Supporting Information

Pyridopyrimidinone Derivatives as Potent and Selective JNK inhibitors

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1. Experimental Section

Commercially available reagents and anhydrous solvents were used without further purification unless otherwise specified. Thin layer chromatography (TLC) analyses were performed with precoated silica gel 60 F254. The mass spectra were recorded by LC/MS with Finnigan LCQ Advantage MAX spectrometer of Thermo Electron®. Flash chromatography was performed on prepacked columns of silica gel (230-400 Mesh, 40-63 μ m) by CombiFlash® with EtOAc/hexane or MeOH/DCM as eluents. The preparative HPLC was performed on SunFire C₁₈ OBD 10 μ m (30 x 250 mm) with CH₃CN + 50% MeOH / H₂O + 0.1% TFA as eluents to purify the targeted compounds. Analytic HPLC was performed on agilent technologies 1200 series with CH₃CN (Solvent B) / H₂O + 0.9% CH₃CN + 0.1% TFA (Solvent A) as eluents and the targeted products were detected by UV in the detection range of 215-310 nm. All compounds were determined to be > 95% pure by this method. NMR spectra were recorded with a Bruker® 400 MHz spectrometer at ambient temperature with the residual solvent peaks as internal standards. The line positions of multiplets were given in ppm (δ) and the coupling constants (J) were given in Hertz. Calibration was performed with an external calibration mixture immediately prior to analysis.

1.1 General synthetic procedures:

A short route (shown in Scheme 1) was used to synthesize inhibitors **1**, and **5-23**. The commercially available ethyl 4-chloro-2-(methylthio)pyrimidine-5-carboxylate **24** was reacted with alkyl amines using Et₃N as base to furnish *N*-substituted pyrimidines, followed with reduction of the ester by LiAlH₄ to give alcohol **25**. After oxidation by MnO₂, the intermediate aldehyde was reacted with Ph₃P=CCO₂Et to yield ester **26**, followed by a cyclization step to afford pyrimidinone **27**. The resulting adduct was then treated with NCS and an amine to furnish products **5-10**. Preparation of inhibitors **11-23** was accomplished by BOC-cleavage and functionalization to amide and urea derivatives. All final products were purified by reverse-phase HPLC to obtain a purity of >95%.

Scheme 1. Reagents and conditions: (a) alkyl amines, Et₃N, THF, rt; (b) LiAlH₄, THF, rt; (c) activated MnO₂, CHCl₃, rt; (d) Ph₃P=CCO₂Et, THF, reflux; (e) DBU, DIPEA, 130°C; (f) amines, NCS, NMP / H₂O (10/1), 80°C. (g) CF₃COOH, CH₂Cl₂. (h) R₂COCl or R₂NCO, CH₂Cl₂.

The mixture of ethyl 4-chloro-2-methylthio-5-pyrimidine carboxylate **24** (12.9 mmol), Et₃N (39.9 mmol), and amines (39.9 mmol) in THF (200 mL) was stirred at room temperature for 3 h. After evaporation of the resulting residue, the mixture was diluted with H₂O and extracted with EtOAc. After evaporation, the crude mixture was purified through silicagel to give amine substituted pyrimidine. To this amine substituted pyrimidine (6.9 mmol) in THF (100 mL) was added LiAlH₄ (13.8 mmol) at 0°C. After stirring at room temperature for 8 h, H₂O (1.81 mL), 2N NaOH (5.9 mL), and H₂O (1.81 mL) were added sequentially. The resulting solids were filtered off and the combined organic layer was evaporated and purified by combiflash to afford alcohol **25** (65 % yield for steps).

The mixture of compound **25** (9.7 mmol) and activated MnO₂ (72.7 mmol) in CHCl₃ (150 mL) was stirred at room temperature for 6 h. After filtering off the black solid, the resulting aldehyde was dissolved in THF (100 mL) and Ph₃P=CCO₂Et (12.9 mmol) was added. The reaction mixture was allowed to react at 65°C for 1 h. The resulting mixture was evaporated, dissolved in water, extracted with EtOAc, and purified by chromatography on silica gel (dichloromethane/methanol) to afford ester **26** (81 % yield for steps).

After adding DBU (51 mmol) and DIPEA (51 mmol) to ester **26** (17.3 mmol), the mixture was stirred at 130 °C for 12 h, evaporated, and purified by silica gel to afford pyrido[2,3-*d*]pyrimidin-7-ones **27** (87 % yield).

After stirring the mixture of compound **27** (2.0 mmol), NCS (2.1 mmol) in NMP (5 mL) and H₂O (0.5 mL) at 80°C for 15 min, amines (4.0 mmol) was added, stirred at 80°C for 8h, and purified by chromatography on silica gel (dichloromethane/methanol) to give compounds **4-10** (80 % yield).

The compounds **28** and **29** (0.2 mmol) in Et₃N (0.4 mmol) in CH₂Cl₂ (1 mL) and DMF (0.1 mL) were further functionalized by the addition of alkyl acid chlorides, aryl acid chlorides, or isocyanates (0.2 mmol) and purified by prep HPLC to give compounds **11-23** (90% yield).

Scheme 2. Reagents and conditions: (a) POCl_3 , PCl_5 , reflux; (b) cyclopropylboronic acid, $\text{Ph(PPh}_3)_4$, K_2CO_3 , dioxane/ H_2O ; (c) pyrrolidine, K_2CO_3 , acetone, 65°C ; (d) $(trans)$ -4-aminocyclohexanol, NCS, NMP / H_2O (10/1), 80°C .

The intermediate **27a** (5 mmol) was slowly added to POCl_3 (5 mL) and PCl_5 (1.2 g). The reaction mixture was heated under reflux for 4 h. After being cooled to about 20°C , it was poured into ice water. The solid thus separated as pale pink needles, was filtered, and washed with ice water to give compound **30**.

The mixture of **30** (2 mmol), cyclopropylboronic acid (4 mmol), and K_2CO_3 (4 mmol) was stirred at 110°C during 20 h in dioxane/ H_2O (3/1, 4 mL) in the presence of $\text{Ph(PPh}_3)_4$ complex under argon affords the corresponding products after addition of water, extraction with ether or dichloromethane, purified by silica gel chromatography to afford compound **31**.

The mixture of **30** (2 mmol), pyrrolidine (4 mmol), DIPEA (4 mmol) and NMP (5 mL) were added to a sealed tube and heated under microwave irradiation to 150°C for 75 minutes, then purified by silica gel chromatography to afford compound **32**.

After stirring the mixture of compound **31** or **32** (1.0 mmol), NCS (1.1 mmol) in NMP (3 mL) and H_2O (0.2 mL) at 80°C for 15 min, amines (2.0 mmol) was added, stirred at 80°C for 8h, and purified by chromatography on silica gel (dichloromethane/methanol) to give compounds **2** or **3**.

Scheme 3. Reagents and conditions: (a) amines, Et_3N , THF, rt; (b) LiOH , THF/ H_2O (1:1), 100°C ; (c) DIEA, NH_3 , 1-Benzotriazolol, MeOH/DMF , rt; (d) I_2 , K_2CO_3 , DMF, 80°C . (e) $(trans)$ -4-aminocyclohexanol, NCS, NMP / H_2O (10/1), 80°C .

The mixture of ethyl 4-chloro-2-methylthio-5-pyrimidine carboxylate **24** (20 mmol), Et_3N (60 mmol), and amines (60 mmol) in THF (200 mL) was stirred at room temperature for 3 h. After evaporation of the resulting residue, the mixture was diluted with H_2O and extracted with EtOAc . After evaporation, the crude mixture was purified through silica gel to give amine substituted pyrimidine. To this amine substituted pyrimidine (15 mmol)

in THF/H₂O (50/50 mL) was added LiOH (20 mmol). After stirring at 100 °C for 4 hrs, the solution was acidified by 2N HCl, resulting in precipitation. The aqueous suspension was extracted with EtOAc and the extracts combined, dried (MgSO₄), and purified by silica gel chromatography to afford acid **33** (71 % yield for steps).

To solution of **33** (20 mmol) in DMF (40 mL) were added HOBt (30 mmol), DIEA (30 mmol), and 2N NH₃/MeOH (30 mmol). The solution was stirred at room temperature for 16 hrs. The mixture was concentrated under reduced pressure and the residue was purified by silica gel chromatography (EtOAc/ hexanes) to afford compound **34**.

To a solution of **34** (10 mmol) dissolved in 20 mL of anhydrous DMF, 3,4,5-trimethoxybenzaldehyde (10 mmol) is added. To the resulting mixture while stirring, iodine (12 mmol) and anhydrous potassium carbonate (10 mmol) are added and then heated at 80°C for overnight. The mixture is poured into crushed ice-water in (100 mL). The precipitated product is filtered, washed with water, and purified by chromatography on silica gel to give compound **35**.

After stirring the mixture of compound **35** (2.0 mmol), NCS (2.1 mmol) in NMP (5 mL) and H₂O (0.5 mL) at 80°C for 15 min, amines (4.0 mmol) was added, stirred at 80°C for 8h, and purified by chromatography on silica gel (dichloromethane/methanol) to give compounds **4** (76 % yield).

1.2 Purity of inhibitors 1-23.

Table1, Purity of inhibitors

Cmpd	RT (min)	Purity,% 230 nm	Purity,% 254 nm		Cmpd	RT (min)	Purity,% 230 nm	Purity,% 254 nm
1	3.21	100	100		13	3.42	98	98
2	3.14	99	100		14	3.55	100	100
3	2.79	100	100		15	3.67	100	100
4	2.83	100	99		16	3.89	98	98
5	3.76	97	100		17	3.54	100	100
6	3.47	100	100		18	3.65	100	99
7	3.75	99	98		19	3.32	99	100
8	3.15	100	100		20	3.23	100	100
9	3.65	100	99		21	3.65	97	100
10	3.35	98	98		22	3.77	98	100
11	3.45	100	100		23	3.34	99	100
12	3.52	100	100					

1.3 Characterization of inhibitors 1-23.

(*trans*)-4-((7-cyclopropylpyrido[2,3-*d*]pyrimidin-2-yl)amino)cyclohexanol (**1**)

25% yield in six steps (TFA salt, white solid). ^1H NMR (DMSO- d_6 , 400 MHz) δ 9.12 (s, 1H), 8.27 (s, 1H), 7.16 (d, $J = 8.0$ Hz, 1H), 3.89-3.83 (m, 2H), 2.36-2.31 (m, 3H), 1.90-1.86 (m, 6H), 1.32-1.24 (m, 6H); LC/MS (ESI) m/z : $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{16}\text{H}_{20}\text{N}_4\text{O}$, 285.17; found, 285.21.

(trans)-4-((7-(pyrrolidin-1-yl)pyrido[2,3-*d*]pyrimidin-2-yl)amino)cyclohexanol (2)

23% yield in six steps (TFA salt, white solid). ^1H NMR (DMSO- d_6 , 400 MHz) δ 8.52 (s, 1H), 8.00 (d, $J = 8.4$ Hz, 1H), 6.75 (d, $J = 10.4$ Hz, 1H), 3.78 – 3.69 (m, 3H), 3.66 – 3.61 (m, 4H), 3.21 – 3.13 (m, 4H), 2.00-1.96 (m, 4H), 1.89-1.83 (m, 4H); LC/MS (ESI) m/z : $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{17}\text{H}_{23}\text{N}_5\text{O}$, 314.19; found, 314.15.

(trans)-7-((4-hydroxycyclohexyl)amino)-2-isopropylpyrimido[4,5-*d*]pyrimidin-4(3*H*)-one (3)

18% yield in six steps (TFA salt, white solid). ^1H NMR (DMSO- d_6 , 400 MHz) δ 12.23 (s, 1H), 7.52 (s, 1H), 3.76 – 3.67 (m, 2H); 2.70 (m, 2H), 2.17 (m, 2H), 1.79-1.58 (m, 5H), 1.30 (d, $J = 6.4$ Hz, 6H); LC/MS (ESI) m/z : $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{15}\text{H}_{21}\text{N}_5\text{O}_2$, 304.17; found, 304.25.

2-(4-Hydroxymethyl-piperidin-1-yl)-8-isopropyl-8*H*-pyrido[2,3-*d*]pyrimidin-7-one (4)

32% yield in six steps (TFA salt, white solid). ^1H NMR (DMSO- d_6 , 400 MHz) δ 8.62 (s, 1H), 7.67 (d, $J = 9.2$ Hz, 1H), 6.21 (d, $J = 9.2$ Hz, 1H), 5.62 (s, br, 1H), 4.76 – 4.72 (m, 2H), 3.78 – 3.69 (m, 1H), 3.29 – 3.26 (m, 2H), 3.02 – 2.94 (m, 2H), 1.77 – 1.71 (m, 2H), 1.52 (d, $J = 6.8$ Hz, 6H), 1.16 – 1.09 (m, 2H); LC/MS (ESI) m/z : $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{16}\text{H}_{22}\text{N}_4\text{O}_2$, 303.17; found, 303.24.

2-(((2*S*,3*S*)-1-hydroxy-3-methylpentan-2-yl)amino)-8-isopropylpyrido[2,3-*d*]pyrimidin-7(8*H*)-one (5)

38% yield in six steps (TFA salt, white solid). ^1H NMR (DMSO- d_6 , 400 MHz) δ 8.56 (s, 1H), 7.65 (d, $J = 9.2$ Hz, 1H), 6.27 – 6.11 (m, 1H), 5.65 (s, br, 1H), 3.94 (s, 1H), 3.56 (qd, $J = 11.0, 3.9$ Hz, 2H), 3.10 (dd, $J = 7.2, 4.9$ Hz, 2H), 1.59-1.45 (m, 7H), 1.19 (dd, $J = 14.2, 7.0$ Hz, 2H), 0.91 (d, $J = 8.0$ Hz, 3H), 0.84 (d, $J = 7.6$ Hz, 3H); LC/MS (ESI) m/z : $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{16}\text{H}_{24}\text{N}_4\text{O}_2$, 304.19; found, 304.23.

(*R*)-2-((8-isopropyl-7-oxo-7,8-dihydropyrido[2,3-*d*]pyrimidin-2-yl)amino)-4-methylpentanoic acid (6)

31% yield in six steps (TFA salt, white solid). ^1H NMR (DMSO- d_6 , 400 MHz) δ 8.59 (s, 1H), 7.67 (d, 6.0 Hz, 1H), 6.22 (s, 1H), 5.62 (s, br, 1H), 4.46 (s, 2H), 1.79 (dd, J = 13.1, 7.3 Hz, 2H), 1.59-1.48 (m, 7H), 0.98 – 0.84 (m, 6H); LC/MS (ESI) m/z : $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{16}\text{H}_{22}\text{N}_4\text{O}_3$, 319.17; found, 319.21.

8-isopropyl-2-((tetrahydro-2H-pyran-4-yl)amino)pyrido[2,3-d]pyrimidin-7(8H)-one (7)

34% yield in six steps (TFA salt, white solid). ^1H NMR (DMSO- d_6 , 400 MHz) δ 8.58 (s, 1H), 7.66 (d, J = 9.4 Hz, 1H), 6.21 (d, J = 9.4 Hz, 1H), 5.63 (s, br, 1H), 4.59-4.51 (m, 1H), 3.99-3.95 (m, 1H), 3.94-3.90 (m, 2H), 3.46-3.36 (m, 2H), 1.89-1.82 (m, 2H), 1.59-1.49 (m, 8H); LC/MS (ESI) m/z : $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{15}\text{H}_{20}\text{N}_4\text{O}_2$, 289.16; found, 289.17.

8-isopropyl-2-((1-methylpiperidin-4-yl)amino)pyrido[2,3-d]pyrimidin-7(8H)-one (8)

35% yield in six steps (TFA salt, white solid). ^1H NMR (DMSO- d_6 , 400 MHz) ^1H NMR (400 MHz, DMSO) δ 8.59 (s, 1H), 7.66 (d, J = 9.2 Hz, 1H), 6.22 (d, J = 8.4 Hz, 1H), 5.68 (s, br, 1H), 4.12-4.04 (m, 1H), 3.39-3.33 (m, 2H), 3.08-3.01 (m, 2H), 2.73 (s, 3H); 2.09-2.03 (m, 2H), 1.76-1.70 (m, 2H), 1.53 (d, J = 6.4 Hz, 6H); LC/MS (ESI) m/z : $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{16}\text{H}_{23}\text{N}_5\text{O}$, 302.17; found, 302.23.

8, 8-isopropyl-2-(piperidin-4-ylamino)pyrido[2,3-d]pyrimidin-7(8H)-one (9)

37% yield in six steps (TFA salt, white solid). ^1H NMR (DMSO- d_6 , 400 MHz) ^1H NMR (400 MHz, DMSO) δ 8.61 (s, 1H), 7.68 (d, J = 9.3 Hz, 1H), 6.23 (d, J = 8.4 Hz, 1H), 5.69 (s, br, 1H), 4.11-4.04 (m, 1H), 3.36 (dd, J = 16.4, 9.2 Hz, 2H), 3.09-3.00 (m, 3H), 2.11-2.03 (m, 2H), 1.77-1.69 (m, 2H), 1.51 (d, J = 6.4 Hz, 6H); LC/MS (ESI) m/z : $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{15}\text{H}_{21}\text{N}_5\text{O}$, 288.17; found, 288.21.

(trans)-2-(4-Amino-cyclohexylamino)-8-isopropyl-8H-pyrido[2,3-d]pyrimidin-7-one (10)

38% yield in six steps (TFA salt, white solid). ^1H NMR (DMSO- d_6 , 400 MHz) δ 8.56 (s, 1H), 7.84 (s, br, 2H), 7.66 (d, J = 9.2 Hz, 1H), 6.23 – 6.16 (m, 1H), 5.65 – 5.62 (m, 1H), 3.79 – 3.68 (m, 2H), 3.04 (s, br, 1H), 2.02 – 1.95 (m, 4H), 1.54 (d, J = 6.8 Hz, 6H), 1.53 – 1.40 (m, 4H); LC/MS (ESI) m/z : $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{16}\text{H}_{23}\text{N}_5\text{O}$, 302.19; found, 302.21.

(trans)-N-[4-(8-Isopropyl-7-oxo-7,8-dihydro-pyrido[2,3-*d*]pyrimidin-2-ylamino)-cyclohexyl]-isobutyramide (11)

33% yield in seven steps (TFA salt, white solid). ^1H NMR (MeOD- d_4 , 400 MHz) δ 8.54 (s, 1H), 7.66 (d, J = 8.4 Hz, 1H), 6.31 (s, br, 1H), 5.77 (s, br, 1H), 3.89 – 3.86 (m, 1H), 3.69 – 3.66 (m, 1H), 2.43 – 2.39 (m, 1H), 2.16 – 2.10 (m, 2H), 2.00 – 1.96 (m, 2H), 1.60 (s, br, 6H), 1.59 – 1.38 (m, 4H), 1.10 (d, J = 6.8 Hz, 6H); LC/MS (ESI) m/z : $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{20}\text{H}_{29}\text{N}_5\text{O}_2$, 372.23; found, 371.22.

(trans)-1-Ethyl-3-[4-(8-isopropyl-7-oxo-7,8-dihydro-pyrido[2,3-*d*]pyrimidin-2-ylamino)-cyclohexyl]-urea (12)

33% yield in seven steps (TFA salt, white solid). ^1H NMR (MeOD- d_4 , 400 MHz) δ 8.53 (s, 1H), 7.66 (d, J = 9.4 Hz, 1H), 6.33 (d, J = 7.6 Hz, 1H), 5.76 (s, br, 1H), (m, 1H), 5.75 (s, br, 1H), 3.93 – 3.85 (m, 1H), 3.55 – 3.48 (m, 1H), 2.15 – 1.99 (m, 4H), 1.59 (s, br, 6H), 1.58 – 1.48 (m, 2H), 1.38 – 1.29 (m, 2H), 1.09 (t, J = 7.2 Hz, 3H); LC/MS (ESI) m/z : $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{19}\text{H}_{28}\text{N}_6\text{O}_2$, 373.22; found, 373.26.

(trans)-1-Isopropyl-3-[4-(8-isopropyl-7-oxo-7,8-dihydro-pyrido[2,3-*d*]pyrimidin-2-ylamino)-cyclohexyl]-urea (13)

31% yield in seven steps (TFA salt, white solid). ^1H NMR (MeOD- d_4 , 400 MHz) δ 8.54 (s, 1H), 7.66 (d, J = 9.4 Hz, 1H), 6.35 – 6.33 (m, 1H), 5.76 (s, br, 1H), 3.88 – 3.76 (m, 2H), 3.55 – 3.48 (m, 1H), 2.15 – 1.99 (m, 4H), 1.60 (s, br, 6H), 1.59 – 1.29 (m, 4H), 1.11 (d, J = 6.6 Hz, 6H); LC/MS (ESI) m/z : $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{20}\text{H}_{30}\text{N}_6\text{O}_2$, 387.24; found, 387.22.

(trans)-1-cyclopentyl-3-(((4-(8-isopropyl-7-oxo-7,8-dihydro-pyrido[2,3-*d*]pyrimidin-2-yl)amino)-cyclohexyl)urea (14)

35% yield in seven steps (TFA salt, white solid). ^1H NMR (DMSO- d_6 , 400 MHz) δ 8.56 (s, 1H), 7.71 (s, 1H), 7.65 (d, J = 9.4 Hz, 1H), 6.19-6.14 (m, 1H), 5.65-5.54 (m, 1H), 3.78-3.71 (m, 2H), 3.37-3.31 (m, 1H), 2.59-2.53 (m, 1H), 1.89-1.72 (m, 8H), 1.70 – 1.54 (m, 8H), 1.33 (d, J = 6.8 Hz, 6H); LC/MS (ESI) m/z : $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{22}\text{H}_{32}\text{N}_6\text{O}_2$, 413.26; found, 413.23.

(*cis*)-*N*-[4-(8-Isopropyl-7-oxo-7,8-dihydro-pyrido[2,3-*d*]pyrimidin-2-ylamino)-cyclohexyl]-isobutyramide (15)

30% yield in seven steps (TFA salt, white solid). ^1H NMR (DMSO- d_6 , 400 MHz) δ 8.57 (s, 1H), 7.76 – 7.51 (m, 2H), 6.24 – 6.15 (m, 1H), 5.75 – 5.54 (m, 1H), 3.72 – 3.64 (m, 2H), 2.47 – 2.42 (m, 1H), 1.81 – 1.62 (m, 6H), 1.54 – 1.43 (m, 8H), 0.99 (d, J = 6.8 Hz, 6H); LC/MS (ESI) m/z : $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{20}\text{H}_{29}\text{N}_5\text{O}_2$, 372.23; found, 372.25.

(*cis*)-1-Ethyl-3-[4-(8-isopropyl-7-oxo-7,8-dihydro-pyrido[2,3-*d*]pyrimidin-2-ylamino)-cyclo-hexyl]-urea (16)

32% yield in seven steps (TFA salt, white solid). ^1H NMR (MeOD- d_4 , 400 MHz) δ 8.56 (s, 1H), 7.67 (d, J = 9.4 Hz, 1H), 6.35 (d, J = 8.8 Hz, 1H), 5.77 (s, br, 1H), 4.07 – 4.03 (m, 1H), 3.75 – 3.71 (m, 1H), 3.15 (q, J = 7.2 Hz, 2H), 1.88 – 1.72 (m, 8H), 1.59 (d, J = 6.8 Hz, 6H), 1.10 (t, J = 7.2 Hz, 3H); LC/MS (ESI) m/z : $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{19}\text{H}_{28}\text{N}_6\text{O}_2$, 373.22; found, 373.23.

2-((1-Isobutyrylpiperidin-4-yl)amino)-8-isopropylpyrido[2,3-*d*]pyrimidin-7(8*H*)-one (17)

32% yield in seven steps (TFA salt, white solid). ^1H NMR (DMSO- d_6 , 400 MHz) δ 8.59 (s, 1H), 7.66 (d, J = 9.2 Hz, 1H), 6.22 – 6.16 (m, 1H), 5.65 (s, br, 1H), 4.39 – 4.28 (m, 2H), 4.13 – 3.91 (m, 2H), 2.94 – 2.86 (m, 1H), 2.83 – 2.64 (m, 1H), 2.01 – 1.82 (m, 2H), 1.58 – 1.34 (m, 8H), 1.02 (d, J = 6.4 Hz, 6H); LC/MS (ESI) m/z : $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{19}\text{H}_{27}\text{N}_5\text{O}_2$, 358.22; found, 358.28.

***N*-ethyl-4-((8-isopropyl-7-oxo-7,8-dihydropyrido[2,3-*d*]pyrimidin-2-yl)amino)piperidine-1-carboxamide (18)**

36% yield in seven steps (TFA salt, white solid). ^1H NMR (MeOD- d_4 , 400 MHz) δ 8.55 (s, 1H), 7.67 (d, $J = 9.3$ Hz, 1H), 6.34 – 6.30 (m, 1H), 5.78 (s, br, 1H), 4.06 – 4.01 (m, 3H), 3.19 (q, $J = 7.2$ Hz, 2H), 3.02 – 2.96 (m, 2H), 2.04 – 1.98 (m, 2H), 1.62 – 1.44 (m, 8H), 1.11 (t, $J = 4.8$ Hz, 3H); LC/MS (ESI) m/z : $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{18}\text{H}_{26}\text{N}_6\text{O}_2$, 359.21; found, 359.28.

4-(8-Isopropyl-7-oxo-7,8-dihydro-pyrido[2,3-*d*]pyrimidin-2-ylamino)-piperidine-1-carboxylic acid isopropylamide (19)

32% yield in seven steps (TFA salt, white solid). ^1H NMR (MeOD- d_4 , 400 MHz) δ 8.54 (s, 1H), 7.67 (d, $J = 9.4$ Hz, 1H), 6.32 (d, $J = 7.7$ Hz, 1H), 5.78 (s, br, 1H), 4.16 – 4.02 (m, 3H), 3.95 – 3.85 (m, 1H), 3.02 – 2.91 (m, 2H), 2.06 – 1.97 (m, 2H), 1.64 – 1.47 (m, 8H), 1.50 (d, $J = 6.6$ Hz, 6H); LC/MS (ESI) m/z : $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{19}\text{H}_{28}\text{N}_6\text{O}_2$, 373.22; found, 373.24

(*trans*)-1-isopropyl-3-(4-((7-oxo-7,8-dihydropyrido[2,3-*d*]pyrimidin-2-yl)amino)cyclohexyl)urea (20)

32% yield in seven steps (TFA salt, white solid). ^1H NMR (DMSO- d_6 , 400 MHz) δ 8.53 (s, 1H), 7.86 (s, 2H), 7.66 (d, $J = 9.2$ Hz, 1H), 6.21 (d, $J = 9.2$ Hz, 1H), 5.50 (s, br, 1H), 3.51 – 3.45 (m, 2H), 3.33 – 3.31 (m, 1H), 1.95 – 1.87 (m, 4H), 1.46 – 1.28 (m, 2H), 1.21 (d, $J = 9.2$ Hz, 3H), 1.03 (d, $J = 6.4$ Hz, 6H); LC/MS (ESI) m/z : $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{17}\text{H}_{24}\text{N}_6\text{O}_2$, 345.20; found, 345.22.

(*trans*)-1-[4-(8-methyl-7-oxo-7,8-dihydro-pyrido[2,3-*d*]pyrimidin-2-ylamino)-cyclohexyl]-3-isopropyl-urea (21)

32% yield in seven steps (TFA salt, white solid). ^1H NMR (DMSO- d_6 , 400 MHz) δ 8.59 (s, 1H), 7.71 (d, $J = 9.2$ Hz, 1H), 6.25 (d, $J = 9.3$ Hz, 1H), 5.57 (br, 1H), 3.52 (s, 3H), 3.50 – 3.43 (m, 2H), 3.36 – 3.30 (m, 1H), 1.96 – 1.86

(m, 4H), 1.44 – 1.30 (m, 2H), 1.20 (d, $J = 9.2$ Hz, 2H), 1.01 (d, $J = 6.4$ Hz, 6H); LC/MS (ESI) m/z : $[M+H]^+$ calcd for $C_{18}H_{26}N_6O_2$, 359.22; found, 359.25.

(*trans*)-1-[4-(8-Cyclopentyl-7-oxo-7,8-dihydro-pyrido[2,3-*d*]pyrimidin-2-ylamino)-cyclohexyl]-3-isopropyl-urea (22)

33% yield in seven steps (TFA salt, white solid). 1H NMR (MeOD- d_4 , 400 MHz) δ 8.54 (s, 1H), 7.66 (d, $J = 9.3$ Hz, 1H), 6.34 – 6.29 (m, 1H), 5.89 (s, br, 1H), 3.89 – 3.75 (m, 2H), 3.58 – 3.47 (m, 2H), 2.49 – 2.22 (m, 2H), 2.14 – 1.97 (m, 4H), 1.91 – 1.79 (m, 2H), 1.78 – 1.61 (m, 2H), 1.57 – 1.42 (m, 2H), 1.37 – 1.24 (m, 4H), 1.11 (d, $J = 6.3$ Hz, 6H); LC/MS (ESI) m/z : $[M+H]^+$ calcd for $C_{22}H_{32}N_6O_2$, 413.26; found, 413.23.

(*trans*)-1-[4-(8-(tetrahydro-2H-pyran-4-yl)-7-oxo-7,8-dihydro-pyrido[2,3-*d*]pyrimidin-2-ylamino)-cyclohexyl]-3-isopropyl-urea (23)

36% yield in seven steps (TFA salt, white solid). 1H NMR (DMSO- d_6 , 400 MHz) δ 8.57 (s, 1H), 7.96 (s, 2H), 7.68 (d, $J = 9.2$ Hz, 1H), 6.23 (d, $J = 9.2$ Hz, 1H), 5.50 (s, br, 1H), 4.05 – 4.00 (m, 4H), 3.46 – 3.29 (m, 4H), 2.98 – 2.93 (m, 1H), 1.98 (d, $J = 12.4$ Hz, 2H), 1.89 (d, $J = 12.2$ Hz, 3H), 1.49 (d, $J = 12.2$ Hz, 2H), 1.38 (d, $J = 13.3$ Hz, 2H), 1.20 (d, $J = 8.5$ Hz, 2H), 1.01 (d, $J = 6.4$ Hz, 6H); LC/MS (ESI) m/z : $[M+H]^+$ calcd for $C_{22}H_{32}N_6O_3$, 429.25; found, 429.27.

Compound 13 H-NMR, DMSO-d₆

Compound 22 H-NMR, DMSO-d₆

2.1 JNK-1/2/3 and p38 assays

Homogeneous Time-resolved Fluorescence Assay—Enzyme inhibition studies were performed in 384-well polystyrene homogeneous time-resolved fluorescence plates (Grainier) for 1 hr. at ambient temperature (~22 °C) with 0.5 μ M biotinylated FL-ATF-2, 1.25 μ M ATP, 0.75 nM activated JNK3 α 1 (with a control in the absence of kinase for determining the basal signal), JNK2, or JNK1 in 10- μ l volumes containing the final concentrations of the following: 50 mM Hepes, pH 7.0, 2.5 mM MgCl₂, 0.1 mg/ml bovine serum albumin, 1 mM DL-dithiothreitol, 0.01% Triton X-100 (all from Sigma-Aldrich), and 5% DMSO (with or without compound). A 10-point titration of all compounds was carried out in 3-fold dilutions from 10 μ M to 0.5 nM. After 22 min, the kinase reaction was terminated by addition of 10 μ l of quenching solution (1X Lance Buffer, detection reagents, streptavidin-xLAPC (200 nM) and europium cryptate-labeled rabbit polyclonal anti-phospho-ATF-2 (1 nM), were from Cis-Bio. The homogeneous time-resolved fluorescence signal was detected using an EnVision plate reader 1 hr post-quenching. The data from four different experiments were averaged and presented as the mean $\pm$ S.D. IC₅₀ values were determined by fitting the data to the equation for a four-parameter logistic.

2.2 Cell Culture: SHSY5Y cells (ATCC) were grown at normal cell culture conditions (37°C and 5% CO₂) in DMEM/F:12 (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin. To assure that the cells were actively growing, only cells at ~80% confluency and between passages five and fifteen were used in our experiments. The cells were serum starved for 24 hrs in DMEM/F:12 media containing 2% FBS before any treatment.

2.3 Mitochondrial membrane depolarization: Mitochondrial membrane depolarization was monitored by MitoTracker® Orange CMTRos (Invitrogen) fluorescence. SHSY5Y cells (60,000 cells/well) were seeded in to black walled, clear bottomed 96 well plate. Cells were incubated with compounds for half an hour before adding 35 μ M 6-hydroxy dopamine (6-OHDA) for 4-5 hrs. After the 4-5 hr incubation, cells were stained with 500 nM MitoTracker® Orange CMTRos for 30 minutes under growth conditions. The cells were washed twice in Hank's Buffer Salt Solution (HBSS), and placed in pre-warmed HBSS for fluorescent recordings. Fluorescence was detected by exciting the fluorophore at 554 nm and monitoring the emission at 576 nm on a SpectraMax e5 plate reader (Molecular Devices). Mitochondrial membrane depolarisation was normalized to cell abundance by staining the cells with Hoechst 33342 (excitation: 350nm; emission: 450nm).

2.4 In cell Western assay: SHSY5Y (60,000 cells/well) were plated in a clear-bottomed Packard View black 96-well plate in 100 μ l of 10% FBS DMEM:F12 medium and were allowed to attach overnight. Next day, the cells were treated with the compounds for 1 hr prior to induction of the JNK pathway activation. The cells were treated with 35 μ M 6-OHDA for 4-5 hrs. Cells were then fixed in 4% paraformaldehyde in PBS for 20 min at room temperature (RT) with no shaking. They were then washed once with 0.1 M glycine to neutralize paraformaldehyde for 5 min. Cells were permeabilized with 0.2% Triton X-100 in PBS for 20 min at RT on orbital shaker after which they were washed once with PBS for 5 min. They were then incubated with Licor Blocking Buffer in PBS (1:1 dilution in PBS) for 1-1.5 h rocking at RT. Cells were incubated with primary antibody p-c-Jun S63 Ab (Cell Signaling # 9261) 1:100 dilution in Licor blocking buffer overnight at 4°C. Next day, they were washed twice with PBS-0.1 % Tween 20 (PBST) washing solution for 5 min each at room temperature on the orbital shaker, followed by one wash with Licor Blocking Buffer containing 0.05% Tween-20 for 5 min on the shaker at RT. The cells were then incubated with secondary antibody goat anti-rabbit IR800 (1:500 dilution) for 1 hr at RT in the dark (covered the plate with foil) in Licor blocking buffer-containing Tween-20. Following this, cells were washed twice with PBST for 5 min each at room temperature and then once with Licor blocking buffer-containing 0.05% Tween-20. The wells were then incubated with ToPro 3 stain (nucleic acid staining), diluted 1:4000 in Licor blocking buffer or Licor blocking buffer with 0.05% Tween-20 for 30 min at room temperature in the dark. Finally the plates were washed twice with PBS and analyzed using the Odyssey LICOR Infrared Scanner.

3.1 Crystallization: Purification of JNK3 39-402 and its crystallization with ATP analogs was done following previously published procedure.¹⁻⁴ **22** was soaked into the crystal by adding 2 mM of the compound into the

crystallization drop and incubating for 24 hrs. The crystals were then transferred to mounting loops and excess soaking solutions were removed and flash frozen by plunging into liquid nitrogen.

3.2 Data collection and refinement: A diffraction dataset was collected at in-house diffraction facility equipped with Micromax-007 HFM (Rigaku Americas) and Mar345dtb (Mar Research). The dataset was processed with autoProc with XDS as the data reduction engine. The PDB ID 1 JNK was used to as the molecular replacement model. Quick molecular replacements using Phaser in Phenix suite properly positioned JNK3, **22** was identified as positive densities in ATP binding pockets. Restraints and coordinates for the compounds were generated using eLBOW in Phenix suite and incorporated into the JNK3 coordinate using the graphics program Coot. The models were then refined using autoBuster with TLS (translation, libration, and screw-motion), water update and unknown ligand search options turned on. The models were manually inspected and adjusted after each refinement cycle using Coot. The refinement was completed after the free and crystallographic R-factors stabilized. Data processing and refinement statistics are given in SI. Structural analysis and figure preparations were done with PyMol.

3.3 The Co-crystal structure of compound 22 in human JNK3: Transparent sphere and wire model of residues involved in hydrophobic pocket formation. The two residues, I70 and A151 sandwich the cyclohexyl ring of compound **22**. Residues are labeled. Residues not visible from the panel are indicated with the arrowed box. **22** is colored orange, I70 and A151 are colored dark grey and the rest residues are colored light grey.

3.4 The Co-crystal structure of compound 13 in human JNK3:

4. Pharmacology experiment

4.1 Pharmacokinetics

Pharmacokinetics studies were conducted in Sprague Dawley rats. The compound was formulated in a generic formulation at 1 mg/mL (e.g. 10:10:80, DMSO:tween 80: water, v:v:v) and dosed at 0.5 mg/kg intravenous into the femoral vein or 3 mg/kg by oral gavage. Blood was obtained at t=5 min, 15 min, 30 min, 1hr, 2hr, 4hr, 6hr, and 8hr. Blood was collected into EDTA containing tubes and plasma was generated by standard centrifugation methods. All procedures and handling were according to standard operating procedures approved by IAPUC at Scripps Florida. In order to assess in vivo pharmacokinetic parameters an LC-MS/MS bioanalytical method was developed where 25 μ L of plasma was treated with 125 μ L of acetonitrile containing an internal standard in a Millipore Multiscreen Slovinter 0.45 micron low binding PTFE hydrophilic filter plate (#MSRLN0450) and allowed to shake at room temperature for five minutes. The plate was then centrifuged for 5 minutes at 4000 rpm in a tabletop centrifuge and the filtrate was collected in a polypropylene capture plate. The filtrate (10 μ L) is injected using an Agilent 1200 HPLC equipped with a Thermo Betasil C18 HPLC column 5 T (50 $\times$ 2.1 mm) #70105-052130. Mobile Phase A was water with 0.1% formic acid. Mobile phase B was acetonitrile with 0.1% formic acid. Flow rate was 375 μ L/min using a gradient of 90%A/10% B from, 0-0.5 min, ramped to 5%A/95%B at 2 min, held at 5%A/95%B until 3.0 min, ramped to 90% A/10% B at 4 min, and held at 90%A/10%B until 7 min. An API Sciex 4000 equipped with a turbo ion spray source was used for all analytical measurement. MRM methods were developed in position ion mode. Peak areas of the analyte ion were measured against the peak areas of the internal standard. Data was fit using WinNonLin using an IV bolus model.⁵⁻¹⁰

4.2 p450s inhibition

p450s inhibition for four major isoforms are evaluated using a cocktail inhibition assay where the metabolism of specific marker substrate (CYP1A2 phenacetin demethylation to acetaminophen); CYP2C9, tolbutamide hydroxylation to hydrocytolbutamide; CYP2D6, bufuralol hydroxylation to 4'-Hydroxybufuralol; and CYP3A4, midazolam hydroxylation to 1'-Hydroxymidazolam) in the presence or absence of 10 TM probe compound is evaluated. The concentration of each marker substrate is approximately its K_m . Conditions were similar to those described by Tesino and Patonay¹¹ except 2C19 was not evaluated as we found that stock solution of the 2C19 probe substrate, omeprazole, had poor stability. Specific inhibitors for each isoform are included in each run to validate the system.

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