Discovery of a novel series of thienopyrimidine as highly potent and selective PI3K inhibitors

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Abstract

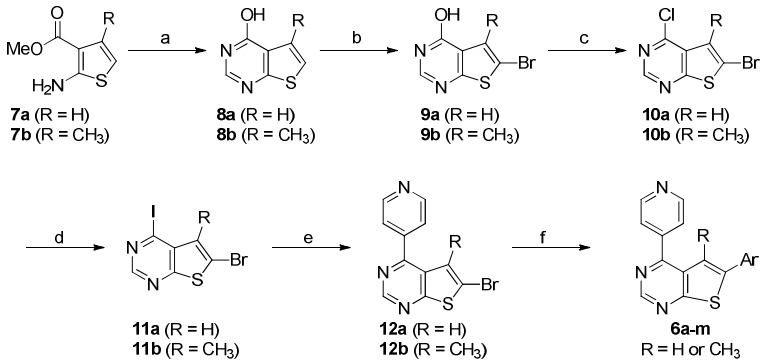
Inhibition of the phosphoinositide 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) signaling pathway provides a promising new approach for cancer therapy. Through a rational design, a novel series of thienopyrimidine was discovered as highly potent and selective PI3K inhibitors. These thienopyrimidine derivatives were demonstrated to bear nanomolar PI3Kα inhibitory potency with over 100-fold selectivity against mTOR kinase. The lead compounds **6g** and **6k** showed good developability profiles in cell-based proliferation and ADME assays. In this communication, their design, synthesis, structure-activity relationship, selectivity, and some developability properties are described.

Supplementary data

1. General experimental procedures

All reagents and solvents were from commercial sources. ^{1}H NMR spectra were recorded on a Varian OXFORD 300 NMR spectrometer (300 MHz) or a Bruker AVANCE^{III} 400 NMR spectrometer (400 MHz) and referenced to tetramethyl silane. ^{13}C NMR spectra were recorded on a Bruker AVANCE^{III} 400 NMR spectrometer (400 MHz). Chemical shifts are expressed as δ units using tetramethylsilane as the external standard (in NMR description, s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; and br, broad peak). Mass spectra were measured with a Shimadzu 6110 MS spectrometer using an ESI source coupled to a HPLC system operating in reverse mode. HRMS spectra were measured with a Bruker Apex IV FTMS spectrometer.

2. General procedure for the synthesis of compounds 6a-m



Scheme 1. Synthesis of compounds **6a-m**. Reagents and conditions: (a) formamidine acetate, NMP, 135 °C, Ar; (b) Br_2, HOAc, rt; (c) $POCl_3$, reflux; (d) NaI, anhydrous dioxane, 105 °C, Ar; (e) pyridine-4-boronic acid,

2N K_2CO_3, $Pd(PPh_3)_2Cl_2$, dioxane, 100 °C, Ar; (f) aryl borate or aryl boronic acid, $PdCl_2(dppf)$, 2N K_2CO_3, dioxane, 100 °C, Ar.

thieno[2,3-*d*]pyrimidin-4-ol **8a**

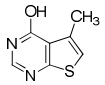
8a

A mixture of methyl 2-aminothiophene-3-carboxylate **7a** (15.72 g, 100 mmol) and formamidine acetate (20.6 g, 200 mmol) in *N*-methylpyrrolidone (80 mL) was stirred at 135 °C under argon atmosphere for 24 hours. After cooling to room temperature, the reaction mixture was diluted with water (400 mL), extracted with ethyl acetate (200 mL×3). The combined organic layers were washed with water (200 mL×3) and brine (200 mL), dried over anhydrous sodium sulfate, filtered and concentrated to afford the crude product **8a** as a brown solid (13 g, 85% yield), which was used directly in the next step without further purification.

^{1}H NMR (400 MHz, DMSO-d_6) δ 12.48 (br s, 1H), 8.12 (d, J = 2.7 Hz, 1H), 7.58 (d, J = 5.8 Hz, 1H), 7.39 (d, J = 5.8 Hz, 1H).

MS (ESI+) m/z 153.1 $[M + H]^+$.

5-methylthieno[2,3-*d*]pyrimidin-4-ol **8b**

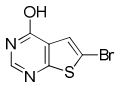


8b

Compound **8b** was prepared according to the procedure described for compound **8a**, using methyl 2-amino-4-methylthiophene-3-carboxylate **7b** instead of methyl 2-aminothiophene-3-carboxylate **7a.**

^{1}H NMR (400 MHz, DMSO-d_6): δ 12.31 (br s, 1H), 8.05 (s, 1H), 7.14 (s, 1H), 2.46 (s, 3H)

6-bromothieno[2,3-*d*]pyrimidin-4-ol **9a**



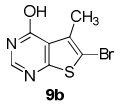
9a

To a mixture of thieno[2,3-*d*]pyrimidin-4-ol **8a** (13 g, 85.43 mmol) in acetic acid (250 mL) was added liquid bromine (8.75 mL, 170.86 mmol), and the resulting reaction mixture was stirred at room temperature for 3 hours. The reaction mixture was partitioned between water (1000 mL) and ethyl acetate (300 mL), and the remnant bromine was quenched with saturated aqueous sodium thiosulfate solution. The resulting yellow solid was collected by suction filtration, dried at 50 °C to afford the crude product (batch I, 13.40 g). The filtrate was transferred to a seperation funnel, and the aqueous layer was extracted with ethyl acetate (300 mL×2). The combined organic layers were washed with water (300 mL×3) and brine (300 mL), dried over anhydrous sodium sulfate, filtered and concentrated to afford the crude product (batch II, 2.80 g). The two batches of crude product **9a** were combined (16.2 g, 82.4% yield), and used directly in the next step without further purification.

^{1}H NMR (400 MHz, DMSO-d_6) δ 12.65 (br s, 1H), 8.15 (s, 1H), 7.56 (s, 1H).

MS (ESI+) m/z 230.9, 232.9 $[M + H]^+$.

6-bromo-5-methylthieno[2,3-*d*]pyrimidin-4-ol **9b**

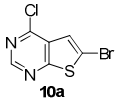


9b

Compound **9b** was prepared according to the procedure described for compound **9a**, using 5-methylthieno[2,3-*d*]pyrimidin-4-ol **8b** instead of thieno[2,3-*d*]pyrimidin-4-ol **8a.**

^{1}H NMR (400 MHz, DMSO-d_6): δ 12.57 (br s, 1H), 8.11 (s, 1H), 2.43 (s, 3H)

6-bromo-4-chlorothieno[2,3-*d*]pyrimidine **10a**



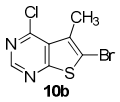
10a

A mixture of 6-bromothieno[2,3-*d*]pyrimidin-4-ol **9a** (13.58 g, 58.77 mmol) in phosphorus oxychloride (70 mL) was stirred under reflux for 2 hours. After removing phosphorus oxychloride in vacuo, the residue was poured into ice-water and neutralized with saturated aqueous sodium bicarbonate solution. The resulting mixture was extracted with ethyl acetate (100 mL×3). The combined organic layers were washed with water (100 mL×2) and brine (100 mL), dried over anhydrous sodium sulfate, filtered and concentrated. The residue was purified with flash column chromatography (silica gel, petroleum ether/ethyl acetate = 50:1) to afford the product **10a** as a pale yellow solid (12.5 g, 84.9% yield).

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.96 (s, 1H), 7.90 (s, 1H).

MS (ESI+) m/z 248.9, 250.9 $[M + H]^+$.

6-bromo-4-chloro-5-methylthieno[2,3-*d*]pyrimidine **10b**

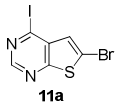


10b

Compound **10b** was prepared according to the procedure described for compound **10a**, using 6-bromo-5-methylthieno[2,3-*d*]pyrimidin-4-ol **9b** instead of 6-bromothieno[2,3-*d*]pyrimidin-4-ol **9a.**

^{1}H NMR (400 MHz, DMSO-d_6): δ 8.92 (s, 1H), 2.60 (s, 3H)

6-bromo-4-iodothieno[2,3-*d*]pyrimidine **11a**



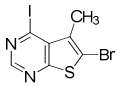
11a

A solution of 6-bromo-4-chlorothieno[2,3-*d*]pyrimidine **10a** (11 g, 44.1 mmol) in anhydrous tetrahydrofuran (80 mL) was bubbled with dry hydrochloride gas for 30 min, and then the volatiles were removed in vacuo. To the residue was added anhydrous 1,4-dioxane (330 mL) and sodium iodide (33.0 g, 200.5 mmol). The resulting mixture was stirred at 110 °C under argon atmosphere for 24 hours. After removing 1,4-dioxane in vacuo, the residue was diluted with water (300 mL) and then treated with saturated aqueous sodium thiosulfate (10 mL). The resulting mixture was extracted with ethyl acetate (100 mL×3). The combined organic layers were washed with water (100 mL×2) and brine (100 mL), dried over anhydrous sodium sulfate, filtered and concentrated. The residue was purified with flash column chromatography (silica gel, petroleum ether/ethyl acetate = 50:1) to afford the product **11a** as a pale yellow solid (10 g, 66% yield).

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.75 (s, 1H), 7.65 (s, 1H).

MS (ESI+) m/z 340.8, 342.8 $[M + H]^+$.

6-bromo-4-iodo-5-methylthieno[2,3-*d*]pyrimidine **11b**

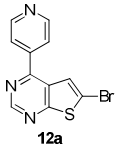


11b

Compound **11b** was prepared according to the procedure described for compound **11a**, using 6-bromo-4-chloro-5-methylthieno[2,3-*d*]pyrimidine **10b** instead of 6-bromo-4-chlorothieno[2,3-*d*]pyrimidine **10a**.

^{1}H NMR (400 MHz, DMSO-d_6): δ 8.69 (s, 1H), 2.61 (s, 3H).

6-bromo-4-(pyridin-4-yl)thieno[2,3-*d*]pyrimidine **12a**



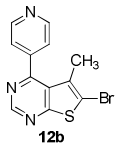
12a

A mixture of 6-bromo-4-iodothieno[2,3-*d*]pyrimidine **11a** (3.41 g, 10 mmol), pyridine-4-boronic acid (1.29g, 10.5 mmol) and 2N aqueous potassium carbonate solutionp (15 mL, 30 mmol) in 1,4-dioxane (75 mL) was degassed, and then bis(triphenylphosphine)palladium(II) chloride (351 mg, 0.5 mmol) was added. The resulting mixture was degassed and back-filled with argon (three cycles), and then stirred at 100 °C under argon atmosphere for 16 hours. The reaction mixture was concentrated in vacuo, diluted with water (100 mL), and extracted with ethyl acetate (50 mL×3). The combined organic layers were washed with water (50 mL×2) and brine (50 mL), dried over anhydrous sodium sulfate, filtered and concentrated. The residue was purified with flash column chromatography (silica gel, petroleum ether/ethyl acetate = 5:1) to afford the product **12a** (0.90 g, 31% yield).

^{1}H NMR (400 MHz, DMSO-d_6) δ 9.23 (s, 1H), 8.83 (dd, J = 4.4, 1.6 Hz, 2H), 8.05 (s, 1H), 7.96 (dd, J = 4.4, 1.6 Hz, 2H).

MS (ESI+) m/z 291.9, 293.9 $[M + H]^+$.

6-bromo-5-methyl-4-(pyridin-4-yl)thieno[2,3-*d*]pyrimidine **12b**

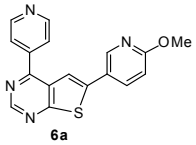


12b

Compound **12b** was prepared according to the procedure described for compound **12a**, using 6-bromo-4-iodo-5-methylthieno[2,3-*d*]pyrimidine **11b** instead of 6-bromo-4-iodothieno[2,3-*d*]pyrimidine **11a**.

^{1}H NMR (400 MHz, DMSO-d_6): δ 9.16 (s, 1H), 8.78 (d, J = 6.0 Hz, 2H), 7.63 (d, J = 6.0 Hz, 2H), 1.88 (s, 3H)

6-(6-methoxypyridin-3-yl)-4-(pyridin-4-yl)thieno[2,3-*d*]pyrimidine **6a**



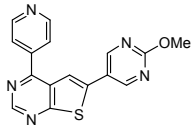
6a

A mixture of 6-bromo-4-(pyridin-4-yl)thieno[2,3-*d*]pyrimidine **12a** (100 mg, 0.34 mmol), 6-methoxypyridine-3-boronic acid (61 mg, 0.4 mmol) and 2N aqueous potassium carbonate solution (0.51 mL, 1.02 mmol) in 1,4-dioxane (5 mL) was degassed, and then bis(triphenylphosphine)palladium(II) chloride (24 mg, 0.034 mmol) was added. The resulting mixture was degassed and back-filled with argon (three cycles), and then stirred at 100 °C under argon atmosphere for 5 hours. The reaction mixture was concentrated in vacuo, diluted with water (20 mL), and extracted with ethyl acetate (20 mL×3). The combined organic layers were washed with water (30 mL×2) and brine (30 mL), dried over anhydrous sodium sulfate, filtered and concentrated. The redidue was purified with flash column chromatography (silica gel, petroleum ether/ethyl acetate = 2:1) to afford the product **6a** (103 mg, 63% yield).

^{1}H NMR (400 MHz, DMSO) δ 9.21 (s, 1H), 8.85 (d, *J* = 6.0 Hz, 2H), 8.76 (d, *J* = 2.4 Hz, 1H), 8.34 (dd, *J* = 8.8, 2.4 Hz, 1H), 8.15 (s, 1H), 8.05 (d, *J* = 6.0 Hz, 2H), 7.00 (d, *J* = 8.8 Hz, 1H), 3.94 (s, 3H).

96.61% by LCMS: *m/z* 321.1 $[M + H]^+$.

6-(2-methoxypyrimidin-5-yl)-4-(pyridin-4-yl)thieno[2,3-*d*]pyrimidine **6b**



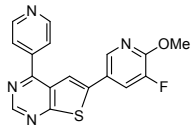
6b

Compound **6b** was prepared according to the procedure described for compound **6a**, using 2-methoxypyrimidine-5-boronic acid instead of 6-methoxypyridine-3-boronic acid**.**

^{1}H NMR (400 MHz, DMSO-d_6) δ 9.24 (s, 1H), 9.21 (s, 2H), 8.86 (d, *J* = 6.1 Hz, 2H), 8.29 (s, 1H), 8.05 (d, *J* = 6.1 Hz, 2H), 4.01 (s, 3H).

99.0% by LCMS: *m/z* 322.1 $[M + H]^+$.

6-(5-fluoro-6-methoxypyridin-3-yl)-4-(pyridin-4-yl)thieno[2,3-*d*]pyrimidine **6c**



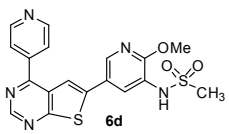
6c

Compound **6c** was prepared according to the procedure described for compound **6a**, using 5-fluoro-6-methoxypyridine-3-boronic acid instead of 6-methoxypyridine-3-boronic acid**.**

^{1}H NMR (400 MHz, DMSO-d_6) δ 9.22 (s, 1H), 8.86 (dd, *J* = 4.4, 1.6 Hz, 2H), 8.53 (d, *J* = 2.1 Hz, 1H), 8.49 (dd, *J* = 11.6, 2.1 Hz, 1H), 8.22 (s, 1H), 8.04 (dd, *J* = 4.4, 1.6 Hz, 2H), 4.02 (s, 3H).

99.46% by LCMS: *m/z* 339.0 $[M + H]^+$.

N-(2-methoxy-5-(4-(pyridin-4-yl)thieno[2,3-*d*]pyrimidin-6-yl)pyridin-3-yl)methanesulfonamide **6d**

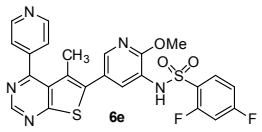


6d

Compound **6d** was prepared according to the procedure described for compound **6a**, using *N*-(2-methoxy-5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)pyridin-3-yl)methanesulfonamide instead of 6-methoxypyridine-3-boronic acid**.**

^{1}H NMR (400 MHz, DMSO-d_6) δ 9.50 (s, 1H), 9.22 (s, 1H), 8.86 (d, J = 6.0 Hz, 2H), 8.61 (d, J = 2.3 Hz, 1H), 8.15 (s, 1H), 8.12 (d, J = 2.3 Hz, 1H), 8.04 (d, J = 6.0 Hz, 2H), 4.00 (s, 3H), 3.11 (s, 3H).
90.76% by LCMS: m/z 414.0 $[M + H]^+$.

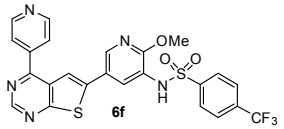
N-(2-methoxy-5-(5-methyl-4-(pyridin-4-yl)thieno[2,3-*d*]pyrimidin-6-yl)pyridin-3-yl)-2,4-difluorobenzenesulfonamide **6e**



6e

Compound **6e** was prepared according to the procedure described for compound **6a**, using *N*-(2-methoxy-5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)pyridin-3-yl)-2,4-difluorobenzenesulfonamide and 6-bromo-5-methyl-4-(pyridin-4-yl)thieno[2,3-*d*]pyrimidine **12b** instead of 6-methoxypyridine-3-boronic acid and 6-bromo-4-(pyridin-4-yl)thieno[2,3-*d*]pyrimidine **12a**, respectively.
^{1}H NMR (400 MHz, DMSO-d_6): δ 10.48 (s, 1H), 9.19 (s, 1H), 8.79 (d, J = 6.0 Hz, 2H), 8.22 (d, J = 1.6Hz, 1H), 7.82 – 7.71 (m, 2H), 7.67 (d, J = 6.0 Hz, 2H), 7.56 (t, J = 10 Hz, 1H), 7.23 (td, J = 8.4, 1.6 Hz, 1H), 3.71 (s, 3H), 1.85 (s, 3H).
98.78% by LCMS: m/z 525.9 $[M+H]^+$.

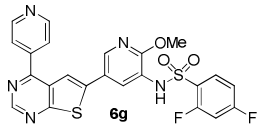
N-(2-methoxy-5-(4-(pyridin-4-yl)thieno[2,3-*d*]pyrimidin-6-yl)pyridin-3-yl)-4-(trifluoromethyl)benzenesulfonamide **6f**



6f

Compound **6f** was prepared according to the procedure described for compound **6a**, using *N*-(2-methoxy-5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)pyridin-3-yl)-4-(trifluoromethyl)benzenesulfonamide instead of 6-methoxypyridine-3-boronic acid**.**
^{1}H NMR(400 MH_Z, DMSO-d_6): δ 10.40 (s, 1H), 9.22 (s, 1H), 8.86 (dd, J = 4.5, 1.6 Hz, 2H), 8.60 (d, J = 2.4 Hz, 1H), 8.16 (s, 1H), 8.15 (d, J = 2.4 Hz, 1H), 8.05 (dd, J = 4.5, 1.6 Hz, 2H), 7.99 (d, J = 8.4 Hz, 2H), 7.94 (d, J = 8.4 Hz, 2H), 3.56 (s, 3H).
99.41% by LCMS: m/z 544.0 $[M+H]^+$.

N-(2-methoxy-5-(4-(pyridin-4-yl)thieno[2,3-*d*]pyrimidin-6-yl)pyridin-3-yl)-2,4-difluorobenzenesulfonamide **6g**



6g

Compound **6g** was prepared according to the procedure described for compound **6a**, using *N*-(2-methoxy-5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)pyridin-3-yl)-2,4-difluorobenzenesulfonamide instead of 6-methoxypyridine-3-boronic acid.
^{1}H-NMR(400 MH_Z, DMSO-d_6): δ 10.44 (s, 1H), 9.21 (s, 1H), 8.86 (d, J = 6.0 Hz, 2H), 8.60 (d, J = 2.0 Hz,

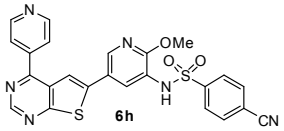
1H), 8.22 – 8.13 (m, 2H), 8.04 (dd, *J* = 6.0 Hz, 2H), 7.77 (td, *J* = 8.4, 6.8 Hz, 1H), 7.59 (t, *J* = 8.8 Hz, 1H), 7.22 (td, *J* = 8.4, 2.4 Hz, 1H), 3.66 (s, 3H).

^{13}C NMR (100 MHz, DMSO-d_6) δ 169.0, 165.1 (dd, $J_{C\text{-}F}$ = 252, 12 Hz), 159.4 (dd, $J_{C\text{-}F}$ = 256, 13 Hz), 158.6, 156.8, 153.1, 150.4, 143.8, 142.8, 140.5, 133.8, 131.8 (d, $^{3}J_{C\text{-}F}$ = 11 Hz), 128.9, 125.1 (dd, $J_{C\text{-}F}$ = 14, 3.7 Hz), 123.3, 122.6, 120.1, 116.2, 111.9 (dd, $J_{C\text{-}F}$ = 22, 3.2 Hz), 105.8 (t, $^{2}J_{C\text{-}F}$ = 24 Hz), 53.7.

98.90% by LCMS: *m/z* 512.0 $[M + H]^+$.

HRMS calcd for $C_{23}H_{16}F_2N_5O_3S_2$ $[M + H]^+$: 512.06571; found 512.06497.

N-(2-methoxy-5-(4-(pyridin-4-yl)thieno[2,3-*d*]pyrimidin-6-yl)pyridin-3-yl)-4-cyanobenzenesulfonamide **6h**



6h

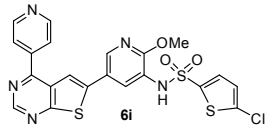
Compound **6h** was prepared according to the procedure described for compound **6a**, using *N*-(2-methoxy-5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)pyridin-3-yl)-4-cyanobenzenesulfonamide instead of 6-methoxypyridine-3-boronic acid.

^{1}H NMR(400 MH$_Z$, DMSO-d_6): δ 10.46 (s, 1H), 9.22 (s, 1H), 8.87 (dd, *J* = 4.8, 2.0 Hz, 2H), 8.55 (s, 1H), 8.1 – 8.02 (m, 6H), 7.90 (d, *J* = 8.5 Hz, 2H), 3.61 (s, 3H).

98.27% by LCMS: *m/z* 501.0 $[M + H]^+$.

HRMS calcd for $C_{24}H_{17}N_6O_3S_2$ $[M + H]^+$: 501.07981; found 501.07855.

N-(2-methoxy-5-(4-(pyridin-4-yl)thieno[2,3-*d*]pyrimidin-6-yl)pyridin-3-yl)-5-chlorothiophene-2-sulfonamid e **6i**



6i

Compound **6i** was prepared according to the procedure described for compound **6a**, using *N*-(2-methoxy-5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)pyridin-3-yl)-5-chlorothiophene-2-sulfonamid e instead of 6-methoxypyridine-3-boronic acid**.**

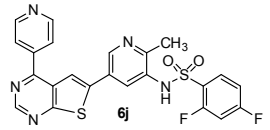
^{1}H NMR(400 MH$_Z$, DMSO-d_6): δ 10.48 (s, 1H), 9.23 (s, 1H), 8.87 (dd, *J* = 4.5, 1.5 Hz, 2H), 8.65 (d, *J* = 2.0 Hz, 1H), 8.16 (s, 1H), 8.14 (d, *J* = 2.0 Hz, 1H), 8.05 (dd, *J* = 4.5, 1.6 Hz, 2H), 7.41 (d, *J* = 4.1 Hz, 1H), 7.25 (d, *J* = 4.1 Hz, 1H), 3.77 (s, 3H).

^{13}C NMR (100 MHz, DMSO-d_6) δ 169.0, 158.3, 156.9, 153.2, 150.4, 143.8, 142.8, 140.5, 138.9, 135.3, 132.9, 132.3, 129.0, 127.9, 123.4, 122.7, 120.2, 116.3, 53.9.

95.46% by LCMS: *m/z* 516.0 $[M + H]^+$.

HRMS calcd for $C_{21}H_{15}ClN_5O_3S_3$ $[M + H]^+$: 516.00201; found 516.00086

N-(2-methyl-5-(4-(pyridin-4-yl)thieno[2,3-d]pyrimidin-6-yl)pyridin-3-yl)-2,4-difluorobenzenesulfonamide **6j**



6j

A mixture of *N*-(5-bromo-2-methylpyridin-3-yl)-2,4-difluorobenzenesulfonamide (87 mg, 0.24 mmol), bis(pinacolato)diboron (73 mg, 0.288 mmol) and potassium acetate (94 mg, 0.96 mmol) in 1,4-dioxane (6 mL) was degassed, and then [1,1′-bis(diphenylphosphino)ferrocene]dichloropalladium(II) (17.6 mg, 0.024 mmol) was added. The resulting mixture was degassed and back-filled with argon (three cycles), stirred at 100 °C under argon atmosphere for 4 hours, and then cooled to room temperature.

To the resulting mixture was added 6-bromo-4-(pyridin-4-yl)thieno[2,3-*d*]pyrimidine (58 mg, 0.2 mmol), 2N aqueous potassium carbonate solution (300 μL, 0.6 mmol) and [1,1′-bis(diphenylphosphino)ferrocene]dichloropalladium(II) (14.6 mg, 0.02 mmol). The resulting mixture was degassed and back-filled with argon (three cycles), and stirred at 100 °C under argon atmosphere for 4 hours. The reaction mixture was cooled to room temperature, diluted with water (30 mL), and extracted with ethyl acetate (30 mL×3). The combined organic layers were washed with brine (30 mL), dried over anhydrous sodium sulfate, filtered and concentrated. The residue was purified with flash column chromatography (silica gel, dichloromethane/10% ammonium hydroxide in methanol = 100:1) to afford the product **6j** as a pale yellow solid (62 mg, 62.6% yield).

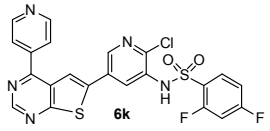
^{1}H NMR (400 MHz, DMSO-d_6) δ 10.59 (br s, 1H), 9.24 (s, 1H), 8.95 (d, J = 1.6 Hz, 1H), 8.87 (dd, J = 4.4, 1.6 Hz, 2H), 8.22 (s, 1H), 8.04 (dd, J = 4.4, 1.6 Hz, 2H), 7.86 (d, J = 2.0 Hz, 1H), 7.80 (td, J = 8.4, 6.3 Hz, 1H), 7.67 – 7.58 (m, 1H), 7.26 (td, J = 8.4, 2.0 Hz, 1H), 2.33 (s, 3H).

^{13}C NMR (100 MHz, DMSO-d_6) δ 169.0, 165.3 (dd, $J_{C\text{-}F}$ = 253, 12 Hz), 159.0 (dd, $J_{C\text{-}F}$ = 256, 14 Hz), 157.3, 155.7, 153.4, 150.4, 144.7, 143.7, 140.1, 132.0 (d, $^3J_{C\text{-}F}$ = 11 Hz), 131.7, 131.0, 128.9, 126.7, 124.7 (dd, J = 14, 3.3 Hz), 123.3, 117.4, 112.5 (dd, $J_{C\text{-}F}$ = 22, 3.2 Hz), 106.3 (t, $^2J_{C\text{-}F}$ = 26 Hz), 20.5.

98.86% by LCMS: *m/z* 496.0 $[M + H]^+$.

HRMS calcd for $C_{23}H_{16}F_2N_5O_2S_2$ $[M + H]^+$: 496.07080; found 496.06978.

N-(2-chloro-5-(4-(pyridin-4-yl)thieno[2,3-*d*]pyrimidin-6-yl)pyridin-3-yl)-2,4-difluorobenzenesulfonamide **6k**



6k

Compound **6k** was prepared according to the procedure described for compound **6j**, using *N*-(5-bromo-2-chloropyridin-3-yl)-2,4-difluorobenzenesulfonamide instead of *N*-(5-bromo-2-methylpyridin-3-yl)-2,4-difluorobenzenesulfonamide**.**

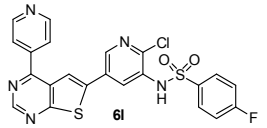
^{1}H NMR (400 MHz, DMSO-d_6) δ 11.01 (br s, 1H), 9.27 (s, 1H), 8.91 (d, J = 2.0 Hz,, 1H), 8.88 (dd, J = 4.4, 1.6 Hz, 2H), 8.37 (s, 1H), 8.29 (d, J = 2.0 Hz, 1H), 8.05 (dd, J = 4.4, 1.6 Hz, 2H), 7.82 (td, J = 8.4, 6.2 Hz, 1H), 7.65 – 7.55 (m, 1H), 7.25 (td, J = 8.4, 2.3 Hz, 1H).

^{13}C NMR (100 MHz, DMSO-d_6) δ 169.3, 165.4 (dd, $J_{C\text{-}F}$ = 253, 12 Hz), 159.3 (dd, $J_{C\text{-}F}$ = 256, 14 Hz), 157.7, 153.7, 150.2, 147.3, 145.3, 143.9, 138.7, 135.3, 131.9 (d, $^3J_{C\text{-}F}$ = 11 Hz), 130.61, 128.8, 128.7, 124.9 (dd, $J_{C\text{-}F}$ = 14, 3.6 Hz), 123.5, 119.0, 112.4 (dd, $J_{C\text{-}F}$ = 22, 3.5 Hz), 106.3 (t, $^2J_{C\text{-}F}$ = 26 Hz).

98.56% by LCMS: *m/z* 515.9 $[M+H]^+$.

HRMS calcd for $C_{22}H_{13}ClF_2N_5O_2S_2$ $[M + H]^+$: 516.01618; found 516.01535.

N-(2-chloro-5-(4-(pyridin-4-yl)thieno[2,3-d]pyrimidin-6-yl)pyridin-3-yl)-4-fluorobenzenesulfonamide **6l**



6l

Compound **6l** was prepared according to the procedure described for compound **6j**, using *N*-(5-bromo-2-chloropyridin-3-yl)-4-difluorobenzenesulfonamide instead of *N*-(5-bromo-2-methylpyridin-3-yl)-2,4-difluorobenzenesulfonamide**.**

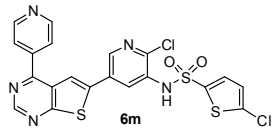
^{1}H NMR (400 MHz, DMSO-d_6) δ 10.64 (br s, 1H), 9.27 (s, 1H), 8.90 (d, J = 2.4 Hz, 1H), 8.88 (dd, J = 4.4, 1.6 Hz, 2H), 8.34 (s, 1H), 8.16 (d, J = 2.3 Hz, 1H), 8.05 (dd, J = 4.4, 1.6 Hz, 2H), 7.88 – 7.77 (m, 2H), 7.45 (t, J = 8.8 Hz, 2H).

^{13}C NMR (100 MHz, DMSO-d_6) δ 169.3, 164.1 (d, $^{1}J_{C\text{-}F}$ = 251 Hz), 157.8, 153.7, 150.4, 146.5, 144.8, 143.7, 138.8, 136.3 (d, $^{4}J_{C\text{-}F}$ = 3.0 Hz), 133.9, 131.0, 129.9 (d, $^{3}J_{C\text{-}F}$ = 10 Hz), 128.8, 128.6, 123.4, 118.9, 116.6 (d, $^{2}J_{C\text{-}F}$ = 23 Hz).

98.90% by LCMS: *m/z* 497.9 $[M + H]^+$.

HRMS calcd for $C_{22}H_{14}ClFN_5O_2S_2$ $[M + H]^+$: 498.02560; found 498.02448.

N-(2-chloro-5-(4-(pyridin-4-yl)thieno[2,3-*d*]pyrimidin-6-yl)pyridin-3-yl)-5-chlorothiophene-2-sulfonamide **6m**



6m

Compound **6m** was prepared according to the procedure described for compound **6j**, using *N*-(5-bromo-2-chloropyridin-3-yl)-5-chlorothiophene-2-sulfonamide instead of *N*-(5-bromo-2-methylpyridin-3-yl)-2,4-difluorobenzenesulfonamide**.**

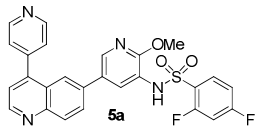
^{1}H NMR (400 MHz, DMSO-d_6) δ 9.27 (s, 1H), 8.93 (d, J = 2.3 Hz, 1H), 8.88 (d, J = 6.0 Hz, 2H), 8.36 (s, 1H), 8.22 (d, J = 2.3 Hz, 1H), 8.06 (d, J = 6.0 Hz, 2H), 7.44 (d, J = 4.1 Hz, 1H), 7.28 (d, J = 4.1 Hz, 1H).

^{13}C NMR (100 MHz, DMSO-d_6) δ 169.3, 157.7, 153.6, 150.2, 147.2, 145.0, 143.9, 138.79, 138.77, 135.8, 134.4, 132.6, 130.8, 128.8, 128.6, 128.3, 123.5, 118.9.

97.66% by LCMS: *m/z* 519.8 $[M + H]^+$.

HRMS calcd for $C_{20}H_{12}Cl_2N_5O_2S_3$ $[M + H]^+$: 519.95247; found 519.95175.

3. *N*-(2-methoxy-5-(4-(pyridin-4-yl)quinolin-6-yl)pyridin-3-yl)-2,4-difluorobenzenesulfonamide 5a



5a

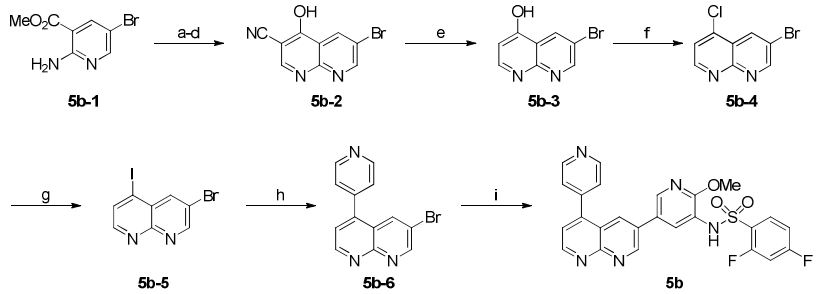
Compound **5a** was prepared according to the procedure described in WO2008144463 (Ref. 16).

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.33 (s, 1H), 9.02 (d, J = 4.4 Hz, 1H), 8.81 (dd, J = 4.4, 1.6 Hz, 2H), 8.37 (d, J = 2.2 Hz, 1H), 8.24 (d, J = 8.8 Hz, 1H), 8.10 (dd, J = 8.8, 1.8 Hz, 1H), 7.94 (d, J = 1.8 Hz, 1H), 7.89 (d, J = 2.2 Hz, 1H), 7.77 – 7.64 (m, 3H), 7.62 – 7.49 (m, 2H), 7.17 (td, J = 8.4, 2.0 Hz, 1H), 3.66 (s, 3H).

^{13}C NMR (100 MHz, DMSO-d_6) δ 165.0 (dd, $J_{C\text{-}F}$ = 252, 12 Hz), 159.3 (dd, $J_{C\text{-}F}$ = 256, 14 Hz), 157.5, 150.4, 150.1, 147.4, 144.8, 144.7, 142.5, 135.1, 133.6, 131.7 (d, $^{3}J_{C\text{-}F}$ = 11 Hz), 130.6, 129.0, 128.6, 125.2, 125.1 (dd, $J_{C\text{-}F}$ = 14, 3.8 Hz), 124.4, 122.0, 119.9, 111.8 (dd, $J_{C\text{-}F}$ = 22, 3.4 Hz), 105.8 (t, $^{2}J_{C\text{-}F}$ = 26 Hz), 53.5.

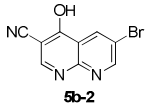
100% by LCMS: *m/z* 505.0 $[M+H]^+$.

4. Procedure for the synthesis of compound 5b



Scheme 2. Synthesis of compounds **5b**. Reagents and conditions: (a). DMF-DMA, 115 °C; (b). CH_3CN, n-BuLi, -78 °C, Ar; (c) -40 °C, Ar; (d) HOAc, -40 °C to rt; (e) conc. HCl, 150 °C; (f) $POCl_3$, reflux; (g) NaI, anhydrous dioxane, 105 °C, Ar; (h) pyridine-4-boronic acid, 2N K_2CO_3, $Pd(PPh_3)_4$, dioxane, 100 °C, Ar; (i) aryl borate, $PdCl_2$(dppf), 2N K_2CO_3, DMF, 100 °C, Ar.

6-bromo-4-hydroxy-1,8-naphthyridine-3-carbonitrile **5b-2**



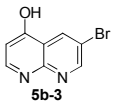
A mixture of methyl 2-amino-5-bromonicotinate **5b-1** (3.314 g, 14.34 mmol) and *N*,*N*-dimethylformamide dimethyl acetal (17.09 g, 143.4 mmol) was stirred at 110 °C for 3 hours. After removing the volatiles in vacuo, the residue was dissolved in anhydrous tetrahydrofuran (30 mL) to form an intermediate solution, which was used immediately in the following step.

To a solution of n-butyl lithium (2.4M in hexane, 13.15 mL, 31.55 mmol) in anhydrous tetrahydrofuran (40 mL) was added dropwise anhydrous acetonitrile (1.64 mL, 31.55 mmol) at -78 °C under argon atmosphere. The resulting reaction mixture was stirred at -78 °C for 20min. To the resulting white suspension was added the intermediate solution described above, and the reaction mixture was stirred at -78 °C for 30 min then at -40 °C for 1 hour. Acetic acid (2.58 mL, 43.02 mmol) was added and the resulting orange thick suspension was warmed to room temperature and stirred for 30 min. The mixture was diluted with water (100 mL), extracted with ethyl acetate (50 mL×3). The combined organic layers were washed with water (50 mL×2) and brine (50 mL×2), dried over anhydrous sodium sulfate, filtered and concentrated. The residue was purified with flash column chromatography (silica gel, petroleum ether/ethyl acetate = 5:1 then 2:1) to afford the product **5b-2** as an orange solid (1.4 g, 39% yield).

^{1}H NMR (400 MHz, DMSO-d_6) δ 13.42 (s, 1H), 8.98 (d, J = 2.5 Hz, 1H), 8.86 (s, 1H), 8.62 (d, J = 2.5 Hz, 1H).

MS (ESI+) m/z 249.9 $[M + H]^+$.

6-bromo-1,8-naphthyridin-4-ol **5b-3**



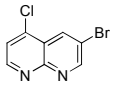
A mixture of 6-bromo-4-hydroxy-1,8-naphthyridine-3-carbonitrile **5b-2** (1.54 g, 6.16 mmol) in concentrated hydrochloride solution (32 mL) in a sealed tube was stirred at 150 °C for 2 hours. After cooling to room

temperature, the reaction mixture was neutralized with ammonium water, extracted with ethyl acetate (50 mL×4). The combined organic layers were washed with water (50 mL×2) and brine (50 mL×2), dried over anhydrous sodium sulfate, filtered and concentrated. The residue was purified with flash column chromatography (silica gel, petroleum ether/ethyl acetate = 1:1 then 1:5) to afford the product **5b-3** as an yellow solid (0.40 g, 30% yield).

^{1}H NMR (400 MHz, DMSO-d_6) δ 12.38 (s, 1H), 8.85 (d, J = 2.5 Hz, 1H), 8.54 (d, J = 2.5 Hz, 1H), 7.99 (dd, J = 7.6, 6.4 Hz, 1H), 6.15 (dd, J = 7.6, 1.2 Hz, 1H).

MS (ESI+) m/z 224.9 $[M + H]^+$.

3-bromo-5-chloro-1,8-naphthyridine **5b-4**



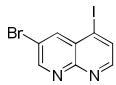
5b-4

A mixture of 6-bromo-1,8-naphthyridin-4-ol **5b-3** (0.34 g, 1.4 mmol) in phosphorus oxychloride (7 mL) was stirred under reflux for 2 hours. After cooling to room temperature, the reaction mixture was poured into ice-water, and the resulting mixture was extracted with ethyl acetate (30 mL×3). The combined organic layers were washed with water (30 mL×2) and brine (30 mL×2), dried over anhydrous sodium sulfate, filtered and concentrated. The residue was purified with flash column chromatography (silica gel, petroleum ether/ethyl acetate = 5:1 then 2:1) to afford the product **5b-4** as a yellow solid (0.085g, 23% yield).

^{1}H NMR (300 MHz, DMSO-d_6) δ 9.26 (d, J = 2.5 Hz, 1H), 9.09 (d, J = 4.8 Hz, 1H), 8.87 (d, J = 2.5 Hz, 1H), 7.96 (d, J = 4.8 Hz, 1H).

MS (ESI+) m/z 242.8, 244.8 $[M + H]^+$.

3-bromo-5-iodo-1,8-naphthyridine **5b-5**

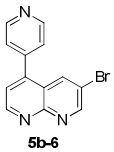


5b-5

To a solution of 3-bromo-5-chloro-1,8-naphthyridine **5b-4** (100 mg, 0.41 mmol) in anhydrous tetrahydrofuran (10 mL) was added 2N hydrochloride in ether (2 mL). The resulting mixture was stirred at room temperature for 30 min and then evaporated to dry in vacuo. To the residue was added anhydrous 1,4-dioxane (3 mL) and sodium iodide (308 mg, 2.05 mmol) and the resulting reaction mixture was stirred at reflux under argon atmosphere for 40 hours. The reaction mixture was cooled to room temperature, diluted with water (30 mL), and extracted with ethyl acetate (30 mL×3). The combined organic layers were washed with water (30 mL×2) and brine (30 mL×2), dried over anhydrous sodium sulfate, filtered and concentrated. The residue was purified with flash column chromatography (silica gel, petroleum ether/ethyl acetate = 2:1) to afford the product **5b-5** as a yellow solid (77 mg, 56% yield).

MS (ESI+) m/z 334.8, 336.8 $[M + H]^+$.

3-bromo-5-(pyridin-4-yl)-1,8-naphthyridine **5b-6**



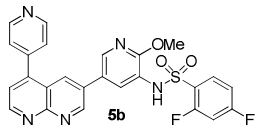
5b-6

A mixture of 3-bromo-5-iodo-1,8-naphthyridine **5b-5** (77 mg, 0.23 mmol), pyridine-4-boronic acid (29 mg, 0.23 mmol) and 2N aqueous potassium carbonate solution (0.35 mL, 0.7 mmol) in 1,4-dioxane (3 mL) was degassed, and then tetrakis(triphenylphosphine)palladium (27 mg，0.023 mmol) was added. The resulting mixture was degassed and back-filled with argon (three cycles), and then stirred at 100 °C under argon atmosphere for 16 hours. The reaction mixture was concentrated in vacuo, diluted with water (20 mL), and extracted with ethyl acetate (20 mL×3). The combined organic layers were washed with water (20 mL) and brine (20 mL), dried over anhydrous sodium sulfate, filtered and concentrated. The residue was purified with flash column chromatography (silica gel, dichloromethane/10% ammonium water in methanol = 40:1) to afford the product **5b-6** as a yellow solid (58 mg, 62% yield).

^{1}H NMR (400 MHz, DMSO-d_6) δ 9.23 (d, J = 4.4 Hz, 1H), 9.20 (d, J = 2.4 Hz, 1H), 8.81 (d, J = 6.0 Hz, 2H), 8.41 (d, J = 2.4 Hz, 1H), 7.73 (d, J = 4.4 Hz, 1H), 7.65 (d, J = 6.0 Hz, 2H).

MS (ESI+) m/z 285.9, 287.9 $[M + H]^+$.

N-(2-methoxy-5-(5-(pyridin-4-yl)-1,8-naphthyridin-3-yl)pyridin-3-yl)-2,4-difluorobenzenesulfonamide **5b**



5b

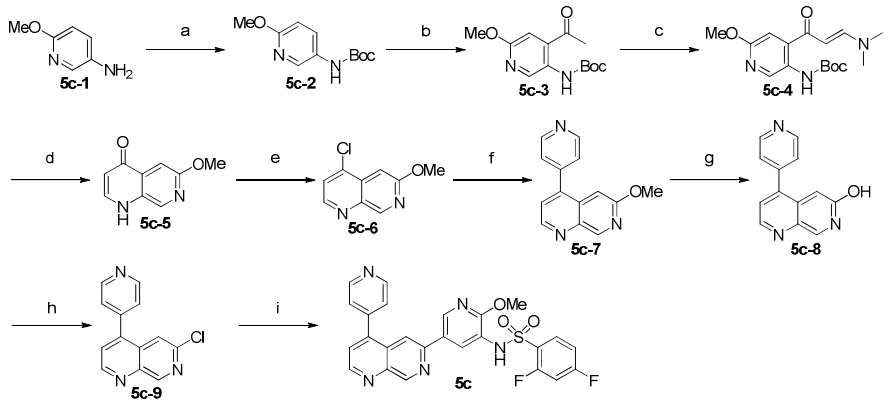
A mixture of 3-bromo-5-(pyridin-4-yl)-1,8-naphthyridine **5b-6** (53 mg, 0.185 mmol), *N*-(2-methoxy-5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)pyridin-3-yl)-2,4-difluorobenzenesulfonamide (95 mg, 0.223 mmol) and 2N aqueous potassium carbonate solution (0.5 mL, 1.0 mmol) in 1,4-dioxane (3 mL) was degassed, and then [1,1′-bis(diphenylphosphino)ferrocene]dichloropalladium(II) (14 mg, 0.0185 mmol) was added. The resulting mixture was degassed and back-filled with argon (three cycles), and then stirred at 100 °C under argon atmosphere for 3.5 hours. The reaction mixture was concentrated in vacuo, diluted with water (15 mL), and extracted with ethyl acetate (15 mL×3). The combined organic layers were washed with water (15 mL) and brine (15 mL), dried over anhydrous sodium sulfate, filtered and concentrated. The residue was purified with flash column chromatography (silica gel, dichloromethane/10% ammonium water in methanol = 60:1) to afford the product **5b** as a yellow solid (53 mg, 57% yield).

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.38 (br s, 1H), 9.44 (d, J = 2.4 Hz, 1H), 9.20 (d, J = 4.4 Hz, 1H), 8.83 (d, J = 6.0 Hz, 2H), 8.49 (d, J = 2.0 Hz, 1H), 8.36 (d, J = 2.4 Hz, 1H), 8.02 (d, J = 2.0 Hz, 1H), 7.77 – 7.67 (m, 4H), 7.56 (td, J = 10.0, 2.0 Hz, 1H), 7.18 (td, J = 8.4, 2.0 Hz, 1H), 3.66 (s, 3H).

^{13}C NMR (100 MHz, DMSO-d_6) δ 165.0 (dd, $J_{C\text{-}F}$ = 252, 11 Hz), 159.8 (dd, $J_{C\text{-}F}$ = 256, 13 Hz), 158.0, 155.0, 153.2, 152.3, 150.2, 146.5, 143.8, 143.1, 134.1, 131.7 (d, $^3J_{C\text{-}F}$ = 11 Hz), 131.2, 130.7, 126.1, 125.2 (dd, J = 14, 3.5 Hz), 124.5, 122.8, 120.1, 119.7, 111.8 (dd, $J_{C\text{-}F}$ = 22, 3.2 Hz), 105.8 (t, $^2J_{C\text{-}F}$ = 26 Hz), 53.5.

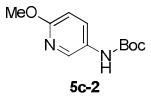
100% by LCMS: *m/z* 505.7 $[M+H]^+$.

5. **Procedure for the synthesis of compound 5c**



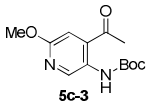
Scheme 3. Synthesis of compound **5c**. Reagents and conditions: (a) $(Boc_2)O$, TEA, DCM, rt; (b) n-butyl lithium, 2,2,6,6-tetramethylpiperidine, *N*,*O*-dimethylacetamide, anhydrous THF, -70 to 0 °C, Ar,; (c) DMF-DEA, toluene, reflux; (d) TFA, DCM, rt; (e) $POCl_3$, 90 °C; (f) pyridine-4-boronic acid, K_2CO_3, $PdCl_2(dppf)$, water, DMF, 100 °C, Ar; (g) $AlCl_3$, 1,2-dichloroethane, reflux; (h) Phenylphosphonic dichloride, 180 °C; (i) aryl borate, K_2CO_3, $PdCl_2(dppf)$, water, DMF, 100 °C, Ar.

tert-butyl (6-methoxypyridin-3-yl)carbamate **5c-2**



To a solution of 5-amino-2-methoxylpyridine **5c-1** (7.4 g, 60 mmol) and triethylamine (25 mL, 180 mmol) in dichloromethane (100 mL) at 0 °C was added di-tert-butyl dicarbonate (19.64 g, 90 mmol), and the resulting reaction mixture was stirred at room temperature overnight. The reaction mixture was then diluted with dichloromethane (100 mL), washed with water (100 mL×2) and brine (100 mL×2), dried over anhydrous sodium sulfate, filtered and concentrated. The residue was purified with flash column chromatography (silica gel, petroleum ether/ethyl acetate = 7:1) to afford the product **5c-2** as a yellow solid (9.06 g, 68% yield).
^{1}H NMR (400 MHz, DMSO-d_6) δ 9.29 (s, 1H), 8.19 (s, 1H), 7.76 (d, J = 7.4 Hz, 1H), 6.75 (d, J = 8.9 Hz, 1H), 3.79 (s, 3H), 1.46 (s, 9H).

tert-butyl (4-acetyl-6-methoxypyridin-3-yl)carbamate **5c-3**

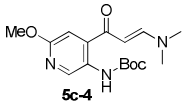


To a solution of tert-butyl (6-methoxypyridin-3-yl)carbamate **5c-2** (224 mg, 1.0 mmol) and 2,2,6,6-tetramethylpiperidine (0.46 mL, 3.0 mmol) in anhydrous THF (5 mL) at -70 °C under argon atmosphere was added dropwise n-butyl lithium (2.4M in hexane, 1.25 mL, 3.0 mmol) and the resulting reaction mixture was stirred at -10 °C for 2 hours. The resulting mixture then cooled to -70 °C and *N*,*O*-dimethylacetamide (206 mg, 2.0 mmol) was added dropwise. The resulting reaction mixture was warmed to 0 °C and stirred for 1 hour. The reaction was quenched with saturated aqueous ammonium hydrochloride (1 mL), diluted with water (30 mL) and extracted with ethyl acetate (30 mL×3). The combined organic layers were washed with water (30 mL×2) and brine (30 mL×2), dried over anhydrous sodium sulfate, filtered and concentrated. The residue was purified with flash column chromatography (silica gel, petroleum ether/ethyl acetate = 20:1) to afford the product **5c-3** as a yellow solid (68 mg, 26%

yield).

^{1}H NMR (400 MHz, $CDCl_3$) δ 9.60 (s, 1H), 9.18 (s, 1H), 7.08 (s, 1H), 3.95 (s, 3H), 2.63 (s, 3H), 1.52 (s, 9H).

(*E*)-tert-butyl (4-(3-(dimethylamino)acryloyl)-6-methoxypyridin-3-yl)carbamate **5c-4**

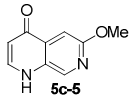


5c-4

A mixture of tert-butyl (4-acetyl-6-methoxypyridin-3-yl)carbamate **5c-3** (856 mg, 3.21 mmol) and *N*,*N*-dimethyformamide diethy acetal (709 mg, 4.82 mmol) in toluene (10 mL) was refluxed for 2 hours, and then evaporated to dry. The residue was dissolved in toluene (10 mL) and concentrated to afford the crude product **5c-4** as a brown oil (900 mg, 87% yield), which was used directly in the next step without further purification.

^{1}H NMR (400 MHz, $CDCl_3$) δ 9.50 (s, 1H), 9.00 (s, 1H), 7.78 (d, *J* = 12.2 Hz, 1H), 6.92 (s, 1H), 5.55 (d, *J* = 12.2 Hz, 1H), 3.94 (s, 3H), 3.19 (s, 3H), 2.94 (s, 3H), 1.50 (s, 9H).

6-methoxy-1,7-naphthyridin-4(1*H*)-one **5c-5**

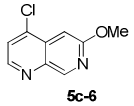


5c-5

A mixture of (*E*)-tert-butyl (4-(3-(dimethylamino)acryloyl)-6-methoxypyridin-3-yl)carbamate **5c-4** (900 mg, 2.80 mmol) and trifluoroacetic acid (20 mL) in dichloromethane (50 mL) was stirred at room temperature for 4 hours and then evaporated to dry in vacuo. The residue was diluted with water (30 mL), neutralized with saturated aqueous sodium bicarbonate, and extracted with ethyl acetate (30 mL×3). The combined organic layers were washed with water (30 mL×2) and brine (30 mL×2), dried over anhydrous sodium sulfate, filtered and concentrated to afford the crude product **5c-5** as a yellow solid (500 mg, 100% yield), which was used directly in the next step without further purification.

^{1}H NMR (400 MHz, DMSO-d_6) δ 12.06 (s, 1H), 8.71 (s, 1H), 7.98 (d, *J* = 7.3 Hz, 1H), 7.23 (s, 1H), 6.03 (d, *J* = 7.3 Hz, 1H), 3.90 (s, 3H).

4-chloro-6-methoxy-1,7-naphthyridine **5c-6**

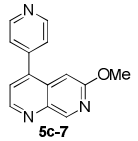


5c-6

A mixture of 6-methoxy-1,7-naphthyridin-4(1*H*)-one **5c-5** (500 mg, 2.84 mmol) in phosphorus oxychloride (5 mL) was stirred at 90 °C for 3 hours. After removing phosphorus oxychloride in vacuo, the residue was diluted in water (30 mL), neutralized with saturated aqueous sodium bicarbonate, and extracted with ethyl acetate (30 mL×3). The combined organic layers were washed with water (30 mL×2) and brine (30 mL×2), dried over anhydrous sodium sulfate, filtered and concentrated. The residue was purified with flash column chromatography (silica gel, petroleum ether/ethyl acetate = 20:1) to afford the product **5c-6** as a yellow solid (412 mg, 75% yield).

^{1}H NMR (400 MHz, DMSO-d_6) δ 9.24 (s, 1H), 8.79 (d, *J* = 4.6 Hz, 1H), 7.92 (d, *J* = 4.6 Hz, 1H), 7.28 (s, 1H), 4.03 (s, 3H).

6-methoxy-4-(pyridin-4-yl)-1,7-naphthyridine **5c-7**

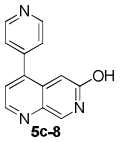


5c-7

A mixture of 4-chloro-6-methoxy-1,7-naphthyridine **5c-6** (350 mg, 1.80 mmol), pyridine-4-boronic acid (285 mg, 2.33 mmol), potassium carbonate (743 mg, 5.39 mmol) and water (1.8 mL) in DMF (10 mL) was degassed, and and then [1,1′-bis(diphenylphosphino)ferrocene]dichloropalladium(II) (131 mg, 0.18 mmol) was added. The resulting mixture was degassed and back-filled with argon (three cycles), and then stirred at 100 °C under argon atmosphere for 3 hours. The reaction mixture was concentrated in vacuo, diluted with water (20 mL), and extracted with ethyl acetate (20 mL×3). The combined organic layers were washed with water (20 mL) and brine (20 mL), dried over anhydrous sodium sulfate, filtered and concentrated. The residue was purified with flash column chromatography (silica gel, petroleum ether/ethyl acetate = 1:1) to afford the product **5c-7** as a yellow solid (322 mg, 74% yield).

^{1}H NMR (300 MHz, DMSO-d_6) δ 9.27 (s, 1H), 8.95 (d, J = 4.2 Hz, 1H), 8.80 (d, J = 5.8 Hz, 2H), 7.69 (d, J = 4.2 Hz, 1H), 7.65 (d, J = 5.8 Hz, 2H), 6.97 (s, 1H), 3.96 (s, 3H).

4-(pyridin-4-yl)-1,7-naphthyridin-6-ol **5c-8**

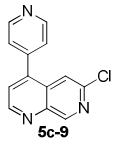


5c-8

To a solution of 6-methoxy-4-(pyridin-4-yl)-1,7-naphthyridine **5c-7** (322 mg,1.36 mmol) in 1,2-dichloroethane (10 mL) was added aluminium trichloride (1.09 g，8.16 mmol) and the resulting mixture was refluxed for 6 hours. After cooling, the mixture was poured into ice-water, and extracted with ethyl acetate (30 mL×2). The aqueous layer was evaporated to dry in vacuo. The residue was purified with flash column chromatography (silica gel, ethyl acetate/methanol = 3:2) to afford the product **5c-8** as a yellow solid (218 mg, 73% yield).

^{1}H NMR (400 MHz, DMSO-d_6) δ 9.15 (s, 1H), 9.01 (d, J = 5.8 Hz, 2H), 8.91 (d, J = 4.2 Hz, 1H), 8.07 (d, J = 5.8 Hz, 2H), 7.69 (d, J = 4.2 Hz, 1H), 6.80 (s, 1H).

6-chloro-4-(pyridin-4-yl)-1,7-naphthyridine **5c-9**

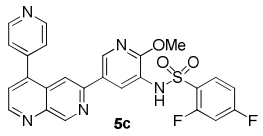


5c-9

A mixture of 4-(pyridin-4-yl)-1,7-naphthyridin-6-ol **5c-8** (160 mg, 0.69 mmol) in Phenylphosphonic dichloride (1.6 mL) was stirred at 180 °C for 24 hours. After cooling to room temperature, the mixture was poured into ice-water, neutralized with saturated aqueous sodium bicarbonate, and extracted with ethyl acetate (30 mL×3). The combined organic layers were washed with water (30 mL×2) and brine (30 mL×2), dried over anhydrous sodium sulfate, filtered and concentrated. The residue was purified with flash column chromatography (silica gel, petroleum ether/ethyl acetate = 1:1) to afford the product **5c-9** as a yellow solid (50 mg, 30% yield).

^{1}H NMR (400 MHz, DMSO-d_6) δ 9.40 (s, 1H), 9.19 (d, J = 4.4 Hz, 1H), 8.82 (d, J = 5.8 Hz, 2H), 7.88 (d, J = 4.4 Hz, 1H), 7.77 (s, 1H), 7.66 (d, J = 5.8 Hz, 2H).

N-(2-methoxy-5-(4-(pyridin-4-yl)-1,7-naphthyridin-6-yl)pyridin-3-yl)-2,4-difluorobenzenesulfonamide **5c**



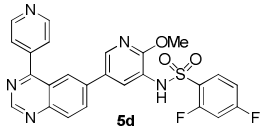
5c

A mixture of 6-chloro-4-(pyridin-4-yl)-1,7-naphthyridine **5c-9** (100 mg, 0.41 mmol), *N*-(2-methoxy-5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)pyridin-3-yl)-2,4-difluorobenzenesulfonamide (210 mg, 0.50 mmol) and potassium carbonate (170 mg, 1.23 mmol) and water (0.62 mL) in DMF (3 mL) was degassed, and then [1,1′-bis(diphenylphosphino)ferrocene]dichloropalladium(II) (30 mg，0.041 mmol) was added. The resulting mixture was degassed and back-filled with argon (three cycles), and then stirred at 100 °C under argon atmosphere for 4 hours. The reaction mixture was concentrated in vacuo, diluted with water (15 mL), and extracted with ethyl acetate (15 mL×3). The combined organic layers were washed with water (15 mL) and brine (15 mL), dried over anhydrous sodium sulfate, filtered and concentrated. The redidue was purified with flash column chromatography (silica gel, petroleum ether/ethyl acetate = 1:2) to afford the product **5c** as a off-white solid (40 mg, 36% yield).

^{1}H NMR (300 MHz, DMSO-d_6) δ 10.35 (s, 1H), 9.60 (s, 1H), 9.15 (d, J = 4.3 Hz, 1H), 8.84 (d, J = 4.4 Hz, 2H), 8.71 (s, 1H), 8.31 (s, 1H), 8.12 (s, 1H), 7.85 (d, J = 4.2 Hz, 1H), 7.80 – 7.67 (m, 3H), 7.57 (t, J = 8.4 Hz, 1H), 7.19 (t, J = 7.8 Hz, 1H), 3.68 (s, 3H).

100% by LCMS: *m/z* 506.1 $[M+H]^+$.

6. *N*-(2-methoxy-5-(4-(pyridin-4-yl)quinazolin-6-yl)pyridin-3-yl)-2,4-difluoro-benzenesulfonamide 5d



5d

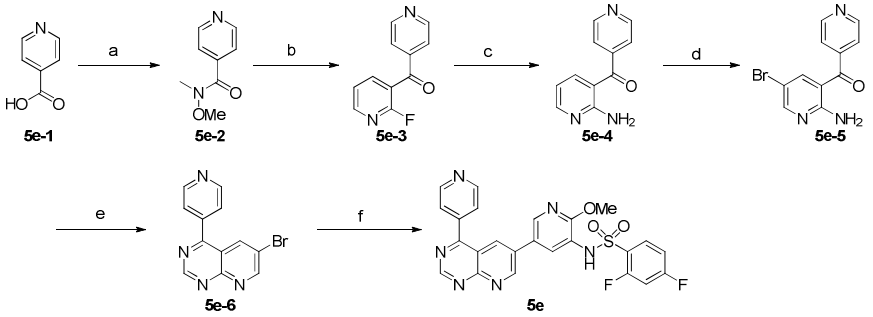
Compound **5d** was prepared according to the procedure described in WO2008157191 (Ref. 19).

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.36 (s, 1H), 9.44 (s, 1H), 8.88 (d, J = 6.0 Hz, 2H), 8.44 (d, J = 1.8 Hz, 1H), 8.38 (dd, J = 8.8, 2.0 Hz, 1H), 8.25 (d, J = 8.8 Hz, 1H), 8.16 (d, J = 1.7 Hz, 1H), 7.97 (d, J = 2.2 Hz, 1H), 7.89 (d, J = 6.0 Hz, 2H), 7.73 (td, J = 8.4, 6.4 Hz, 1H), 7.62 – 7.44 (m, 1H), 7.17 (td, J = 8.4, 2.2 Hz, 1H), 3.67 (s, 3H).

^{13}C NMR (101 MHz, DMSO) δ 166.0, 165.5 (dd, $J_{C\text{-}F}$ = 252, 11 Hz), 159.8 (dd, $J_{C\text{-}F}$ = 256, 13 Hz), 158.2, 154.9, 150.6, 150.3, 144.3, 143.3, 136.9, 134.4, 133.9, 132.2 (d, $^3J_{C\text{-}F}$ = 11 Hz), 129.9, 128.8, 125.6 (dd, J = 15, 3.7 Hz), 124.8, 123.6, 122.7, 120.5, 112.3 (dd, $J_{C\text{-}F}$ = 22, 3.2 Hz), 106.3 (t, $^2J_{C\text{-}F}$ = 26 Hz), 53.9.

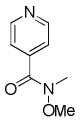
98.24% by LCMS: *m/z* 505.7 $[M + H]^+$.

7. Procedure for the synthesis of compound 5e



Scheme 4. Synthesis of compound **5e**. Reagents and conditions: (a) EDC-HCl, *N*,*O*-dimethylhydroxylamine hydrochloride, DIEPA, DCM, rt; (b) 2-fluoro-3-bromopyridine, n-BuLi, THF, -78 °C then 0 °C, Ar; (c) ammoniun water, NMP, 90 °C; (d) NBS, acetone, rt; (e) $HCONH_2$, HCOOH, 150 °C, Ar; (f) aryl borate, $PdCl_2$(dppf), K_2CO_3, DMF, H_2O, 100 °C, Ar.

N-Methoxy-*N*-methylisonicotinamide **5e-2**

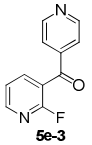


5e-2

To a mixture of isonicotinic acid **5e-1** (12.3 g, 100 mmol), 1-ethyl-3-(3-dimethylaminopropyl)carbodiie hydrochlide (28.76 g, 150 mmol), *N*,*O*-dimethylhydroxylamine hydrochloride (11.70 g, 120 mmol), and 4-dimethylaminopyridine (1.22 g, 10 mmol) in dichloromethane (200 mL) was added diisopropylethylamine (38.8 g, 300 mmol) dropwise at 0 °C. The resulting mixture was stirred at room temperature overnight. The reactions mixture was diluted with water (200 mL) and extracted with dichloromethane (200 mL×3). The combined organic layers were washed with water (200 mL×2) and brine (200 mL), dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified by column chromatography (silica gel, petroleum ether/ethyl acetate = 1:1) to give the product **5e-2** as a yellow solid (9.95 g, 60% yield).

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.69 (dd, J = 4.4, 1.5 Hz, 2H), 7.52 (dd, J = 4.4, 1.5 Hz, 2H), 3.55 (s, 3H), 3.28 (s, 3H)

(2-Fluoropyridin-3-yl)(pyridin-4-yl)methanone **5e-3**



5e-3

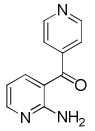
To a solution of 2-fluoro-3-bromopyridine (1.76 g, 10 mmol) in anhydrous THF (35 mL)was added n-BuLi (2.4M in hexane, 4.58 mL, 11 mmol) at -78 °C under Argon atmosphere, and the reaction solution was stirred at -78 °C for 15 min. A solution of compound **5e-2** (1.66 g, 10 mmol) in anhydrous THF (15 mL) was added dropwise and the resulting mixture was warmed to 0 °C and stirred for 3 hrs. The reaction was quenched with sat. NH_4Cl solution, diluted with water (50 mL) and extracted with ethyl acetate (50 mL×3). The combined organic layers were washed with water (50 mL×2) and brine (50 mL×2), dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified by column chromatography (silica gel, petroleum ether/ethyl acetate = 1:1) to give the product **5e-3** as a

yellow oil (1.07 g, 53% yield).

^{1}H NMR (300 MHz, DMSO-d_6) δ 8.83 (d, J = 5.4 Hz, 2H), 8.52 (m, 1H), 8.33 – 8.21 (m, 1H), 7.69 (d, J = 5.4 Hz, 2H), 7.63 – 7.55 (m, 1H)

MS (ESI+) m/z 203.1 $[M + H]^+$

(2-Aminopyridin-3-yl)(pyridin-4-yl)methanone **5e-4**



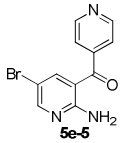
5e-4

A mixture of compound **5e-3** (2.63 g, 13 mmol) in ammonia wate (20 mL) and *N*-methylpyrollidone (20 mL) was stirred at 90 °C for 4 hrs. The reaction mixture was diluted with water (100 mL), neutralized with concentrated hydrochloride and then extracted with ethyl acetate (50 mL×3). The combined organic layers were washed with water (50 mL×2) and brine (50 mL×2), dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified by column chromatography (silica gel, dichloromethane/methanol = 60:1) to give the product **5e-4** as a yellow solid (1.68 g, 65% yield).

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.76 (d, J = 6.0 Hz, 2H), 8.29 (dd, J = 4.6, 1.8 Hz, 1H), 7.80 (br s, 2H), 7.62 (dd, J = 8.0, 1.8 Hz, 1H), 7.51 (d, J = 6.0 Hz, 2H), 6.62 (dd, J = 8.0, 4.6 Hz, 1H)

MS (ESI+) m/z 200.1 $[M + H]^+$

(2-Amino-5-bromopyridin-3-yl)(pyridin-4-yl)methanone **5e-5**



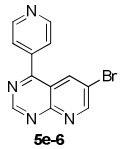
5e-5

To a mixture of compound **5e-4** (1.44 g, 7.23 mmol) in acetone (50 mL) was added *N*-bromosuccinimide (1.40 g, 7.83 mmol), and the rection mixture was stirred at room temperature for 1 hr. The reaction mixture was diluted with water (200 mL) and extracted with ethyl acetate (50 mL×3). The combined organic layers were washed with water (50 mL×2) and brine (50 mL×2), dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified by column chromatography (silica gel, dichloromethane/methanol = 100:1) to give the product **5e-5** as a yellow solid (1.60 g, 80% yield).

^{1}H NMR (300 MHz, DMSO-d_6) δ 8.79 (d, J = 5.9 Hz, 2H), 8.38 (d, J = 2.5 Hz, 1H), 7.92 (br s, 2H), 7.68 (d, J = 2.5 Hz, 1H), 7.57 (d, J = 5.9 Hz, 2H)

MS (ESI+) m/z 277.9 $[M + H]^+$

6-bromo-4-(pyridin-4-yl)pyrido[2,3-*d*]pyrimidine **5e-6**



5e-6

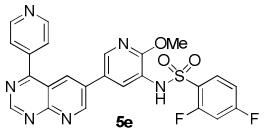
A mixture of (2-Amino-5-bromopyridin-3-yl)(pyridin-4-yl)methanone **5e-5** (556mg, 2 mmol), HCOOH (0.22 mL) and $HCONH_2$ was stirred at 150 under argon atmosphere for 7 hours. After cooling, the mixture was poured into water and extracted with ethyl acetate (30 mL×3). The combined organic layers were washed with water (30 mL×2) and brine (30 mL×2), dried over anhydrous Na_2SO_4, filtered and

concentrated. The residue was purified by column chromatography (silica gel, petroleum ether/ethyl acetate 1:1) to give the product **5e-6** as a yellow solid (0.22 g, 38% yield).

^{1}H NMR (400 MHz, DMSO-d_6) δ 9.63 (s, 1H), 9.44 (d, J = 2.5 Hz, 1H), 8.87 (d, J = 5.9 Hz, 2H), 8.67 (d, J = 2.5 Hz, 1H), 7.82 (d, J = 5.9 Hz, 2H).

MS (ESI+) m/z 286.9 $[M + H]^+$.

N-(2-methoxy-5-(4-(pyridin-4-yl)pyrido[2,3-*d*]pyrimidin-6-yl)pyridin-3-yl)-2,4-difluorobenzenesulfonamide **5e**



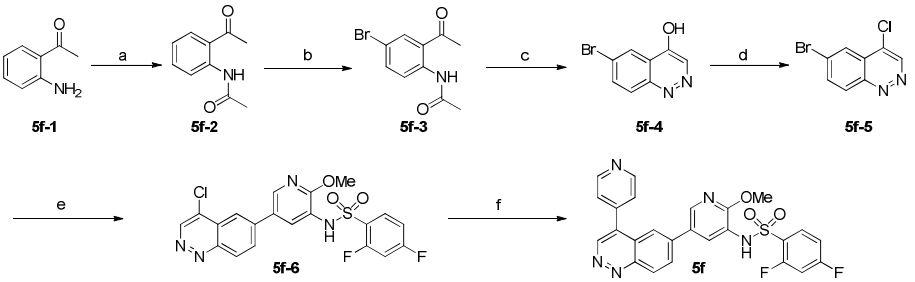
5e

Compound **5e** was prepared according to the procedure described for compound **5b**, using 6-bromo-4-(pyridin-4-yl)pyrido[2,3-*d*]pyrimidine **5e-6** instead of 3-bromo-5-(pyridin-4-yl)-1,8-naphthyridine **5b-6.**

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.38 (br s, 1H), 9.66 (d, J = 2.5 Hz, 1H), 9.60 (s, 1H), 8.88 (d, J = 5.8 Hz, 2H), 8.59 (d, J = 2.5 Hz, 1H), 8.56 (d, J = 2.1 Hz, 1H), 8.11 (d, J = 2.1 Hz, 1H), 7.91 (d, J = 5.8 Hz, 2H), 7.77-7.70 (m, 1H), 7.57 (td, J = 10.0, 2.0 Hz, 1H), 7.18 (td, J = 8.4, 2.0 Hz, 1H), 3.66 (s, 3H).

96.86% by LCMS: *m/z* 507.1 $[M + H]^+$.

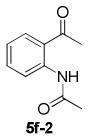
8. Procedure for the synthesis of compound 5f



5f-1 **5f-2** **5f-3** **5f-4** **5f-5** **5f-6** **5f**

Scheme 5 Synthesis of compound **5f**. Reagents and conditions: (a) acetyl chloride, TEA, DCM, rt; (b) Br_2, HOAc, rt; (c) $NaNO_2$, concentrated HCl, H_2O, THF; (d) $POCl_3$, 85 °C; (e) aryl borate, Pd $(PPh_3)_4$, K_2CO_3, dioxane, H_2O, 100 °C, Ar; (f) pyridine-4-boronic acid, $PdCl_2$(dppf), K_2CO_3, DMF, H_2O, 100 °C, Ar.

N-(2-acetylphenyl)acetamide **5f-2**

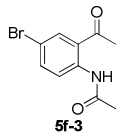


5f-2

To a solution of 1-(2-aminophenyl)ethanone (8.10g g, 60 mmol) **5f-1** and triethylamine (9.11g, 90 mmol) in DCM (100 mL) was added dropwise acetyl chloride (7.07 g, 90 mmol) and the reaction mixture was stirred at room temperature for 4 hours. The reaction mixture was diluted with DCM (100 mL) and washed with water (50 mL×2) and brine (50 mL×2), dried over anhydrous Na_2SO_4, filtered and concentrated to afford the crude product **5f-2** as a yellow solid (7.7 g, 72.4% yield), which was used directly in the next step without further purification.

^{1}H NMR (400 MHz, DMSO-d_6) δ 11.17 (s, 1H), 8.28 (d, J = 8.0 Hz, 1H), 7.97 (dd, J = 8.0, 1.6 Hz, 1H), 7.64 – 7.49 (m, 1H), 7.29 – 7.11 (m, 1H), 2.61 (s, 3H), 2.11 (s, 3H).

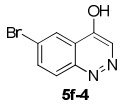
N-(2-acetyl-4-bromophenyl)acetamide **5f-3**



5f-3

To a solution of *N*-(2-acetylphenyl)acetamide **5f-2** (5.0 g, 28.2 mmol) in HOAc (50 mL) was added Br_2 (3.3 mL, 64.4 mmol) and the resulting mixture was stirred at room temperature for 1 hour. The reaction was quenched with saturated aqueous sodium thiosulfate, diluted with water (300 mL) and extracted with ethyl acetate (100 mL×3). The combined organic layers were washed with water (50 mL×2) and brine (50 mL×2), dried over anhydrous Na_2SO_4, filtered and concentrated to afford the crude product **5f-3** as a yellow solid (7.25 g, 100% yield), which was used directly in the next step without further purification.
^{1}H NMR (300 MHz, DMSO-d_6) δ 10.96 (s, 1H), 8.11 (d, J = 8.8 Hz, 1H), 8.05 (d, J = 2.4 Hz, 1H), 7.75 (dd, J = 8.8, 2.4 Hz, 1H), 2.60 (s, 3H), 2.10 (s, 3H).

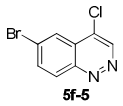
6-bromocinnolin-4-ol **5f-4**



5f-4

A mixture of *N*-(2-acetyl-4-bromophenyl)acetamide **5f-3** (5.1 g, 20 mmol), concentrated HCl (12mL) and water (12 mL) in THF (60 mL) was stirred at 85 °C for 1.5 hours. After removing THF from the reaction mixture, concentrated HCl (4mL) and water (4 mL) were added. The resulting reaction mixture was stirred at 100 °C for 3 hours. The reaction was diluted with water (100 mL), neutralized with saturated aqueous sodium bicarbonate and extracted with ethyl acetate (50 mL×3). The combined organic layers were washed with water (50 mL×2) and brine (50 mL×2), dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified by column chromatography (silica gel, petroleum ether/ethyl acetate = 1:1) to afford the product **5f-4** as a yellow solid (1.56 g, 34.8% yield).
^{1}H NMR (300 MHz, DMSO-d_6) δ 13.66 (s, 1H), 8.13 (d, J = 2.2 Hz, 1H), 7.95 (dd, J = 8.8, 2.2 Hz, 1H), 7.82 (s, 1H), 7.57 (d, J = 8.8 Hz, 1H).
MS (ESI+) m/z 224.9, 226.9 $[M + H]^+$.

6-bromo-4-chlorocinnoline **5f-5**

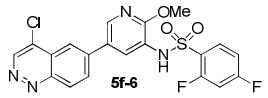


5f-5

A mixture of 6-bromocinnolin-4-ol **5f-4** (1.0 g, 4.4 mmol) in phosphorus oxychloride (10 mL) was stirred at 85 °C for 3.5 hours. After cooling, the reaction mixture was poured into ice-water, neutralized with saturated aqueous sodium bicarbonate and extracted with ethyl acetate (30 mL×3). The combined organic layers were washed with water (30 mL×2) and brine (30 mL×2), dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified by column chromatography (silica gel, petroleum ether/ethyl acetate = 10:1) to afford the product **5f-5** as a yellow solid (0.59 g, 54.7% yield).

^{1}H NMR (300 MHz, DMSO-d_6) δ 9.66 (s, 1H), 8.52 (d, J = 9.0 Hz, 1H), 8.43 (d, J = 2.1 Hz, 1H), 8.23 (dd, J = 9.0, 2.1 Hz, 1H),.
MS (ESI+) m/z 242.9, 244.9 $[M + H]^+$.

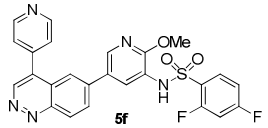
N-(5-(4-chlorocinnolin-6-yl)-2-methoxypyridin-3-yl)-2,4-difluorobenzenesulfonamide **5f-6**



5f-6

A mixture of 6-bromo-4-chlorocinnoline **5f-5** (260 mg, 1.07 mmol), *N*-(2-methoxy-5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)pyridin-3-yl)-2,4-difluorobenzenesulfonamide (320 mg, 0.75 mmol) and 2N aqueous potassium carbonate solution (1.60 mL, 3.2 mmol) in 1,4-dioxane (5 mL) was degassed, and then tetrakis-(triphenylphosphine)palladium(0) (123 mg, 0.107 mmol) was added. The resulting mixture was degassed and back-filled with argon (three cycles), and then stirred at 100 °C under argon atmosphere for 3 hours. The reaction mixture was concentrated in vacuo, diluted with water (15 mL), and extracted with ethyl acetate (15 mL×3). The combined organic layers were washed with water (15 mL) and brine (15 mL), dried over anhydrous sodium sulfate, filtered and concentrated. The redidue was purified with flash column chromatography (silica gel, petroleum ether/ethyl acetate = 2:1) to afford the product **5f-6** as a yellow solid (56 mg, 11% yield).
^{1}H NMR (300 MHz, DMSO-d_6) δ 10.45 (s, 1H), 9.62 (s, 1H), 8.66 – 8.62 (m, 2H), 8.40 (dd, J = 8.8, 1.8 Hz, 1H), 8.30 (d, J = 1.2 Hz, 1H), 8.13 (d, J = 2.0 Hz, 1H), 7.79 (td, J = 8.4, 6.6 Hz, 1H), 7.65 – 7.55 (m, 1H), 7.23 (td, J = 8.6, 2.0 Hz, 1H), 3.71 (s, 3H).
MS (ESI+) m/z 462.8 $[M + H]^+$.

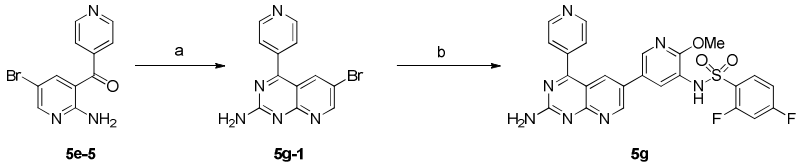
N-(2-methoxy-5-(4-(pyridin-4-yl)cinnolin-6-yl)pyridin-3-yl)-2,4-difluorobenzenesulfonamide **5f**



5f

A mixture of *N*-(5-(4-chlorocinnolin-6-yl)-2-methoxypyridin-3-yl)-2,4-difluorobenzenesulfonamide **5f-6** (100 mg, 0.22 mmol), pyridine-4-boronic acid (32 mg, 0.26 mmol) and 2N aqueous potassium carbonate solution (0.33 mL, 0.66 mmol) in DMF (5 mL) was degassed, and then [1,1′-bis(diphenylphosphino)ferrocene]dichloropalladium(II) (16 mg, 0.022 mmol) was added. The resulting mixture was degassed and back-filled with argon (three cycles), and then stirred at 100 °C under argon atmosphere for 3 hours. The reaction mixture was concentrated in vacuo, diluted with water (15 mL), and extracted with ethyl acetate (15 mL×3). The combined organic layers were washed with water (15 mL) and brine (15 mL), dried over anhydrous sodium sulfate, filtered and concentrated. The redidue was purified with flash column chromatography (silica gel, dichloromethane/methanol = 70:1) to afford the product **5f** as a yellow solid (20 mg, 18.3% yield).
^{1}H NMR (300 MHz, DMSO-d_6) δ 10.38 (s, 1H), 9.43 (s, 1H), 8.85 (d, J = 6.0 Hz, 2H), 8.69 (d, J = 8.9 Hz, 1H), 8.49 (d, J = 2.2 Hz, 1H), 8.33 (dd, J = 9.0, 1.7 Hz, 1H), 8.06 (d, J = 1.4 Hz, 1H), 8.01 (d, J = 2.2 Hz, 1H), 7.82 (d, J = 6.0 Hz, 2H), 7.73 (td, J = 8.6, 6.4 Hz, 1H), 7.60 – 7.50 (m, 1H), 7.22 – 7.11 (m, 1H), 3.67 (s, 3H).
98.23% by LCMS: *m/z* 505.9 $[M + H]^+$.

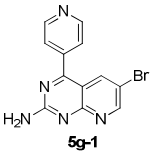
9. Procedure for the synthesis of compound 5g



5e-5 5g-1 5g

Scheme 6. Synthesis of compound **5g**. Reagents and conditions: (a) 2M HCl in ether, cyanamide, 90 °C; (b) aryl borate, $PdCl_2(dppf)$, K_2CO_3, DMF, H_2O, 100 °C, Ar.

6-Bromo-4-(pyridin-4-yl)pyrido[2,3-d]pyrimidin-2-amine **5g-1**



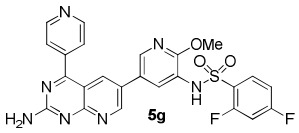
5g-1

A mixture of compound **5e-5** (0.33 g, 4.0 mmol) in 2M HCl in ether (15mL) was stirred at room temperature for 20 min and then evaporated. To the residue was added Cyanamide (5 mL) and the reaction mixture was stirred at 90 ℃ overnight. The reaction was diluted with water (50 mL) and extracted with ethyl acetate (30 mL×3). The combined organic layers were washed with water (30 mL×2) and brine (30 mL×2), dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified by column chromatography (silica gel, dichloromethane/methanol = 20:1) to give the product **5g-1** (100 mg, 30% yield) as a yellow solid.

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.97 (d, J = 2.6 Hz, 1H), 8.82 (d, J = 5.8 Hz, 2H), 8.13 (d, J = 2.6 Hz, 1H), 7.70 (d, J = 5.8 Hz, 2H), 7.59 (s, 2H)

MS (ESI+) m/z 302.0, 304.0 $[M + H]^+$

N-(5-(2-Amino-4-(pyridin-4-yl)pyrido[2,3-*d*]pyrimidin-6-yl)-2-methoxypyridin-3-yl)-2,4-difluorobenzenesulfonamide **5g**



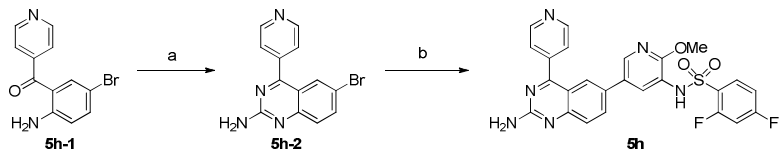
5g

Compound **5g** was prepared according to the procedure described for compound **5b**, using 6-Bromo-4-(pyridin-4-yl)pyrido[2,3-d]pyrimidin-2-amine **5g-1** instead of 3-bromo-5-(pyridin-4-yl)-1,8-naphthyridine **5b-6.**

^{1}H NMR (400 MHz, DMSO-d_6) δ 9.21 (d, J = 2.1 Hz, 1H), 8.84 (d, J = 5.5 Hz, 2H), 8.37 (s, 1H), 8.12 (d, J = 2.4 Hz, 1H), 7.91 (d, J = 1.8 Hz, 1H), 7.79 (d, J = 5.6 Hz, 2H), 7.76 – 7.69 (m, 1H), 7.59 – 7.49 (m, 3H), 7.29 (dd, J = 16.6, 8.2 Hz, 1H), 7.16 (t, J = 7.6 Hz, 1H), 3.64 (s, 3H).

96.48% by LCMS: *m/z* 522.1 $[M + H]^+$.

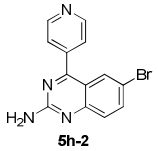
10. Procedure for the synthesis of compound 5h



5h-1 5h-2 5h

Scheme 7. Synthesis of compound **5h**. Reagents and conditions: (a) 2M HCl in ether, cyanamide, 90 ^{o}C; (b) aryl borate, $PdCl_2$(dppf), K_2CO_3, DMF, H_2O, 100 ^{o}C, Ar.

6-bromo-4-(pyridin-4-yl)quinazolin-2-amine **5h-2**



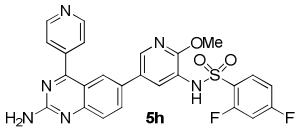
5h-2

Compound **5h-2** was prepared according to the procedure described for compound **5g-1**, using (2-amino-5-bromophenyl)(pyridin-4-yl)methanone **5h-1** (prepared according to the procedure described in WO2008157191) instead of (2-Amino-5-bromopyridin-3-yl)(pyridin-4-yl)methanone **5e-5.**

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.81 (dd, J = 4.4, 1.6 Hz, 2H), 7.84 (dd, J = 9.0, 2.2 Hz, 1H), 7.70 (d, J = 2.2 Hz, 1H), 7.68 (dd, J = 4.4, 1.6 Hz, 2H), 7.48 (d, J = 9.0 Hz, 1H), 7.19 (s, 2H).

MS (ESI+) m/z 301.0, 303.0 $[M + H]^+$

N-(5-(2-amino-4-(pyridin-4-yl)quinazolin-6-yl)-2-methoxypyridin-3-yl)-2,4-difluorobenzenesulfonamide **5h**



5h

Compound **5h** was prepared according to the procedure described for compound **5b**, using 6-bromo-4-(pyridin-4-yl)quinazolin-2-amine **5h-2** instead of 3-bromo-5-(pyridin-4-yl)-1,8-naphthyridine **5b-6.**

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.29 (s, 1H), 8.82 (d, J = 5.7 Hz, 2H), 8.29 (d, J = 2.3 Hz, 1H), 7.99 (dd, J = 8.8, 2.0 Hz, 1H), 7.80 (d, J = 2.3 Hz, 1H), 7.78 – 7.66 (m, 4H), 7.63 (d, J = 8.8 Hz, 1H), 7.58 – 7.47 (m, 1H), 7.16 (td, J = 8.4, 2.0 Hz, 1H), 7.10 (s, 2H), 3.63 (s, 3H).

^{13}C NMR (100 MHz, DMSO-d_6) δ 167.5, 165.0 (dd, $J_{C\text{-}F}$ = 252, 12 Hz), 160.2, 159.3 (dd, $J_{C\text{-}F}$ = 256, 13 Hz), 157.1, 152.8, 149.9, 144.1, 142.1, 133.4, 132.9, 131.7 (d, $^3J_{C\text{-}F}$ = 11 Hz), 130.1, 129.1, 126.4, 125.1 (dd, J = 14, 3.7 Hz), 123.9, 123.5, 119.7, 117.0, 111.8 (dd, $J_{C\text{-}F}$ = 22, 3.5 Hz), 105.8 (t, $^2J_{C\text{-}F}$ = 26 Hz), 53.4.

100% by LCMS: *m/z* 520.7 $[M + H]^+$.

11. PI3Kα Biochemical Assay

Compounds were evaluated for potency against PI3Kα using an *in vitro* kinase assay. PI3Kα enzymatic activity was determined by measuring the amount of ADP produced following the kinase reaction using a luciferase-based luminescence assay. ADP-Glo^{TM} kinase detection kit was from Promega. PI3Kα kinase was from Crownbio. The substrate PIP2/PS was from Invitrogen. All assays were performed in a OptiPlate^{TM}-384 white 384-well plate at room temperature. The kinase buffer contained 50 mM Hepes (pH 7.5), 3 mM $MgCl_2$, 100 mM NaCl, 1 mM EGTA, 0.03% CHAPS and 2 mM DTT. PI3Kα kinase mixture were prepared by diluting PI3Kα in the kinase buffer to 0.375 ng/μL. The ATP/substrate mixture contained 5 μM PIP2/PS and 25 μM ATP. Compounds for testing were diluted into 10 mM in 100% DMSO then serially diluted 3-fold in 100% DMSO at 10 different concentrations. Then the diluted compounds in 100% DMSO were 20-fold diluted in ddH_2O. 2 μL of diluted compound and 4 μL of ATP/substrate mixture were added to individual wells of 384-well assay plates. The reactions were started by adding 4 μL of PI3Kα kinase mixture per well. The final volume for the reaction was 10 μL, ATP concentration was 10 μM, PIP2/PS concentration

was 2 μM, and PI3Kα kinase concentration was 0.15 ng/μL. The assay plates were covered and reactions were allowed to proceed for 1 hour, after which 10 μL of Kinase Glo™ reagent per well was added. The plates were incubated for 40 minutes, and then 20μL of kinase detection reagent per well was added. The plates then were equilibrated in the dark for 30 minutes, after which luminescence was measured using an Envision plate reader.

The percentage of inhibition was calculated based on the following equation:

%inhibition = $[1 - (RLU_{compound} - RLU_{min})/(RLU_{max} - RLU_{min})] \times 100$,

where $RLU_{compound}$ is the luminescence reading at a given compound concentration, RLU_{min} is the luminescence reading at the highest concentration (2.5 μM) of positive control to completely inhibit PI3Kα kinase activity, and RLU_{max} is luminescence reading in the absence of a compound. IC_{50} values were determined by curve fitting using XLfit program of Excel software (Microsoft, USA).

12. PI3Kβ Biochemical Assay

Compounds were evaluated for potency against PI3Kβ using an *in vitro* kinase assay. PI3Kβ enzymatic activity was determined by measuring the amount of ADP produced following the kinase reaction using a luciferase-based luminescence assay. ADP-Glo™ kinase detection kit was from Promega. PI3Kβ kinase was from Crownbio. The substrate PIP2/PS was from Invitrogen. All assays were performed in a OptiPlate™-384 white 384-well plate at room temperature. The kinase buffer contained 50 mM Hepes (pH 7.5), 3 mM $MgCl_2$, 100 mM NaCl, 1 mM EGTA, 0.03% CHAPS and 2 mM DTT. PI3Kβ kinase mixture were prepared by diluting PI3Kβ in the kinase buffer to 11 ng/μL. The ATP/substrate mixture contained 25 μM PIP2/PS and 75 μM ATP. Compounds for testing were diluted into 10 mM in 100% DMSO then serially diluted 3-fold in 100% DMSO at 10 different concentrations. Then the diluted compounds in 100% DMSO were 20-fold diluted in ddH_2O. 2 μL of diluted compound and 4 μL of ATP/substrate mixture were added to individual wells of 384-well assay plates. The reactions were started by adding 4 μL of PI3Kβ kinase mixture per well. The final volume for the reaction was 10 μL, ATP concentration was 30 μM, PIP2/PS concentration was 10 μM, and PI3Kβ kinase concentration was 4.4 ng/μL. The assay plates were covered and reactions were allowed to proceed for 1 hour, after which 10 μL of Kinase Glo™ reagent per well was added. The plates were incubated for 40 minutes, and then 20μL of kinase detection reagent per well was added. The plates then were equilibrated in the dark for 30 minutes, after which luminescence was measured using an Envision plate reader.

The percentage of inhibition was calculated based on the following equation:

%inhibition = $[1 - (RLU_{compound} - RLU_{min})/(RLU_{max} - RLU_{min})] \times 100$,

where $RLU_{compound}$ is the luminescence reading at a given compound concentration, RLU_{min} is the luminescence reading at the highest concentration (20 μM) of positive control to completely inhibit PI3Kβ kinase activity, and RLU_{max} is luminescence reading in the absence of a compound. IC_{50} values were determined by curve fitting using XLfit program of Excel software (Microsoft, USA).

13. PI3Kδ Biochemical Assay

Compounds were evaluated for potency against PI3Kδ using an *in vitro* kinase assay. PI3Kδ enzymatic activity was determined by measuring the amount of ADP produced following the kinase reaction using a luciferase-based luminescence assay. ADP-Glo™ kinase detection kit was from Promega. PI3Kδ kinase was from Crownbio. The substrate PIP2/PS was from Invitrogen. All assays were performed in a OptiPlate™-384 white 384-well plate at room temperature. The kinase buffer contained 50 mM Hepes (pH 7.5), 3 mM $MgCl_2$, 100 mM NaCl, 1 mM EGTA, 0.03% CHAPS and 2 mM DTT. PI3Kδ kinase mixture

were prepared by diluting PI3Kδ in the kinase buffer to 10 ng/μL. The ATP/substrate mixture contained 5 μM PIP2/PS and 25 μM ATP. Compounds for testing were diluted into 10 mM in 100% DMSO then serially diluted 3-fold in 100% DMSO at 10 different concentrations. Then the diluted compounds in 100% DMSO were 20-fold diluted in ddH_2O. 2 μL of diluted compound and 4 μL of ATP/substrate mixture were added to individual wells of 384-well assay plates. The reactions were started by adding 4 μL of PI3Kδ kinase mixture per well. The final volume for the reaction was 10 μL, ATP concentration was 10 μM, PIP2/PS concentration was 2 μM, and PI3Kδ kinase concentration was 4 ng/μL. The assay plates were covered and reactions were allowed to proceed for 1 hour, after which 10 μL of Kinase Glo™ reagent per well was added. The plates were incubated for 40 minutes, and then 20μL of kinase detection reagent per well was added. The plates then were equilibrated in the dark for 30 minutes, after which luminescence was measured using an Envision plate reader.

The percentage of inhibition was calculated based on the following equation:

$\%inhibition = [1 - (RLU_{compound} - RLU_{min})/(RLU_{max} - RLU_{min})] \times 100$,

where $RLU_{compound}$ is the luminescence reading at a given compound concentration, RLU_{min} is the luminescence reading at the highest concentration (20 μM) of positive control to completely inhibit PI3Kδ kinase activity, and RLU_{max} is luminescence reading in the absence of a compound. IC_{50} values were determined by curve fitting using XLfit program of Excel software (Microsoft, USA).

14. PI3Kγ Biochemical Assay

Compounds were evaluated for potency against PI3Kγ using an *in vitro* kinase assay. PI3Kγ enzymatic activity was determined by measuring the amount of ADP produced following the kinase reaction using a luciferase-based luminescence assay. ADP-Glo™ kinase detection kit was from Promega. PI3Kγ kinase was from Crownbio. The substrate PIP2/PS was from Invitrogen. All assays were performed in a OptiPlate™-384 white 384-well plate at room temperature. The kinase buffer contained 50 mM Hepes (pH 7.5), 3 mM $MgCl_2$, 100 mM NaCl, 1 mM EGTA, 0.03% CHAPS and 2 mM DTT. PI3Kγ kinase mixture were prepared by diluting PI3Kγ in the kinase buffer to 4.5 ng/μL. The ATP/substrate mixture contained 5 μM PIP2/PS and 25 μM ATP. Compounds for testing were diluted into 10 mM in 100% DMSO then serially diluted 3-fold in 100% DMSO at 10 different concentrations. Then the diluted compounds in 100% DMSO were 20-fold diluted in ddH_2O. 2 μL of diluted compound and 4 μL of ATP/substrate mixture were added to individual wells of 384-well assay plates. The reactions were started by adding 4 μL of PI3Kγ kinase mixture per well. The final volume for the reaction was 10 μL, ATP concentration was 10 μM, PIP2/PS concentration was 2 μM, and PI3Kγ kinase concentration was 1.8 ng/μL. The assay plates were covered and reactions were allowed to proceed for 1 hour, after which 10 μL of Kinase Glo™ reagent per well was added. The plates were incubated for 40 minutes, and then 20μL of kinase detection reagent per well was added. The plates then were equilibrated in the dark for 30 minutes, after which luminescence was measured using an Envision plate reader.

The percentage of inhibition was calculated based on the following equation:

$\%inhibition = [1 - (RLU_{compound} - RLU_{min})/(RLU_{max} - RLU_{min})] \times 100$,

where $RLU_{compound}$ is the luminescence reading at a given compound concentration, RLU_{min} is the luminescence reading at the highest concentration (20 μM) of positive control to completely inhibit PI3Kγ kinase activity, and RLU_{max} is luminescence reading in the absence of a compound. IC_{50} values were determined by curve fitting using XLfit program of Excel software (Microsoft, USA).

15. mTOR Biochemical Assay

Compounds were evaluated for potency against mTOR using an *in vitro* kinase assay. mTOR activity is measured *in vitro* by determining the level of phosphorylation of the kinase substrate 4EBP-1. mTOR kinase, substrate GFP-4E-BP1, antibody Tb-anti-p4E-BP1(pThr46) and TR-FRET dilution buffer were from Invitrogen.

All assays were performed in a OptiPlateTM-384 white 384-well at room temperature. The kinase buffer contained 50 mM Hepes (PH7.5), 1 mM EGTA, 10 mM $MnCl_2$, 0.01% Tween20 and 2 mM DTT. mTOR kinase mixture were prepared by diluting mTOR in kinase buffer to 0.5 ng/μL. The ATP/substrate mixture contained 1 μM GFP-4E-BP1 and 25 μM ATP. TR-FRET detection solution contained 20mM EDTA and 4nM Tb-anti-p4E-BP1 in TR-FRET dilution buffer。Compounds for testing were diluted into 10 mM in 100% DMSO then serially diluted 3-fold in 100% DMSO at 10 different concentrations. Then the diluted compounds in 100% DMSO were 20-fold diluted in ddH_2O. The final starting concentration was 2.5 μM and the final lowest concentration was 0.5 nM, and DMSO concentration was 1%. 2 μL of diluted compound solution and 4 μL of ATP/substrate mixture were added to individual wells of 384-well assay plates. The reactions were started by adding 4 μL of mTOR kinase mixture per well. The final volume for the reaction was 10 μL, ATP concentration was 10 μM, GFP-4E-BP1 concentration was 0.4 μM, and mTOR kinase concentration was 0.2 ng/μL. The assay plates were covered and reactions were allowed to proceed at room temperature for 1 hour, after which 10 μL of TR-FRET detection solution was added to stop the reaction and detect the product. The plates were incubated in the dark for 30 minutes. The FRET signal at 495 nm and 520 nm after excitation at 340 nm was measured on an Envision 2014 plate reader.

The percentage of inhibition was calculated based on the following equation:

$$\%\text{inhibition} = [1 - (FRET_{compound} - FRET_{min})/(FRET_{max} - FRET_{min})] \times 100,$$

where $FRET_{compound}$ is the ratio of FRET reading at 520 nm to FRET reading at 495 nm at a given compound concentration, $FRET_{min}$ is the ratio of FRET reading at 520 nm to FRET reading at 495 nm at the highest concentration (2.5 μM) of positive control to completely inhibit mTOR kinase activity, and $FRET_{max}$ is the ratio of FRET reading at 520 nm to FRET reading at 495 nm in the absence of a compound. IC_{50} values were determined by curve fitting using XLfit program of Excel software (Microsoft, USA).

16. Parallel artificial membrane permeation assay (PAMPA)

Prior to use, the pre-coated PAMPA plate system (BD Gentest) should be warmed to room temperature (25□) for at least 30 minutes. While the plate system is warming, the compound solutions can be prepared by diluting stock solutions into a buffer by the following steps.

a) Preparation of dialysis buffer (HyClone™ Phosphate Buffered Saline (PBS), pH 7.0-7.2)

b) Preparation of incubation solution.
   14 μL of stock solution (10 mM in DMSO) + 1400 μL of PBS = 100 μM working solution A
   or 140 μL of working solution A + 1260 μL of PBS(1% DMSO) = 10 μM working solution B

c) Add triplicate 300 μL of working solutions A or B per well in the receiver plate (donor plate).

d) Add triplicate 200 μL of blank buffer per well in the filter plate (acceptor plate).

e) Place the filter plate on the receiver plate by slowly lowering the pre-coated PAMPA plate until it sits on the receiver plate. Incubate the assembly at room temp for 5 hours.

f) Add triplicate 100 μL of working solutions A or B per well to C0 plate, then add 300 μL of acetonitrile (ACN) containing internal standard (IS, 200 ng/mL Tolbutamide), shook and stored at 4□.

g) After incubation, separate the pre-coated PAMPA plate and the receiver plate. Transfer aliquots of 100 μL from donor and acceptor plate into the sample collection plate.

h) Add 300 μL of acetonitrile (ACN) containing internal standard (IS, 200 ng/mLTolbutamide), then

shook sample collection plate at 800 rpm for 20 min, then centrifuged at 4000 rpm for 20 min.

i) Transfer an aliquot of supernatant (200 µL) in each well and mixed with 400 µL Milli-Q water, mixed. Acceptor sample collection plate was sent to LC/MS/MS analysis.

j) Transfer 50 µL donor and C0 plate solution of step 9 mixed with ACN:water 1:3 (IS, 50 ng/mL Tolbutamide) in each well respectively, mixed. Then they were delivered to LC/MS/MS analysis on Waters UPLC system coupling to an API 4000 MS instrument, using Waters UPLC AQURITY BEH C18 column (1.7 µm, 2.1*50 mm). The moblie phase A is 0.1% Formic Acid in water and the moblie phase B is 0.1% Formic Acid in acetonitrile.

Data Analysis:

Permeability (in unit of cm/s):

$$P_e = \frac{-\ln[1 - C_A(t)/C_{equilibrium}]}{A*(1/V_D + 1/V_A)*t}$$

Mass Retention:

$$R = 1 - [C_D(t)*V_D + C_A(t)*V_A]/(C_0*V_D)$$

Where:

C_0 = initial compound concentration in donor well (mM)

$C_D(t)$ = compound concentration in donor well at time t. (mM)

$C_A(t)$ = compound concentration in acceptor well at time t. (mM)

V_D = donor well volume = 0.3 mL

V_A = acceptor well volume = 0.2 mL

$C_{equilibrium} = [C_D(t)*V_D + C_A(t)*V_A]/(V_D + V_A)$

A = filter area = 0.3 cm^2

t = incubation time = 18000 s (= 5 hr)

17. Caco-2 permeability assay

1). Caco-2 Culture

Caco-2 cells purchased from ATCC were seeded onto polyethylene membranes (PET) in 24-well Corning Transwell plates at 1 x 10^5 cells/cm^2, and refreshed medium every other day until to the 21st day for confluent cell monolayer formation.

2). Cell monolayer integrity

The standard transport buffer in the study was HBSS at pH 7.4. Before and after the transport studies, the monolayer integrity was evaluated by measuring transepithelial electrical resistance (TEER). The pre-transport and post-transport TEER were required to be no less than 200 Ω•cm^2.

3). Transport study

The standard transport buffer in the study was HBSS at pH 7.4. Test compounds were diluted with transport buffer from a 10 mM stock solution to a concentration of 10 µM . The bidirectional permeability of the test compounds were investigated by adding the compound solution either in the apical chamber or the basolateral chamber. Efflex ratio was used to identify whether the test compounds were Pgp substrate or not. Test and reference compounds were quantified by LC-MS/MS analysis.

a) On day 21, transfer 50 µL of 10 µM dosing solution(compound solution is diluted from DMSO

stock in HBSS, pH 7.4, with a final DMSO concentration of less than 1%) into a pre-labeled 96 deep well plate containing 200 μL of ACN with internal standard (Tolbutamide at 500 nM) and mix thoroughly. This is the C_0 sample.

b) Remove cell culture medium from the insert and wash the cell monolayer twice with HBSS (Ca.30 mL).

c) For A→B (Apical to Basolateral) transport study, add 100 μL dosing solution into the Apical sides, and 600 μL transport buffer(HBSS) into the Basolateral sides.

d) For B→A transport study, add 600 μL dosing solution into the Basolateral sides, and 100 μL transport buffer (HBSS) into the Apical sides.

e) Plates are incubated for 1.5 hours in the incubator at 37°C, 95% humidity, 5% CO_2.

f) After incubation, remove plates from the incubator and transfer 50 μL of each sample (A→B, B→A receiver and donor) into a pre-labeled 96 deep well plate containing 200 μL ACN with internal standard, mix thoroughly.

g) Centrifuge the sample plates at 3750 rpm for 20 minutes to precipitate protein, then transfer 180 μL/well supernatant, mix throughly with 120 μL Milli-Q water and analyze samples by LC/MS/MS.

4). Data analysis

The Papp value is derived using the following equation and has the dimension of a rate (10-6cm/s).

$$P_{app} = C_{rec} \times V_{rec} / (A \times t \times C_0)$$

Where C_{rec} is the compound concentration in the receiver chamber at the measurement time t; V_{rec} is the volume of the receiver chamber; A is the area of the permeability barrier, which corresponds to the surface area of the filter (0.33 cm^2 for Corning 24- insert plates); C_0 is the initial concentration in the donor chamber (μM).

The Efflux ratio is derived using the following equation:

$$\text{Efflux Ratio} = P_{app}(B \rightarrow A)/P_{app}(A \rightarrow B)$$

18. pAKT inhibition study in T47D cells

T47D cells were cultured in RPMI1640 medium (Hyclone) supplemented with 10% FBS (Gibco), in the temperature of 37 °C, 5% CO_2 and 95% humidity. T47D cells were treated with compound **6g** of for 4 hours, then detecting the the pAKT inhibition by western blot.

Day 1 - Cell seeding

1) Harvest the cells during the logarithmic growth period and counted with Vi-Cell (Beckman Coulter), seed in average 8×10^5 cells/2 mL each well in 6-well plate.

Day 2- Drug treatment and protein lysate preparation

2) Dispense 0.5 mL serial diluted different compounds (10 uM, 3 uM, 1 uM, 300 nM, 100 nM, 30 nM, 10 nM, 2 nM and 0 nM in medium containing 0.5% DMSO) in each well, then the final DMSO concentration in each assay well is 0.1%, and 0 nM as vehicle control.

3) After 4h drug treatment, remove medium and wash cells with cold PBS (Gibco, PH 7.2) twice. Add 100 μL cell lysis buffer, which were prepared by adding 1 complete-EDTA free tablet (Roche), 100 uL of Phosphatase inhibitor cocktail II (Sigma), and 100 uL of Phosphatase inhibitor cocktail III (Sigma) into 10 mL of 1×CelLytic lysis buffer (Sigma).

4) Incubate the plates added lysis buffer on ice for 10 minutes to induce cell lysis.

5) Scrape off cells and transfer the lysates to 1.5 mL Eppendorf tubes. Vortex the cell lysates and spin at 13000 rpm for 20 min at 4 °C to remove cell debris.

Day 2- Protein quantification

This part refer to the "Instruction of Pierce 23227 BCA Protein Assay Kit" page 2: the part of "Preparation of Standards and Working Reagent ", and page 3, the part of "Microplate Procedure (Sample to WR ratio = 1:8)"

6) Prepare BCA standard (0, 25 μg/mL, 125 μg/mL, 250 μg/mL, 500 μg/mL, 750 μg/mL, 1000 μg/mL, 1500 μg/mL, 2000 μg/mL) according to the user manual.
7) Dilute cell lysate sample with PBS at 1:5 ratio.
8) Pipette 25 μL of each standard or sample duplicate into 96-well plate.
9) Add 200 μL of the working reagent (50:1 of Reagent A:B) to each well and mix plate thoroughly on a plate shaker for 30 sec.
10) Cover plate and incubate at 37 °C for 30 min.
11) Cool plate to RT. Measure the absorbance at or near 562 nm on Spectramax 384PLUS or Envision. Calculate total protein amount of each sample.

Day 3/4- Western blotting

12) Preparation of sample: Mix each sample with 4X LDS sample buffer plus reducing agent. Boil at 70°C for 10 mins. Cool down on ice before centrifuge briefly. Ready to use.
13) Preparation of gel: Unpack Bio-rad 4-15% precast gel and wash wells with running buffer. Assemble the gel into the cassette. Fill the inner and outer chamber with running buffer.
14) Running the gel: Load samples, 30□g/lane. Run the gel with constant voltage (100V). Stop running when the dye is close to the lower edge. About 1.5hr.
15) Membrane transfer: Pre-activate PVDF membrane in ethanol for 1 or 2 min, followed by pre-wet it and filter paper in cold transfer buffer. Assemble 'sandwich' for wet transfer blot, down to up: filter paper–gel–membrane–filer paper; wet transfer, 1.5 hr at 300 mA.
16) Blocking: when transfer finished, immerse PVDF membrane in 5% milk TBS/0.1%T, and generally shake, 1hr, RT.
17) Incubate with primary antibody in 0.5% milk TBS/0.1%T at 4ºC overnight, the dilution ratio is 1:1000 of pAkt, and Akt.
18) Wash membrane in TBS/0.1%T, 5mins ×3.
19) Incubate the blots with IRDye 800/680 anti-rabbit/ mouse antibody (Licor), 1:5000 diluted in 0.5% milk TBS/0.1%T solution, 1hr, RT.
20) Wash membrane in TBS/0.1%T, 5 mins ×3.
21) Detect with Odyssey (LI-COR Biosciences Co) for pAkt and Akt: rinse membrane once with TBS then scan with Odyssey.

19. Proliferation assays

Compounds were evaluated for antiproliferative potency against U87MG, T47D, SKOV3, H1975, H460 and A549 tumor cell lines using an CellTiter-Glo® Luminescent Cell Viability Assay from Promega. The human tumor cell lines used were obtained from the ATCC. All the mediums and FBS were Gibco. T47D, H1975 and H460 tumor cells were cultured in RPMI1640 medium supplemented with 10% FBS. SKOV3 ovarian cancer cells were cultured in McCoy's 5a medium supplemented with 10% FBS. U87MG were cultured in DMEM medium supplemented with 10%FBS. A549 were cultured in Ham's F12K medium supplemented with 10%FBS.Tumor cells were seeded at a volume of 80 μL/well and a density of 3000 cells/well in 96-well plates and subsequently incubated at 37 °C and 5% CO_2 for 2 hours. Compounds for testing were diluted into 10 mM in 100% DMSO then serially diluted 3.16-fold in 100% DMSO at 10 different

concentrations. Then the diluted compounds in 100% DMSO were 20-fold diluted in 100% DMSO. The tumor cells were then treated with serially diluted compound or DMSO control in the incubator at 37 °C and 5% CO_2 for 4 days, prior to the addition of CellTiter-Glo reagents (Promega) and reading of luminescence using a PerkinElmer Envision plate reader. IC_{50} values were determined by curve fitting using XLfit program of Excel software (Microsoft, USA).

The percentage of inhibition was calculated based on the following equation:

$$\%\text{inhibition} = [1 - (V_{compound} - V_{medium})/(V_{vehicle\ control} - V_{medium})] \times 100,$$

where $V_{compound}$ is the reading at a given compound concentration, V_{medium} is the reading in the absence of a compound and DMSO, and $V_{vehicle\ control}$ is the reading of vehicle control group. IC_{50} values were determined by curve fitting using XLfit program of Excel software (Microsoft, USA).