (400 MHz, CDCl₃) δ 9.18 (s, 1H), 9.01 (d, *J* = 1.4, 1H), 7.93 (d, *J* = 1.1, 1H), 7.84 (s, 1H), 4.51 (q, *J* = 7.1, 3H), 1.46 (t, *J* = 7.1, 4H).

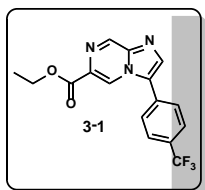


Ethyl 3-bromoimidazo[1,2-a]pyrazine-6-carboxylate (7). NBS (139 mg, 0.785 mmol) was added in one portion to a solution of ethyl pyrrolo[1,2-a]pyrazine-3-carboxylate **6** (100 mg, 0.523 mmol) in dichloromethane (2.0 mL) at room temperature. After three hours, the reaction was filtered and the filtrate was purified by flash chromatography (silica, 10-100% ethyl acetate/hexanes) to give pure **7** as a white colored solid (133 mg, 94%).



yield). ^1H NMR (400 MHz, DMSO) δ 9.15 (s, 1H), 8.83 (s, 1H), 8.10 (s, 1H), 4.40 (q, J = 7.2, 2H), 1.37 (t, J = 7.2, 3H); ^{13}C NMR (101 MHz, DMSO) δ 163.5, 142.4, 140.9, 130.5, 121.7, 99.9, 61.5, 14.1; ESIMS: m/z [$\text{C}_9\text{H}_8\text{BrN}_3\text{O}_2 + \text{H}$] $^+$ 270.0.

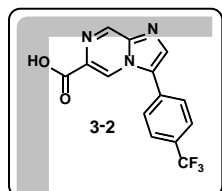
Ethyl 3-(4-(trifluoromethyl)phenyl)imidazo[1,2-a]pyrazine-6-carboxylate (3-1). $\text{Pd}(\text{PPh}_3)_4$ (73 mg, 0.0629 mmol) was added to a degassed mixture of 3-bromoimidazo[1,2-a]pyrazine-6-carboxylate **7** (170 mg, 0.629 mmol), 4-trifluoromethylphenyl boronic acid (237 mg, 1.25 mmol), KF (0.70 mL of a 2.0 M solution in water). The reaction was



heated in the microwave at 110 °C for 30 minutes. The reaction was cooled to room temperature, filtered and concentrated. The crude mixture was purified by flash chromatography (silica, 0-10% methanol/chloroform) to give **3-1** as a white

colored solid (126 mg, 61%). ^1H NMR (400 MHz, DMSO) δ 9.23 (d, J = 1.4, 1H), 9.07 (d, J = 1.4, 1H), 8.29 (s, 1H), 8.06-7.95 (m, 4H), 4.37 (q, J = 7.1, 2H), 1.34 (t, J = 7.1, 3H); ^{13}C NMR (101 MHz, DMSO) δ 163.8, 142.8, 141.0, 131.4, 130.4, 128.6, 126.6, 126.4, 126.3, 125.4, 122.7, 122.0, 61.3, 14.1; ESIMS: m/z [$\text{C}_{16}\text{H}_{12}\text{F}_3\text{N}_3\text{O}_2 + \text{H}$] $^+$ 336.1.

3-(4-(trifluoromethyl)phenyl)imidazo[1,2-a]pyrazine-6-carboxylic acid (3-2) NaOH (1.25 mL of a 1.0 N solution in water, 1.25 mmol) was added to a solution of ethyl 3-(4-(trifluoromethyl)phenyl)imidazo[1,2-

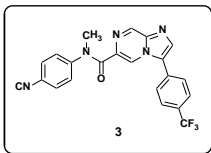


a]pyrazine-6-carboxylate **3-1** (200 mg, 0.629 mmol) in THF (1.25 mL). The reaction mixture was stirred at room temperature for 3 h. The solvent was removed *in vacuo*. The crude reaction mixture was taken up in water and acidified to pH 3, resulting in a white precipitate that was collected by vacuum filtration to give **3-2** as a white colored solid (106 mg, 55%). ^1H NMR (400 MHz,

DMSO) δ 9.13 (s, 1H), 9.01 (s, 1H), 8.23 (s, 1H), 8.05-7.95 (m, 4H) ppm; ^{13}C NMR (101 MHz, DMSO) δ 165.3, 142.3, 141.1, 133.3, 131.6, 129.0, 128.7, 128.5, 126.3, 126.1, 125.4, 120.6; ESIMS: m/z [$\text{C}_{14}\text{H}_8\text{F}_3\text{N}_3\text{O}_2 + \text{H}$] $^+$ 308.1.

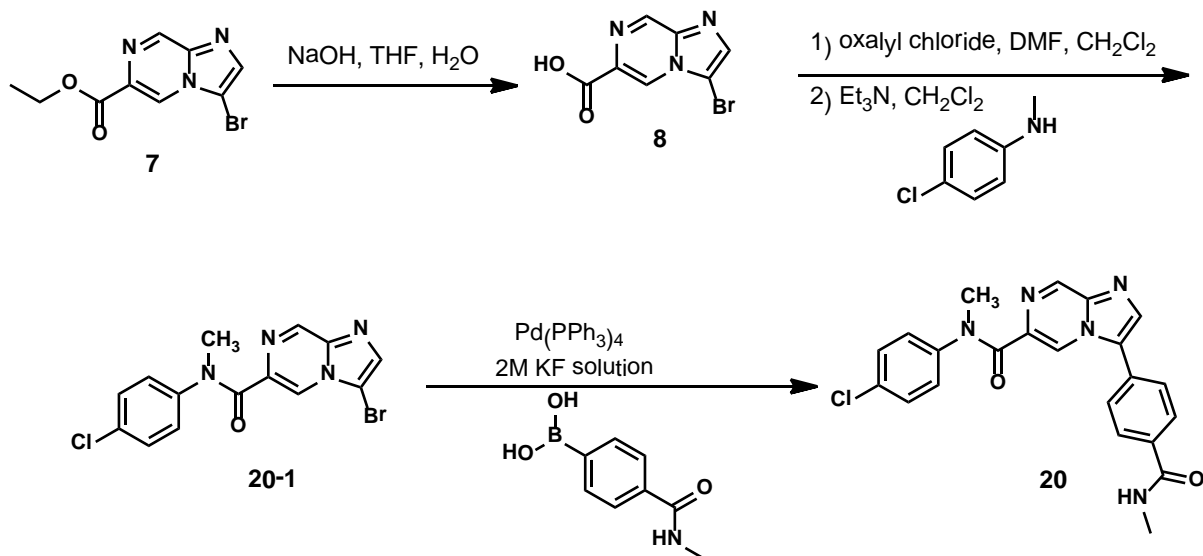
N-(4-cyanophenyl)-N-methyl-3-(4-(trifluoromethyl)phenyl)imidazo[1,2-a]pyrazine-6-carboxamide (3).

Oxalyl chloride (0.47 mL, 5.40 mmol) was added drop wise to a solution of 3-(4-(trifluoromethyl)phenyl)imidazo[1,2-a]pyrazine-6-carboxylic acid **3-4** (826 mg, 2.70 mmol), DMF (0.05 mL) and dry dichloromethane (20 mL) at room temperature (gas evolution). After 30 minutes at room temperature, the solvent was removed *in vacuo*. The crude acid chloride was dissolved in dry

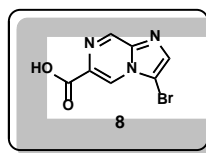


dichloromethane (15 mL) and a solution of 4-(methylamino)benzonitrile (533 mg, 4.04 mmol) in dichloromethane (5 mL) was added drop wise at room temperature. Triethylamine (1.13 mL, 8.10 mmol) was added and the reaction stirred at room temperature for 3 hours. The solvent was removed *in vacuo* and the crude material was purified by flash chromatography (silica, 10-100% ethyl acetate/hexanes). The product was further recrystallized from methanol to give pure **3** as white needles (568 mg, 50%). ^1H NMR (400 MHz, DMSO) δ 9.72 (d, $J = 1.4$, 1H), 9.65 (d, $J = 1.3$, 1H), 9.06 (s, 1H), 8.80 (s, 4H), 8.57 (d, $J = 8.7$, 2H), 8.27 (d, $J = 8.6$, 2H), 4.28 (s, 3H); ^{13}C NMR (151 MHz, DMSO) δ 165.8, 148.6, 141.2, 140.3, 136.5, 135.7, 133.0, 131.4, 128.4, 126.7, 126.2, 126.2, 125.0, 123.2, 120.6, 118.5, 108.2, 37.6; HRMS: m/z calcd for $[\text{C}_{22}\text{H}_{14}\text{F}_3\text{N}_5\text{O} + \text{H}]^+$ 422.1223, found: 422.1225.

Synthesis of Compound 20

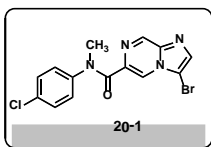


3-bromoimidazo[1,2-a]pyrazine-6-carboxylic acid (8). NaOH (4.0 mL of a 1.0N aqueous solution) was added to a mixture of ethyl 3-bromoimidazo[1,2-a]pyrazine-6-carboxylate **7** (650 mg, 2.42 mmol) in tetrahydrofuran (4.0 mL). The reaction mixture was heated to 60 °C for 90 minutes. After cooling to room temperature, the solvent was removed *in vacuo*. The crude product was taken up in water and acidified to pH 3 with 1.0 N HCl resulting in the formation of 3-bromoimidazo[1,2-a]pyrazine-6-carboxylic acid **8** (328 mg, 56%) as a white precipitate that was collected by vacuum filtration. ¹H NMR (400 MHz, DMSO) δ 9.15 (d, *J* = 1.4, 1H), 8.83 (d, *J* = 1.4, 1H), 8.13 (s, 1H); ¹³C NMR (100 MHz, DMSO) δ 165.1, 141.7, 140.8, 136.4, 120.0, 99.0; ESIMS: *m/z* [C₇H₄BrN₃O₂ + H]⁺ 242.0.



3-bromo-N-(4-chlorophenyl)-N-methylimidazo[1,2-a]pyrazine-6-carboxamide

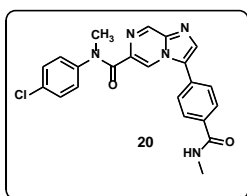
(20-1) Oxalyl chloride (0.83 mL of a 2.0 M solution in dichloromethane, 1.66 mmol) was added drop wise to a solution of 3-bromoimidazo[1,2-a]pyrazine-6-carboxylic acid **8** (200 mg, 0.833 mmol), DMF (1 drop) and dry dichloromethane (5.0 mL) at room temperature (gas evolution). After 30 minutes at room



temperature, the solvent was removed *in vacuo*. The crude acid chloride was dissolved in dry dichloromethane (5.0 mL) and a solution of 4-(methanimino)chlorobenzene (235 mg, 1.670 mmol) in 2.0 mL of dichloromethane was added drop wise at room temperature. Triethylamine (0.25 mL, 2.44 mmol)

was added and the reaction stirred at room temperature for 3 hours. The solvent was removed *in vacuo* and the crude material was purified by flash chromatography to give 3-bromo-N-(4-chlorophenyl)-N-methylimidazo[1,2-a]pyrazine-6-carboxamide **20-1** (191 mg, 63%) as a tan solid (silica, 10-100% ethyl acetate/hexanes). ^1H NMR (400 MHz, CDCl_3) δ 8.62 (d, J = 1.3, 1H), 8.59 (s, 1H), 7.78 (s, 1H), 7.21 (d, J = 8.6, 2H), 7.04 (d, J = 7.7, 2H), 3.51 (s, 3H); ^{13}C NMR (100 MHz, DMSO) δ 165.5, 143.1, 140.8, 139.9, 136.6, 136.3, 130.5, 128.8, 128.2, 119.7, 99.1, 37.9; ESIMS: m/z [$\text{C}_{14}\text{H}_{10}\text{BrClN}_4\text{O} + \text{H}$] $^+$ 365.0.

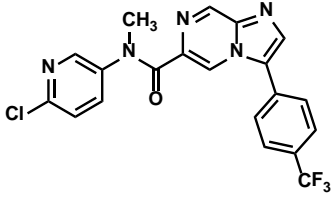
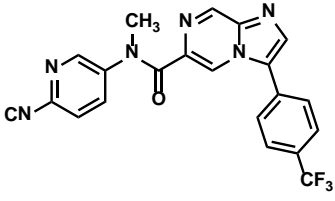
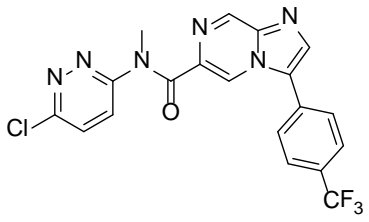
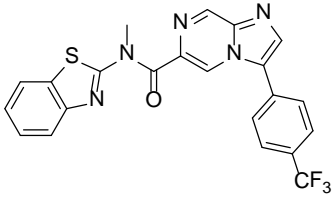
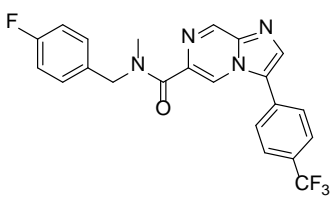
Synthesis of N-(4-chlorophenyl)-N-methyl-3-(4-(methylcarbamoyl)phenyl)imidazo[1,2-a]pyrazine-6-carboxamide (20). To a solution of 3-bromo-N-(4-chlorophenyl)-N-methylimidazo[1,2-a]pyrazine-6-

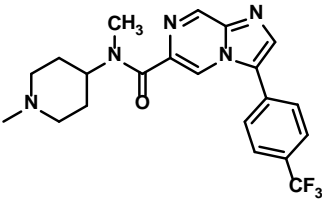
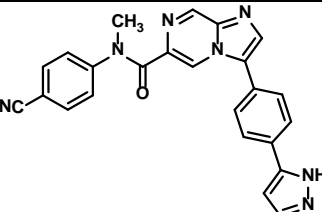
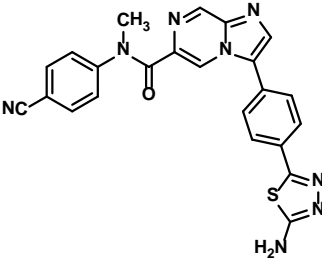
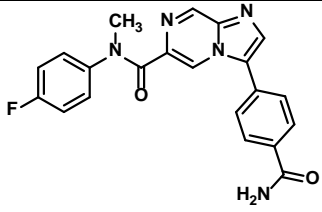
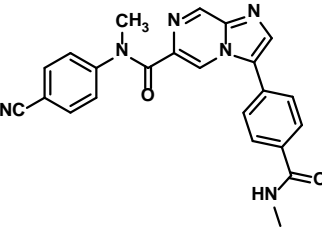


carboxamide **20-1** (1.52 g, 4.10 mmol) in 1,4-dioxane/1,2-dimethoxyethane (3.6 ml/4.8 ml) was added 4-(N-methylaminocarbonyl)phenylboronic acid (1.47 g, 8.20 mmol), $\text{Pd}(\text{PPh}_3)_4$ (0.23 g, 0.20 mmol) and an aqueous solution of potassium fluoride (0.48 g in 8.20 ml water; 8.20 mmol). The mixture was pre-stirred for 10 seconds and subjected to microwave heating at 110°C for 30

minutes. Saturated aqueous sodium bicarbonate solution was added. The resultant mixture was extracted with ethyl acetate (3×50 mL). The combined organic extracts were washed with water and brine, dried with sodium sulfate and concentrated under reduced pressure. The residue was subjected to column chromatography (0%-5% methanol in dichloromethane) to yield N-(4-chlorophenyl)-N-methyl-3-(4-(methylcarbamoyl)phenyl)imidazo[1,2-a]pyrazine-6-carboxamide as white foam **20** (1.38 g, 80.2%). ^1H NMR (400 MHz, DMSO) δ 8.86 (s, 1H), 8.81 (d, J = 1.3, 1H), 8.62 (d, J = 4.6, 1H), 8.19 (s, 1H), 8.05 (d, J = 8.4, 2H), 7.79 (d, J = 8.4, 2H), 7.35 (d, J = 8.8, 2H), 7.30 (d, J = 8.8, 2H), 3.42 (s, 3H), 2.84 (d, J = 4.5, 3H); ^{13}C NMR (75 MHz, $\text{DMSO}-d_6$) δ 178.1, 141.6, 134.0, 133.9, 132.7, 129.1, 125.6, 125.4, 125.3, 124.9, 112.9, 111.9, 111.7, 111.3, 104.3, 61.9, 44.2, 21.6, 20.8; HRMS: m/z calcd for [$\text{C}_{22}\text{H}_{18}\text{ClN}_5\text{O}_2 + \text{H}$] $^+$ 420.1222, found: 420.1227.

Analytical data of analogues 10-19: The following compounds were prepared according to the procedures described for the synthesis of compounds **3** or **20**.

| Compound | Structure | Analytical Data |
|-----------|---|---|
| 10 |  | ^1H NMR (400 MHz, DMSO) δ 8.90 (s, 1H), 8.85 (s, 1H), 8.30 (s, 1H), 8.25 (s, 1H), 8.0 (m, 4H), 7.80 (m, 1H), 7.76 (m, 1H); 3.45 (s, 3H). M/Z = 432.8 (M+H) $^+$ |
| 11 |  | ^1H NMR (400 MHz, DMSO) δ 9.00 (d, J = 1.4, 1H), 8.85 (d, J = 1.4, 1H), 8.79 (d, J = 1.6, 1H), 8.29 (s, 1H), 8.17 (dd, J = 2.3, 8.6, 1H), 8.02 (q, J = 8.4, 4H), 7.47 (d, J = 8.6, 1H), 3.55 (s, 3H); M/Z = 423.1 (M+H) $^+$ |
| 12 |  | ^1H NMR (400 MHz, DMSO- d_6) δ 8.78 (d, J = 2.4 Hz, 1H), 8.03 (dd, J = 9.1, 2.5 Hz, 1H), 7.91-7.83 (m, 2H), 7.44 (s, 1H), 7.34 (s, 1H), 7.04-6.96 (m, 2H), 6.56 (d, J = 8.9 Hz, 1H), 3.79 (s, 3H). M/Z = 433.8 (M+H) $^+$ |
| 13 |  | ^1H NMR (400 MHz, DMSO- d_6) δ 9.29 (d, J = 1.4 Hz, 1H), 9.16 (d, J = 1.5 Hz, 1H), 8.35 (s, 1H), 8.06 (dd, J = 7.8, 1.8 Hz, 3H), 7.97 (d, J = 8.3 Hz, 2H), 7.88 (d, J = 8.1 Hz, 1H), 7.50 (td, J = 8.2, 7.7, 1.3 Hz, 1H), 7.38 (td, J = 7.6, 1.2 Hz, 1H), 3.80 (s, 3H). M/Z = 454.4 (M+H) $^+$ |
| 14 |  | ^1H NMR (400 MHz, DMSO- d_6) δ 9.19 (d, J = 9.8 Hz, 1H), 8.86 (d, J = 7.4 Hz, 1H), 8.26 (s, 1H), 8.07-7.90 (m, 4H), 7.41 (d, J = 6.9 Hz, 2H), 7.26-7.12 (m, 2H), 4.70 (s, 2H), 3.39 (s, 3H, overlap with water peak). M/Z = 429.0 (M+H) $^+$ |

| | | |
|----|---|--|
| 15 |  | ^1H NMR (400 MHz, DMSO- d_6) δ ppm: 9.17 (s, 1H), 8.78 (s, 1H), 8.27 (s, 1H), 7.97 (dt, J = 13.2 Hz, J = 8.1 Hz, 4H), 3.31 (s, 3H, overlap with water peak), 2.98 (br, 1H), 2.88 (s, 3H), 2.29 (br, 1H), 2.21 (br, 2H), 2.00-1.68 (m, 5H). M/Z = 418.0 ($M+H$) $^+$ |
| 16 |  | ^1H NMR (400 MHz, DMSO) δ 8.89 (d, J = 1.4 Hz, 1H), 8.81 (d, J = 1.2 Hz, 1H), 8.17 (s, 1H), 8.06 (s, 2H), 7.81-7.73 (m, 5H), 7.54-7.43 (m, 2H), 6.87 (d, J = 2.2 Hz, 1H), 3.45 (s, 3H). M/Z = 420.2 ($M+H$) $^+$ |
| 17 |  | ^1H NMR (400 MHz, DMSO) δ 9.25 (s, 1H), 8.91 (dd, J = 1.3, 3.7, 1H), 8.82 (d, J = 1.3, 1H), 8.21 (d, J = 2.8 Hz, 1H), 8.01 (m, 2H), 7.83 (m, 2H), 7.77 (d, J = 8.6 Hz, 2H), 7.48 (dd, J = 2.7, 8.6 Hz, 2H), 3.48 (s, 3H). M/Z = 453.1 ($M+H$) $^+$ |
| 18 |  | ^1H NMR (400 MHz, DMSO) δ 8.85 (s, 1H), 8.79 (s, 1H), 8.18 (s, 1H), 8.16 (s, 1H), 8.09 (d, J = 8.4, 2H), 7.78 (d, J = 8.3, 2H), 7.54 (s, 1H), 7.40 – 7.26 (m, 2H), 7.12 (t, J = 8.6, 2H), 3.41 (s, 3H); MS m/z 390.1 ($M+H$) $^+$ |
| 19 |  | ^1H NMR (400 MHz, DMSO) δ 8.90 (d, J = 1.4 Hz, 1H), 8.82 (d, J = 1.3 Hz, 1H), 8.63 (d, J = 4.6 Hz, 1H), 8.22 (s, 1H), 8.06 (d, J = 8.4 Hz, 2H), 7.85 (d, J = 8.4, 2H), 7.80-7.73 (m, 2H), 7.47 (d, J = 8.6 Hz, 2H), 3.48 (s, 3H), 2.85 (d, J = 4.5 Hz, 3H). M/Z = 411.1 ($M+H$) $^+$ |

X-ray Structure of 20 (KDU691):

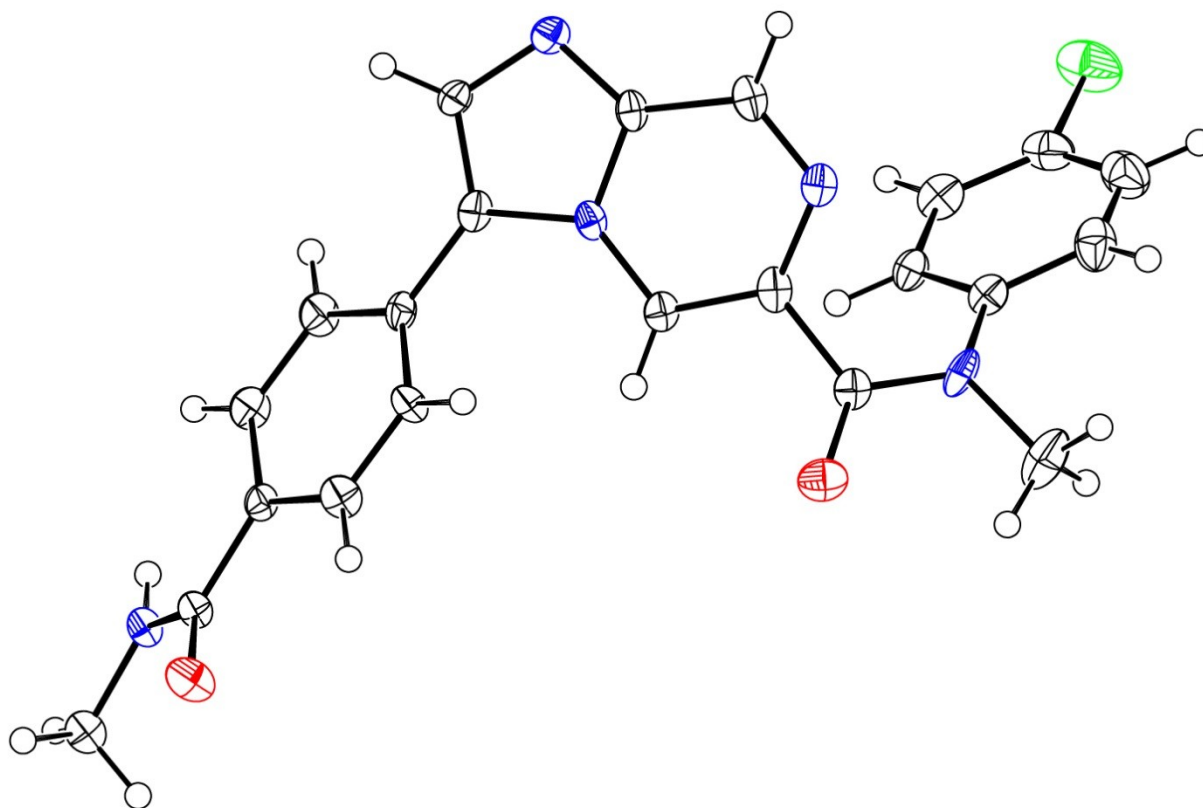


Table 1. Crystal data and structure refinement for SIN01c.

| | | |
|----------------------|--|----------------------|
| Identification code | SIN01c [#3471] [NVC-KDU691-NX-3] [BZL/PYR] | |
| Empirical formula | C ₂₂ H ₁₈ Cl N ₅ O ₂ | |
| Formula weight | 419.86 | |
| Temperature | 100(2) K | |
| Wavelength | 1.54178 Å | |
| Crystal system | Triclinic | |
| Space group | P-1 | |
| Unit cell dimensions | a = 9.828(7) Å | α = 86.47(4)° |
| | b = 14.349(8) Å | β = 89.96(5)° |

| | | |
|-----------------------------------|---|---------------------------|
| | $c = 28.676(18) \text{ \AA}$ | $\gamma = 83.32(4)^\circ$ |
| Volume | 4009(4) \AA^3 | |
| Z | 8 | |
| Density (calculated) | 1.391 g/cm ³ | |
| Absorption coefficient | 1.936 mm ⁻¹ | |
| F(000) | 1744 | |
| Crystal size | 0.15 x 0.02 x 0.02 mm ³ | |
| Theta range for data collection | 1.54 to 66.77° | |
| Index ranges | -11 ≤ h ≤ 11, -16 ≤ k ≤ 17, 0 ≤ l ≤ 34 | |
| Reflections collected | 15516 | |
| Independent reflections | 15516 [R(int) = 0.0000] | |
| Completeness to theta = 66.77° | 94.0 % | |
| Absorption correction | Semi-empirical from equivalents | |
| Max. and min. transmission | 0.9623 and 0.7600 | |
| Refinement method | Full-matrix least-squares on F ² | |
| Data / restraints / parameters | 15516 / 720 / 1089 | |
| Goodness-of-fit on F ² | 1.046 | |
| Final R indices [I > 2σ(I)] | R1 = 0.0830, wR2 = 0.2247 | |
| R indices (all data) | R1 = 0.1135, wR2 = 0.2484 | |
| Largest diff. peak and hole | 0.642 and -0.643 e. \AA^{-3} | |

Cytotoxicity data for representative compounds 3, 18, and 20.

| Compound | HepG2 IC ₅₀ (μM) | THP-1 IC ₅₀ (μM) |
|-----------|-----------------------------|-----------------------------|
| 3 | 42 | >50 |
| 18 | >50 | 23 |
| 20 | 35 | 19 |

CCK8 Cytotoxicity Assay in HepG2 Cells

HepG2 cells were trypsinized, washed, counted and diluted to 1.6×10^4 cells/ml in DMEM-W/O Glucose, supplemented with 10% Fetal bovine serum (FBS), 1% Penicillin/ Streptomycin, 2mM HEPES, 1mM Sodium Pyruvate, 10mM Galactose & 2mM Glutamine. 25μl of the media containing 400 cells per well were dispensed in clear 384-well tissue culture plate and incubated at room temperature for 30 minutes. The plate was then transferred and placed at 37°C, 5% CO₂ humidified incubator overnight. On the next day, serial-diluted compound plates were prepared and 125nl of compounds at various concentrations were then dispensed into the tissue culture well (200 × dilution). The plates were then transferred to 37°C, 5% CO₂ humidified incubator for additional 96 hours. Cytotoxicity was measured by CCK-8 assay. Briefly, CCK-8 was thawed on bench top and diluted 2.5 × with the growth media. 35ul of the pre-diluted CCK-8 was then introduced into each well and the plates were then further incubated in 37°C, 5% CO₂ humidified incubator for 3 h. The absorbance was read by Envision at 450nm. Dose response curves were calculated as in the previous section. The CC₅₀ is estimated as the concentration of the compound which will inhibit 50% of the signal. A positive control (puromycin) was used to ensure the quality of the data.

CCK8 Cytotoxicity Assay in THP-1 Cells

4 day cytotoxicity assay using THP-1 cells: THP-1 cells grown in suspension were counted and diluted to 8×10^4 cells/ml in RPMI-1640 media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. 25ul of the THP-1 containing media consisting of 2000 cells were dispensed in 384-well tissue culture plate and pre-incubated at room temperature for 30 minutes, followed by 37°C, 5% CO₂ overnight in the humidified incubator. On the next day, serial-diluted compound plates were prepared and 125nl of compounds at various concentrations were then dispensed into the tissue culture well (200 × dilution). The plates were then transferred to 37°C, 5% CO₂ humidified incubator for additional 96 hours. The plates were then transferred to 37°C, 5% CO₂ humidified incubator for additional 96 hours. Cytotoxicity was measured by CCK-8 assay. Briefly, CCK-8 was thawed on bench top and diluted 2.5 × with the growth media. 35ul of the pre-diluted CCK-8 was then introduced into each well and the plates were then further incubated in 37°C, 5% CO₂ humidified incubator for 3 hours. The absorbance was read by Envision at 450nm. Dose response curves were calculated as in the previous section (n=2). The CC₅₀ is estimated as the concentration of the compound which will inhibit 50% of the signal. A positive control (puromycin) was used to ensure the quality of the data.

Solubility assay

Solubility of all the compounds are measured in a high-throughput and miniaturized equilibrium solubility assay. The HT-equilibrium solubility assay consists of (1) compound dispensing, (2) incubation/phase separation, and (3) quantification of saturated solution. As an alternative for solid dispensing, evaporation of DMSO solutions is utilized for dispensing with negligible sample loss and degradation. LC/UV provides accurate quantification for equilibrium solubility determination and is configured in the high-throughput format.

Mouse microsome stability assay

Metabolic stability of all the compounds in mouse liver microsomes was determined using the compound depletion approach, quantified by LC/MS. The assay measures the rate and extent of metabolism of the compounds as determined by the disappearance of the parent compound, offering in vitro half-life ($t_{1/2}$), intrinsic clearance (CL_{int}) and also predicted metabolic clearance (CL_h) in mouse species. The extraction ration was determined using the ration of predicted metabolic clearance (CL_h) and hepatic blood flow. Scaling Factors for CL_{int} → CL_h : 45 mg microsomal protein/g liver; 87.5 g liver/kg body weight; 90 mL portal blood flow/min/kg; Protein binding not considered.