Discovery of a Novel Series of CHK1 Kinase Inhibitors with a Distinctive Hinge Binding Mode

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Supporting Information

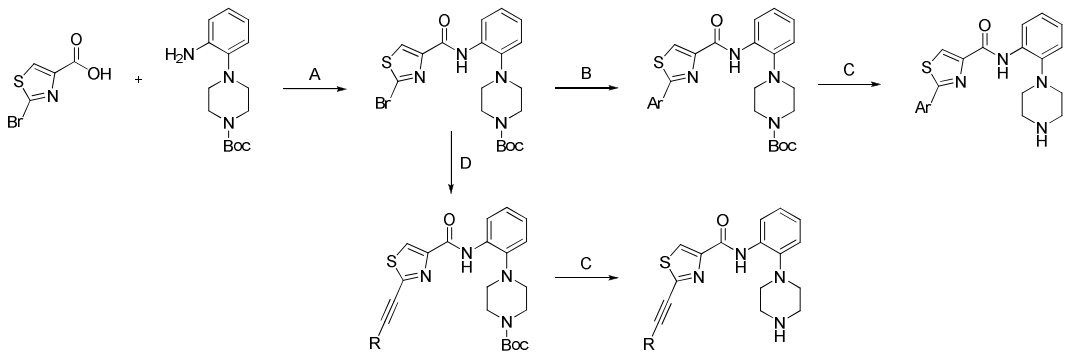
Commonly used abbreviations

MeCN	Acetonitrile
AcOH	Acetic acid
DCM	Dichloromethane
DIEA	*N,N*-Diisopropylethylamine
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
EtOAc	Ethyl acetate
HATU	*O*-(7-Azabenzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate
Hex	hexanes
HPLC	High pressure liquid chromatography
LCMS	Liquid chromatography mass spectrometry
NMR	Nuclear magnetic resonance
RT	Room temperature
TEA	Triethylamine
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
S-Phos	2-Dicyclohexylphosphino-2',6'-dimethoxybiphenyl
X-Phos	2-Dicyclohexylphosphino-2',4',6'-triisopropylbiphenyl

Section I: Synthetic Chemistry and Key Compound Characterization

The purity of all the compounds in the manuscript was ≥95%, determined by NMR, LC/MS.

Scheme 1. Synthesis of 2-Aryl-*N*-(2-(piperazin-1-yl)phenyl)thiazole-4-carboxamide



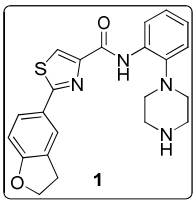
Reagents and conditions: (A) HATU, DIEA, DMF, rt; (B) $Pd(OAc)_2$, S-Phos, $ArB(OH)_2$, K_3PO_4, Dioxane, 100 °C; (C) TFA; (D) $Pd(OAc)_2$, X-Phos, Alkyne, Cs_2CO_3, MeCN, 85 °C.

Step A: Synthesis of *tert*-butyl 4-(2-(2-bromothiazole-4-carboxamido)phenyl)piperazine-1-carboxylate

To a solution of 2-bromo-thiazole-4-carboxylic acid (2.0 mmol, 0.42 g), HATU (2.0 mmol, 0.76 g) and DIEA (3.0 mmol, 0.52 mL) in DMF (10 mL) was added 4-(2-aminophenyl)-piperazine-1-carboxylic acid *tert*-butyl ester (2.0 mmol, 0.56 g). The reaction mixture was stirred at 80 °C for 3 h, and then concentrated *in vacuo*. The residue was purified by column chromatography on silica gel using Hexane/EtOAc (4.5/1) to afford the desired product as a yellow solid (0.67 g, 72% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.38 (s, 1H), 8.49 (dd, J = 8.0, 1.2 Hz, 1H), 8.14 (s, 1H), 7.23-7.10 (m, 3 H), 3.72 (br s, 4H), 2.89-2.87 (br s, 4H), 1.50 (s, 9H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 157.3, 154.9, 150.7, 141.7, 136.1, 133.1, 127.2, 125.7, 124.4, 120.6, 119.5, 80.0, 52.2, 45.0, 43.9, 28.6.

Step B and C: Suzuki cross coupling and Boc deprotection

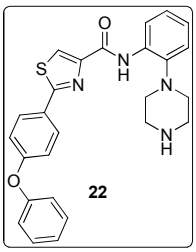
A mixture of *tert*-butyl 4-(2-(2-bromothiazole-4-carboxamido)phenyl)piperazine-1-carboxylate (47 mg, 0.10 mmol), palladium(II) acetate (1.1 mg, 5.0 μmol), S-Phos (4.1 mg, 10 μmol), potassium phosphate (43 mg, 0.20 mmol) and arylboronic acid (0.15 mmol) in dioxane (0.5 mL) was stirred under an atmosphere of argon at 100 °C for 15 h. The reaction mixture was cooled to room temperature and filtered through celite. The filtrate was concentrated *in vacuo*, and to the residue was added TFA (0.5 mL, neat). The reaction mixture was stirred at room temperature for 15 minutes. TFA was removed *in vacuo*, and the residue was purified by reverse phase HPLC to afford the final compound.



1

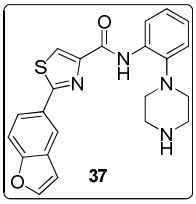
Compound **1**, 2-(2,3-Dihydrobenzofuran-5-yl)-*N*-(2-(piperazin-1-yl)phenyl)thiazole-4-carboxamide: Following steps B and C, compound **1** was prepared from *tert*-butyl 4-(2-(2-bromothiazole-4-carboxamido)phenyl)piperazine-1-carboxylate in 85% yield as an HCl salt. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.28 (s, 1H), 9.47 (br s, 2H), 8.43 (s, 1H), 8.42 (dd, J = 8.0, 1.5 Hz, 1H), 7.91 (s, 1H), 7.84 (dd, J = 8.5, 2.0 Hz, 1H), 7.32 (dd, J = 8.0, 1.5 Hz, 1H), 7.25 (dt, J = 7.5, 1.5 Hz, 1H), 7.17 (dt, J = 8.0, 1.5 Hz, 1H), 7.08 (d, J = 8.0 Hz, 1H), 4.65 (t, J = 8.8 Hz, 2H), 3.35 (t, J = 8.8 Hz, 2H), 3.30 (br s, 4H), 3.13 (t, J =

4.8 Hz, 4H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 168.3, 162.2, 158.1, 149.9, 140.8, 132.7, 129.4, 126.8, 125.7, 124.9, 124.3, 123.5, 120.9, 119.0, 110.3, 71.9, 48.7, 43.8, 28.7. Mass calcd for $C_{22}H_{23}N_4O_2S^+$ $(M+H)^+$ 407.15362; Found 407.15520.



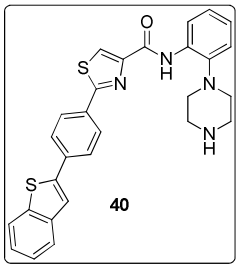
22

Compound **22**, 2-(4-phenoxyphenyl)-*N*-(2-(piperazin-1-yl)phenyl)thiazole-4-carboxamide: Following steps B and C, compound **22** was prepared from *tert*-butyl 4-(2-(2-bromothiazole-4-carboxamido)phenyl)piperazine-1-carboxylate in 77% yield as an HCl salt. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.25 (s, 1H), 9.47 (br s, 2H), 8.52 (s, 1H), 8.46 (dd, J = 8.0, 1.5 Hz, 1H), 8.08-8.03 (m, 2H), 7.50-7.44 (m, 2H), 7.33 (dd, J = 8.0, 1.5 Hz, 1H), 7.31-7.21 (m, 4H), 7.20-7.13 (m, 3H), 3.32 (br s, 4H), 3.13 (t, J = 4.8 Hz, 4H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 167.5, 159.2, 158.0, 155.6, 150.3, 140.7, 132.7, 130.4, 128.3, 127.2, 125.7, 125.3, 124.4, 124.3, 121.0, 119.6, 119.1, 119.0, 48.7, 43.9. Mass calcd for $C_{26}H_{25}N_4O_2S^+$ $(M+H)^+$ 457.16927; Found 457.16769.



37

Compound **37**, 2-(benzofuran-5-yl)-*N*-(2-(piperazin-1-yl)phenyl)thiazole-4-carboxamide: Following steps B and C, compound **37** was prepared from *tert*-butyl 4-(2-(2-bromothiazole-4-carboxamido)phenyl)piperazine-1-carboxylate in 85% yield as an HCl salt. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.31 (s, 1H), 9.50 (br s, 2H), 8.53 (s, 1H), 8.46 (dd, J = 8.5, 1.5 Hz, 1H), 8.34 (d, J = 2.0 Hz, 1H), 8.15 (d, J = 2.0 Hz, 1H), 8.07 (dd, J = 9.0, 2.0 Hz, 1H), 7.98 (d, J = 8.5 Hz, 1H), 7.34 (dd, J = 8.0, 1.5 Hz, 1H), 7.28-7.24 (m, 1H), 7.21-7.18 (m, 1H), 7.18 (dd, J = 8.0, 1.5 Hz, 1H), 3.35 (br s, 4H), 3.15 (t, J = 4.8 Hz, 4H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 168.4, 158.1, 155.6, 150.2, 147.7, 140.8, 132.8, 128.3, 127.5, 125.7, 125.4, 124.3, 122.7, 121.0, 119.9, 119.1, 113.1, 107.5, 48.8, 43.9. Mass calcd for $C_{22}H_{21}N_4O_2S^+$ $(M+H)^+$ 405.13797; Found 405.13708.



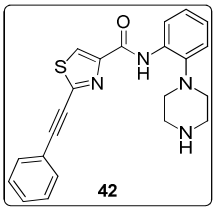
40

Compound **40**, 2-(benzo[*b*]thiophen-2-yl)-*N*-(2-(piperazin-1-yl)phenyl)thiazole-4-carboxamide: Following steps B and C, compound **40** was prepared from *tert*-butyl 4-(2-(2-bromothiazole-4-carboxamido)phenyl)piperazine-1-carboxylate in 86% yield as an HCl salt. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.33 (s, 1H), 9.63 (br s, 2H), 8.58 (s, 1H),

8.49 (dd, J = 8.0, 1.5 Hz, 1H), 8.36-8.32 (m, 1H), 8.26 (s, 1H), 7.97 (dd, J = 7.0, 1.5 Hz, 1H), 7.53-7.45 (m, 2H), 7.36 (dd, J = 8.0, 1.5 Hz, 1H), 7.29-7.25 (m, 1H), 7.20-7.16 (m, 1H), 3.47 (br s, 4H), 3.15 (t, J = 4.8 Hz, 4H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 162.0, 157.6, 149.9, 140.5, 139.8, 139.4, 135.2, 133.0, 126.3, 126.2, 126.0, 125.6, 125.3, 124.7, 124.2, 124.0, 121.2, 118.5, 48.9, 43.8. Mass calcd for $C_{22}H_{21}N_4OS_2^+$ $(M+H)^+$ 421.11513; Found 421.11417.

Step D and C: Sonogashira cross coupling and Boc deprotection

A mixture of *tert*-butyl 4-(2-(2-bromothiazole-4-carboxamido)phenyl)piperazine-1-carboxylate (47 mg, 0.10 mmol), palladium(II) acetate (1.1 mg, 5.0 μmol), X-Phos (4.8 mg, 10 μmol), cesium carbonate (98 mg, 0.30 mmol) and phenylacetylene (0.15 mmol, 17μL) in acetonitrile (0.5 mL) was stirred under an atmosphere of argon at 85 °C for 15 h. The reaction mixture was cooled to room temperature and filtered through celite. The filtrate was concentrated *in vacuo*, and to the residue was added TFA (0.5 mL, neat). The reaction mixture was stirred at room temperature for 15 minutes. TFA was removed *in vacuo*, and the residue was purified by reverse phase HPLC to afford the final compound.



42

Compound **42**, 2-(phenylethynyl)-*N*-(2-(piperazin-1-yl)phenyl)thiazole-4-carboxamide: Following steps D and C, compound **42** was prepared from *tert*-butyl 4-(2-(2-bromothiazole-4-carboxamido)phenyl)piperazine-1-carboxylate in 93% yield as an HCl salt. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.08 (s, 1H), 9.31 (br s, 2H), 8.64 (s, 1H), 8.36 (dd, J = 8.0, 1.5 Hz, 1H), 7.82-7.78 (m, 2H), 7.59-7.50 (m, 3H), 7.30 (dd, J = 8.0, 1.5 Hz, 1H), 7.26-7.22 (m, 1H), 7.18 (dt, J = 7.5, 1.5 Hz, 1H), 3.35 (br s, 4H), 3.13 (t, J = 4.8 Hz, 4H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 157.4, 150.2, 148.1, 140.9, 132.4, 132.3, 130.5, 129.0, 127.8, 125.4, 124.5, 120.6, 119.9, 119.2, 94.9, 81.4, 48.5, 43.6. Mass calcd for $C_{22}H_{21}N_4OS^+$ $(M+H)^+$ 389.14306; Found 389.14212.

Section II: CHK1 SPA Assay

An in vitro assay was developed that utilizes recombinant His-CHK1 expressed in the baculovirus expression system as an enzyme source and a biotinylated peptide based on CDC25C as substrate (biotin-RSGLYRSPSMPENLNRPR).

Materials and Reagents:

1) CDC25C Ser 216 C-term Biotinylated peptide substrate (25 mg), stored at -20 °C, Custom Synthesis by Research Genetics: biotin-RSGLYRSPSMPENLNRPR 2595.4 MW
2) His-CHK1 In House lot P976, 235 μg/mL, stored at -80 °C.
3) D-PBS (without CaCl and MgCl): GIBCO, Cat.# 14190-144
4) SPA beads: Amersham, Cat.# SPQ0032: 500 mg/vial
   Add 10 mL of D-PBS to 500 mg of SPA beads to make a working concentration of 50 mg/mL. Store at 4 °C. Use within 2 week after hydration.
5) 96-Well White Microplate with Bonded GF/B filter: Packard, Cat.# 6005177
6) Top seal-A 96 well Adhesive Film: Perkin Elmer, Cat.# 6005185

7) 96-well Non-Binding White Polystyrene Plate: Corning, Cat. # 6005177
8) $MgCl_2$: Sigma, Cat.# M-8266
9) DTT: Promega, Cat.# V3155
10) ATP, stored at 4 °C: Sigma, Cat.# A-5394
11) γ^{33}P-ATP, 1000-3000 Ci/mMol: Amersham, Cat.# AH9968
12) NaCl: Fisher Scientific, Cat.# BP358-212
13) H_3PO_4 85% Fisher, Cat.#A242-500
14) Tris-HCL pH 8.0: Bio-Whittaker, Cat. # 16-015V
15) Staurosporine, 100 μg: CALBIOCHEM, Cat. # 569397
16) Hypure Cell Culture Grade Water, 500 mL: HyClone, Cat.# SH30529.02

Reaction Mixtures:

1) Kinase Buffer: 50 mM Tris pH 8.0; 10 mM $MgCl_2$; 1 mM DTT
2) His-CHK1, In House Lot P976, MW ~30KDa, stored at -80 °C.

6 nM is required to yield positive controls of ~5,000 CPM. For 1 plate (100 rxn): dilute 8 μL of 235 μg/mL (7.83 μM) stock in 2 mL Kinase Buffer. This makes a 31 nM mixture. Add 20 μL/well. This makes a final reaction concentration of 6 nM.

3) CDC25C Biotinylated peptide.

Dilute CDC25C to 1 mg/mL (385 μM) stock and store at -20 °C. For 1 plate (100 rxn): dilute 10 μL of 1 mg/mL peptide stock in 2 mL Kinase Buffer. This gives a 1.925 μM mix. Add 20 μL/rxn. This makes a final reaction concentration of 385 nM.

4) ATP Mix.

For 1 plate (100 rxn): dilute 10 μL of 1 mM ATP (cold) stock and 2 μL fresh P33-ATP (20 μCi) in 5 mL Kinase Buffer. This gives a 2 μM ATP (cold) solution; add 50 μL/well to start the reaction. Final volume is 100 μL/rxn so the final reaction concentrations will be 1 μM ATP (cold) and 0.2 μCi/rxn.

5) Stop Solution:

For 1 plate add: To 10 mL Wash Buffer 2 (2M NaCl 1% H_3PO_4) : 1mL SPA bead slurry (50 mg); Add 100 μL/well

6) Wash buffer 1: 2 M NaCl
7) Wash buffer 2: 2 M NaCl, 1% H_3PO_4

Assay Procedure:

Assay Component	Final Concentration	Volume
CHK1	6nM	20 μl/rxn
Compound (10% DMSO)	- -	10 μl/rxn
CDC25C	0.385 μM	20 μl/rxn
γ^{33}P-ATP Cold ATP	0.2 μCi/rxn 1 μM	50μl/rxn
Stop solution SPA beads	0.5 mg/rxn	**100 μl/rxn***
		200 μl/rxn**

* Total reaction volume for assay.** Final reaction volume at termination of reaction (after addition of stop solution).

1) Dilute compounds to desired concentrations in water/10% DMSO - this will give a final DMSO concentration of 1% in the rxn. Dispense 10 μL/rxn to appropriate wells. Add 10 μL 10% DMSO to positive (CHK1+CDC25C+ATP) and negative (CHK1+ATP only) control wells.
2) Thaw enzyme on ice -- dilute enzyme to proper concentration in kinase buffer (see Reaction Mixtures) and dispense 20 μL to each well.
3) Thaw the Biotinylated substrate on ice and dilute in kinase buffer (see Reaction Mixtures). Add 20 μL/well except to negative control wells. Instead, add 20 μL Kinase Buffer to these wells.
4) Dilute ATP (cold) and P33-ATP in kinase buffer (see Reaction Mixtures). Add 50 μL/well to start the reaction.
5) Allow the reaction to run for 2 hours at room temperature.
6) Stop reaction by adding 100 μL of the SPA beads/stop solution (see Reaction Mixtures) and leave to incubate for 15 minutes before harvest
7) Place a blank Packard GF/B filter plate into the vacuum filter device (Packard plate harvester) and aspirate 200 mL water through to wet the system.
8) Take out the blank and put in the Packard GF/B filter plate.
9) Aspirate the reaction through the filter plate.
10) Wash: 200 mL each wash; 1X with 2M NaCl; 1X with 2M NaCl/ 1% H_3PO_4
11) Allow filter plate to dry 15 min.
12) Put TopSeal-A adhesive on top of filter plate.
13) Run filter plate in Top Count

Settings: Data mode: CPM
Radio nuclide: Manual SPA:P33
Scintillator: Liq/plast
Energy Range: Low

IC_{50} DETERMINATIONS: Dose-response curves were plotted from inhibition data generated, each in duplicate, from 8 point serial dilutions of inhibitory compounds. Concentration of compound was plotted against % kinase activity, calculated by CPM of treated samples divided by CPM of untreated samples. To generate IC_{50} values, the dose-response curves were then fitted to a standard sigmoidal curve and IC_{50} values were derived by nonlinear regression analysis.

Section III: CDK2 ASSAY

BACULOVIRUS CONSTRUCTIONS: Cyclin E was cloned into pVL1393 (Pharmingen, La Jolla, California) by PCR, with the addition of 5 histidine residues at the amino-terminal end to allow purification on nickel resin. The expressed protein was approximately 45kDa. CDK2 was cloned into pVL1393 by PCR, with the addition of a haemaglutinin epitope tag at the carboxy-terminal end (YDVPDYAS). The expressed protein was approximately 34kDa in size.

ENZYME PRODUCTION: Recombinant baculoviruses expressing cyclin E and CDK2 were co-infected into SF9 cells at an equal multiplicity of infection (MOI=5), for 48 hrs. Cells were harvested by centrifugation at 1000 RPM for 10 minutes, then pellets lysed on ice for 30 minutes in five times the pellet volume of lysis buffer containing 50mM Tris pH 8.0, 150mM NaCl, 1% NP40, 1mM DTT and protease inhibitors (Roche Diagnostics GmbH, Mannheim, Germany). Lysates were spun down at 15000 RPM for 10 minutes and the supernatant retained. 5mL of nickel beads (for one liter of SF9 cells) were

washed three times in lysis buffer (Qiagen GmbH, Germany). Imidazole was added to the baculovirus supernatant to a final concentration of 20mM, then incubated with the nickel beads for 45 minutes at 4 °C. Proteins were eluted with lysis buffer containing 250mM imidazole. Eluate was dialyzed overnight in 2 liters of kinase buffer containing 50mM Tris pH 8.0, 1mM DTT, 10mM $MgCl_2$, 100μM sodium orthovanadate and 20% glycerol. Enzyme was stored in aliquots at -70 °C.

In Vitro Kinase Assay

Cyclin E/CDK2 kinase assays were performed in low protein binding 96-well plates (Corning Inc, Corning, New York). Enzyme was diluted to a final concentration of 50 μg/mL in kinase buffer containing 50mM Tris pH 8.0, 10 mM $MgCl_2$, 1 mM DTT, and 0.1 mM sodium orthovanadate. The substrate used in these reactions was a biotinylated peptide derived from Histone H1 (from Amersham, UK). The substrate was thawed on ice and diluted to 2 μM in kinase buffer. Compounds were diluted in 10% DMSO to desirable concentrations. For each kinase reaction, 20 μL of the 50 μg/mL enzyme solution (1 μg of enzyme) and 20 μl of the 2 μM substrate solution were mixed, then combined with 10 μL of diluted compound in each well for testing. The kinase reaction was started by addition of 50 μL of 2 μM ATP and 0.1 μCi of 33P-ATP (from Amersham, UK). The reaction was allowed to run for 1 hour at room temperature. The reaction was stopped by adding 200 μL of stop buffer containing 0.1% Triton X-100, 1 mM ATP, 5mM EDTA, and 5 mg/mL streptavidine coated SPA beads (from Amersham, UK) for 15 minutes. The SPA beads were then captured onto a 96-well GF/B filter plate (Packard/Perkin Elmer Life Sciences) using a Filtermate universal harvester (Packard/Perkin Elmer Life Sciences.). Non-specific signals were eliminated by washing the beads twice with 2M NaCl then twice with 2 M NaCl with 1% phosphoric acid. The radioactive signal was then measured using a TopCount 96 well liquid scintillation counter (from Packard/Perkin Elmer Life Sciences).

IC_{50} DETERMINATIONS: Dose-response curves were plotted from inhibition data generated, each in duplicate, from 8 point serial dilutions of inhibitory compounds. Concentration of compound was plotted against % kinase activity, calculated by CPM of treated samples divided by CPM of untreated samples. To generate IC_{50} values, the dose-response curves were then fitted to a standard sigmoidal curve and IC_{50} values were derived by nonlinear regression analysis.

Section IV: γ-H2AX Assay

U2OS cells were exposed to an antimetabolite (hydroxyurea, Sigma, St. Louis, MO) at 1 mM for 15 hours to induce the activation of CHK1. Control populations were left untreated. Compounds were then titrated onto cells over a 2-hour exposure window in the presence of the antimetabolite (hydroxyurea). Following the 2-hour coexposure to compounds, cells were fixed and permeabilized (70% ethanol) before staining with a fluorescein isothiocyanate (FITC)-conjugated anti-γ-H2AX monoclonal antibody (cell signaling). Cells were counterstained with propidium iodide and subsequently analyzed using Discovery 1 immunofluorescence platform (Molecular Devices). Experiments were typically done in triplicate and data are presented as the percentage of γ-H2AX positive cells, and thus reflect the overall penetrance of the γ-H2AX phenotype.