Supplementary Information

The Crystal Structure of Cancer Osaka Thyroid Kinase Reveals an Unexpected Kinase Domain Fold*

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*Running title: *Structural Basis for Cancer Osaka Thyroid Kinase Inhibition*

Figure 1.

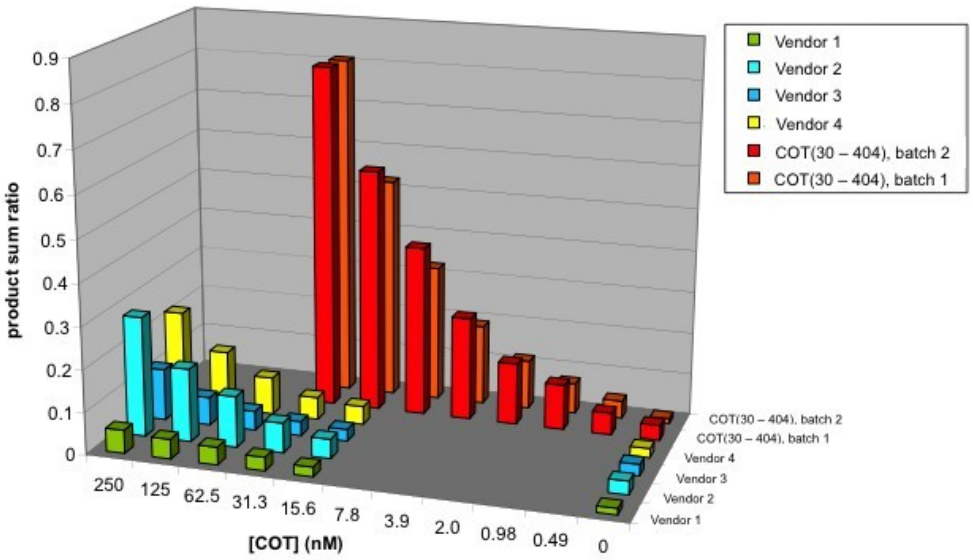
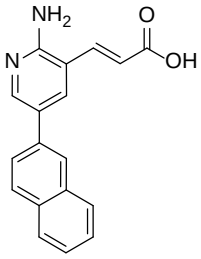


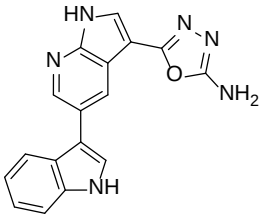
Figure 1. Enzymatic activity of COT kinase in the *in vitro* phosphorylation assay. Enzymatic activity of different batches of COT kinase were compared in a Caliper based *in vitro* phosphorylation assay. The ratios of phosphorylated and non-phosphorylated substrate peptides are reported for each batch of enzyme. Two batches of COT kinase produced by procedures described here were compared to commercially available material from four different vendors.

Chemical synthesis of compounds 1, 2 and 3.

Chemical structures of compounds 1, 2 and 3:



1



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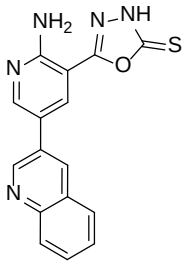
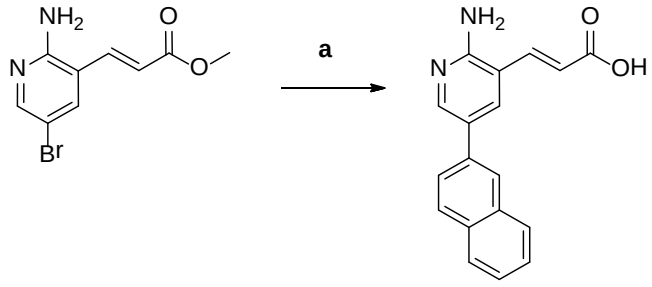


3

Scheme 1. A) Synthesis of (*E*)-3-(2-amino-5-(naphthalen-2-yl)pyridin-3-yl)acrylic acid (**1**), B) synthesis of 5-(5-(1H-indol-3-yl)-1H-pyrrolo[2,3-b]pyridin-3-yl)-1,3,4-oxadiazol-2-amine (**2**) and C) synthesis of 5-(2-amino-5-(quinolin-3-yl)pyridin-3-yl)-1,3,4-oxadiazole-2(3H)-thione (**3**) .

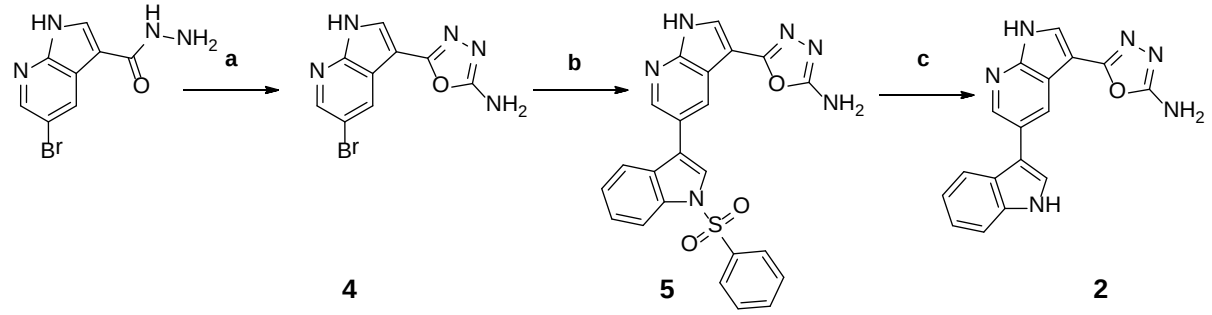
A



1

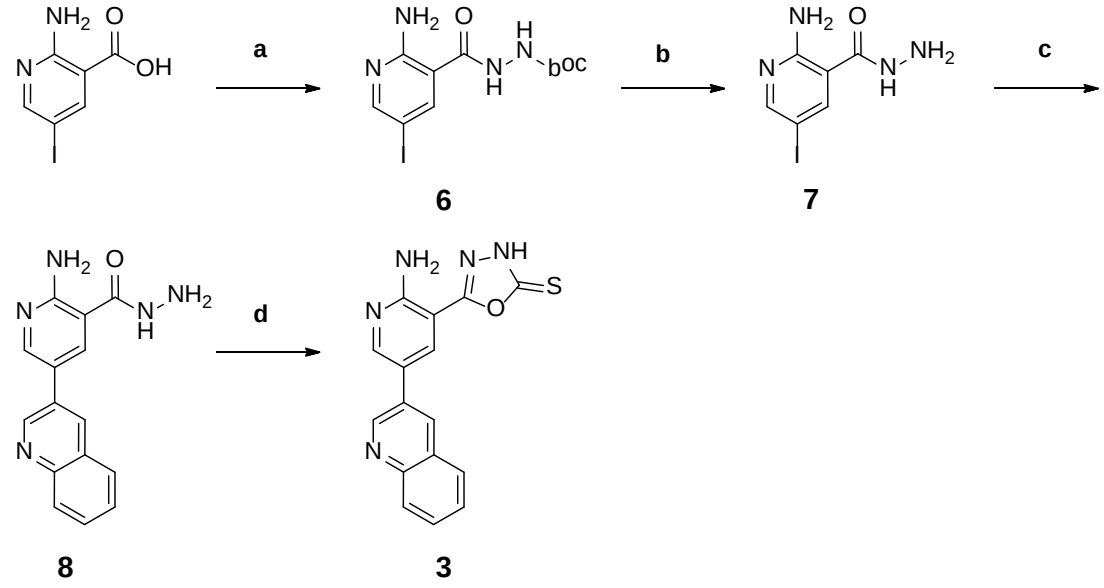
a) 2-naphtylboronic acid, $Pd(PPh_3)_4$, $NaHCO_3$, DME, 12h, 140°C; 1M NaOH, rt 2.5h.

B



a) Cyanogen bromide, aq. $NaHCO_3$, 1,4-dioxane, rt, 49%; b) 1-(phenylsulfonyl)-1H-indol-3-ylboronic acid, $PdCl_2(PPh_3)_2$, Na_2CO_3, DME/water/EtOH, 1.5h, 130°C, mw, 47%; c) Cs2CO3, MeOH, 70°C, 1h, 45%.

C



a) tert-Butyl carbazate, EDAC, HOBt, TEA, DCM, 24h rt, 100%; b) HCl in MeOH, 17h, rt, 66%; c) 3-quinolinboronic acid, $Pd(PPh_3)_4$, Na_2CO_3, DME/water/EtOH, 0.5h, 140°C, mw, 99%; d) carbon disulfide, aq NaOH, EtOH, 80°C, 15h, 21%.

C

Experimental Procedures:

All reagents and solvents were purchased from commercial suppliers and used without further purification or were prepared according to published procedures. All reactions were performed under inert conditions (argon) unless otherwise stated. 1H -NMR spectra were recorded on a Bruker 400 MHz or a Bruker 600 MHz NMR spectrometer. Chemical shifts are reported in parts per million (ppm) relative to an internal solvent reference. Significant peaks are tabulated in the order multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; quintet; m, multiplet; br, broad), coupling constants, and number of protons. Purity of final compounds was assessed by analytical liquid chromatography with one of the following methods.

Method A: HP-1100 LC–MS; column Zorbax SB-C18 1.8 μm, 3 mm × 30 mm; A, water + 0.05% trifluoroacetic acid; B, acetonitrile + 0.05% trifluoroacetic acid; 10–100% B in 3.25 min, 100% B 0.75 min, flow 0.7 ml/min; column temperature 35 °C.

Method B: Acquity UPLC/SQD; LC–MS; column Acquity HSS T3 1.8 μm, 2.1 mm × 50 mm; A, water + 0.05% formic acid + 3.75 mM ammonium acetate; B, acetonitrile + 0.04% formic acid; 10-95% B in 1.50 min, 95% B 2.50 min, flow 1.0 ml/min; column temperature 60 °C.

Method C: Acquity UPLC/SQD; LC–MS; column Acquity HSS T3 1.8 μm, 2.1 mm × 50 mm; A, water + 0.05% formic acid + 3.75 mM ammonium acetate; B, acetonitrile + 0.04% formic acid; 5-98% B in 1.40 min, 98% B 1.80 min, flow 1.0 ml/min; column temperature 60 °C.

A) Synthesis of (*E*)-3-(2-amino-5-(naphthalen-2-yl)pyridin-3-yl)acrylic acid (1).

A solution of (*E*)-methyl 3-(2-amino-5-bromopyridin-3-yl)acrylate (635 mg, 2.47 mmol), 2-naphtylboronic acid (482 mg, 2.72 mmol), tetrakis(triphenylphosphine)palladium (288 mg, 0.247 mmol) and 1 M aqueous sodium hydrogen carbonate (12.35 ml) in dimethoxyethane (18 ml) were stirred at 140°C for 12h. The reaction mixture was cooled to rt, diluted with 1M sodium hydroxide (10 ml) and

stirred for another 2.5h at rt. Resulting reaction mixture was filtered and the filter cake washed with ethyl acetate (10 ml) and 1 M sodium hydroxide (10 ml). Separation of the aqueous layer followed by acidification to pH 5-6 using 4 M hydrochloric acid and isolation of the newly formed white precipitate afforded (*E*)-3-(2-amino-5-(naphthalen-2-yl)pyridin-3-yl)acrylic acid (380 mg, 2.47 mmol, 53%) in high purity (100%). LC-MS: Rt: 0.86 min (Method C), MS (ESI): *m/z* 291.3 [M^+H]. 1H-NMR (400 MHz, DMSO-d

6): 8.51 (d, 1 H), 8.26 (d, J=2.27 Hz, 1 H), 8.24 (s, 1H), 8.02-7.84 (m, 5H), 7.79 (d, J=15.66 Hz, 1H), 7.59-7.40 (m, 3H), 6.65 (d, J=15.66 Hz, 1H), 6.52 (s, br, 2H).

B) Synthesis of 5-(5-(1H-indol-3-yl)-1H-pyrrolo[2,3-b]pyridin-3-yl)-1,3,4-oxadiazol-2-amine (2)

5-(5-bromo-1H-pyrrolo[2,3-b]pyridin-3-yl)-1,3,4-oxadiazol-2-amine (4)

An aqueous solution of sodium hydrogen carbonate (1.5 ml, 1.111 mmol) was added at rt to a suspension of 5-bromo-1H-pyrrolo[2,3-b]pyridine-3-carbohydrazide (300 mg, 1.059 mmol) in 1,4-dioxane (3 ml). The mixture was stirred for 10min before cyanogen bromide (118 mg, 1.111 mmol) was added. Resulting orange solution was stirred at room temperature for 2h40 before water (5 ml) was added. Resulting brown precipitate was extracted with ethyl acetate (adding NaCl to saturate the aqueous phase made the extraction of the product easier). Combined organic layers were dried over Na_2SO_4 and the solvent was evaporated to yield 200 mg of a brown solid. Trituration of the solid in MeOH afforded 5-(5-bromo-1H-pyrrolo[2,3-b]pyridin-3-yl)-1,3,4-oxadiazol-2-amine (**4**) (160 mg, 0.514 mmol, 49%) as a beige powder (90% purity). LC-MS: Rt: 2.343 min (Method A), MS (ESI): *m/z* 280.0, 282.0 [M^+H]; 1H-NMR (DMSO-d6): 12.58 (s, br, 1H), 8.46 (d, J = 2.1 Hz, 1H), 8.42 (d, J = 2.1 Hz, 1H), 8.05 (s, 1H), 7.10 (s, 2H).

5-(5-(1-(phenylsulfonyl)-1H-indol-3-yl)-1H-pyrrolo[2,3-b]pyridin-3-yl)-1,3,4-oxadiazol-2-amine (5)

A solution of 5-(5-bromo-1H-pyrrolo[2,3-b]pyridin-3-yl)-1,3,4-oxadiazol-2-amine (**4**) (122 mg, 0.436 mmol), 1-(phenyl-sulfonyl)-1H-indol-3-ylboronic acid (176 mg, 0.566 mmol), bis(triphenylphosphine)palladium(II)chloride (31.2 mg, 0.044 mmol) and sodium carbonate (106 mg,

1.006 mmol) in dimethoxyethane/water/ethanol (2.7 ml, 7/3/2) were stirred in at 130°C in the microwave for 1.5h. The reaction mixture was cooled to rt, diluted with water (4 ml) and extracted with ethyl acetate (2x 5 ml). Combined organic layers were dried over Na_2SO_4 and the solvent evaporated to yield a brown solid. Trituration in Et_2O afforded 5-(5-(1-(phenylsulfonyl)-1H-indol-3-yl)-1H-pyrrolo[2,3-b]pyridin-3-yl)-1,3,4-oxadiazol-2-amine (**5**) (109 mg, 0.203 mmol; 47%) as a pale pink powder (85% purity). LC-MS: Rt: 3.169 min (Method A), MS (ESI): *m/z* 425.0 [M^+H].

5-(5-(1H-indol-3-yl)-1H-pyrrolo[2,3-b]pyridin-3-yl)-1,3,4-oxadiazol-2-amine (2)

Caesium carbonate (331 mg, 1.015 mmol) was added at rt to a solution of 5-(5-(1-(phenylsulfonyl)-1H-indol-3-yl)-1H-pyrrolo[2,3-b]pyridin-3-yl)-1,3,4-oxadiazol-2-amine (**5**) (109 mg, 0.203 mmol) in MeOH (3 ml). After stirring at 70°C for 1h, the reaction mixture was cooled to rt and the solvent evaporated to yield a brown solid. Purification by preparative HPLC (Waters Sunfire C18 5 µm, 30 mm × 100 mm; A, water + 0.1% trifluoroacetic acid; B, acetonitrile; 15–35% B in 16 min, flow 50 ml/min) afforded 5-(5-(1H-indol-3-yl)-1H-pyrrolo[2,3-b]pyridin-3-yl)-1,3,4-oxadiazol-2-amine (**2**) (40mg, 0.091 mmol, 45%) as a yellow solid (>98% purity). LC-MS: Rt: 2.585 min (Method A), MS (ESI): *m/z* 425.0 [M^+H]; 1H-NMR (DMSO-d6): 12.33 (d, J = 3.0 Hz, 1H), 11.41 (s, 1H), 8.59 (d, J = 2.1 Hz, 1H), 8.65 (d, J = 2.1 Hz, 1H), 7.97 (d, J = 2.8 Hz, 1H), 7.84 (d, J = 7.8 Hz, 1H), 7.75 (d, J = 2.5 Hz, 1H), 7.47 (d, J = 7.9 Hz, 1H), 7.29 (s, 2H), 7.15 (dt, J = 19.4, 7.0 Hz, 2H).

C) Synthesis of 5-(2-amino-5-(quinolin-3-yl)pyridin-3-yl)-1,3,4-oxadiazole-2(3H)-thione (3).

tert-butyl 2-(2-amino-5-iodonicotinoyl)hydrazinecarboxylate (6)

tert-Butyl carbazate (8.00 g, 60.5 mmol) was added to a prestirred solution of 2-amino-5-iodonicotinic acid (10.0 g, 37.9 mmol), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (11.0 g, 77.9 mmol), hydroxybenzotriazole (8.70 g, 56.8 mmol) and triethylamine (11.5 g, 114 mmol) in dichloromethane (200 ml) and stirred for 24 h at rt. The reaction mixture was washed with water (200 ml) and the aqueous phase extracted with dichloromethane (3x 150 ml). Combined organic layers were dried

over Na_2SO_4 and the solvent evaporated to afford 2-amino-5-iodonicotinohydrazide **(6)** (15.29 g, 40.4 mmol, 107%) as a brown solid (95% purity). LC-MS: Rt: 0.76 min (Method B), MS (ESI): *m/z* 253.3 $[M^+H]$.

2-amino-5-iodonicotinohydrazide (7)

2-amino-5-iodonicotinohydrazide **(6)** (15.29 g, 40.4 mmol) was stirred in a concentrated hydrogen chloride methanol solution (30 ml) for 17h at rt. The formed precipitate was filtered, transferred to a mixture of MeOH (5 ml) and aqueous saturated Na_2CO_3(20 ml) and resulting solution was stirred for 1h at rt. The newly formed precipitate is filtered and dried to afford 2-amino-5-(quinolin-3-yl)nicotinohydrazide (**7**) (7.4g, 26.6 mmol, 66%) as a white solid (99% purity). LC-MS: Rt: 0.33 min (Method A), MS (ESI): *m/z* 279.1 $[M^+H]$.

2-amino-5-(quinolin-3-yl)nicotinohydrazide (8)

A solution of 2-amino-5-(quinolin-3-yl)nicotinohydrazide (**7**) (500 mg, 1.79 mmol), 3-quinolinboronic acid (467 mg, 2.70 mmol), tetrakis(triphenylphosphine)palladium (229 mg, 0.198 mmol) and sodium carbonate (305 mg, 2.88 mmol) in dimethoxyethane/water/ethanol (17 ml, 7/3/2) were stirred in at 140°C in the microwave for 0.5h. The reaction mixture was cooled to rt, diluted with water (10 ml) and extracted with ethyl acetate (2x 25 ml). Combined organic layers were dried over Na_2SO_4 and the solvent evaporated to afford crude 2-amino-5-(quinolin-3-yl)nicotinohydrazide (**8**) (590 mg, 2.11 mmol, 117%) as a red solid (purity 50%) which was used without further purification. LC-MS: Rt: 0.56 min (Method C), MS (ESI): *m/z* 280.2 $[M^+H]$.

5-(2-amino-5-(quinolin-3-yl)pyridin-3-yl)-1,3,4-oxadiazole-2(3H)-thione (3)

0.1N aqueous sodium hydroxide (23.24 ml, 2.32 mmol) and carbon disulfide (0.140 ml, 2.32 mmol) were added to a solution of crude 2-amino-5-(quinolin-3-yl)nicotinohydrazide (**8**) (590 mg, 2.11 mmol) in

ethanol (40 ml). The reaction mixture was stirred at 80°C for 15h, cooled to rt and the ethanol evaporated. The pH of resulting yellow suspension was adjusted to pH5 by addition of 1N aqueous HCl. The precipitate was filtered and purified by flash chromatography (25g SiO_2, DCM/MeOH, 100:0 to 80:20 in 85 min) to afford 5-(2-amino-5-(quinolin-3-yl)pyridin-3-yl)-1,3,4-oxadiazole-2(3H)-thione (**2**) (140 mg, 0.44 mmol, 21%) as a yellow solid (>98% purity). LC-MS: Rt: 0.80 min (Method C), MS (ESI): *m/z* 322.3 [M^+H]; 1H-NMR (DMSO-d6): 9.26 (d, J = 2.3 Hz, 1H), 8.74 (d, J = 2.4 Hz, 1H), 8.67 (d, J = 2.3 Hz, 1H), 8.34 (d, J = 2.4 Hz, 1H), 8.05-8.01 (m, 2H), 7.75-7.71 (m, 1H), 7.64-7.60 (m, 1H), 7.23 (s, br, 2H).