Supporting Information

Examining the mechanism of action of a kinesin inhibitor using Stable Isotope Labeled Inhibitors for Crosslinking (SILIC)

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1. Methods

1.1 General Synthesis Information

General Procedures. Reactions were run in capped 1 dram vials (4 mL) stirred with Teflon®-coated magnetic stir bars. Moisture- and air-sensitive reactions were performed in flame-dried round bottom flasks, fitted with rubber septa or glass gas adapters, under a positive pressure of nitrogen. Moisture- and air-sensitive liquids or solutions were transferred via nitrogen-flushed syringe. Where necessary, solutions were deoxygenated by bubbling with nitrogen using a gas dispersion tube. Concentration of solvents was accomplished by rotary evaporation using a Büchi rotary evaporator, equipped with a dry ice-acetone condenser, at 5-75 mm Hg at temperatures between 35°C and 50°C. Experiments were monitored by thin layer chromatography (TLC) or liquid chromatography mass spectrometry (LC-MS). The maintenance of 30°C to 150°C reaction temperatures was accomplished by the use of an oil bath, or a 12-well (16 mm) aluminum heating block which could achieve temperatures up to 200°C.

Materials. Unless otherwise noted, materials were obtained from commercial suppliers and used without purification. Removal of solvent under reduced pressure refers to distillation with a Büchi rotary evaporator attached to a vacuum pump. Products obtained as solids or high boiling oils were dried under vacuum (~1 mmHg). Methylene chloride (CH_2Cl_2) and tetrahydrofuran (THF) were dried using a Pure-Solv 400 Solvent Purification System (activated columns).

Chromatography. Analytical TLC was performed using Whatman 250 micron aluminum backed UV F_{254} precoated silica gel flexible plates. Subsequent to elution, ultraviolet illumination at 254 nm allowed for visualization of UV active materials. Staining with *p*-anisaldehyde, basic potassium permanganate solution, or Verghn's reagents allowed for further visualization. The retardation factor (R_f) is the ratio of the distance traveled by the compound to the distance traveled by the eluent.

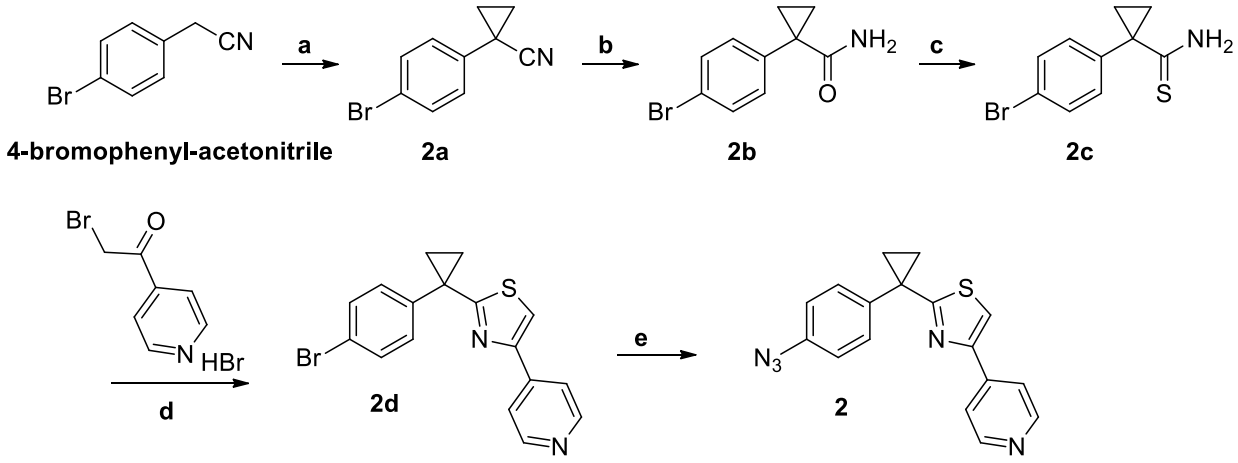
Flash column chromatography was performed using a Teledyne ISCO CombiFlash® Companion® chromatography instrument and RediSep® Columns (machine-packed with 35 to 60 micron silica gel). Preparative high performance liquid chromatography (HPLC) was carried out using a Waters Prep LC System Controller and Pump System equipped with a Waters Delta Prep 4000 injector and a Waters 486 Tunable Absorbance Detector. Solvents were degassed using helium through a bubbling stone, and 3-mL injections were made into a 5-mL injection loop.

Physical Data. Proton nuclear magnetic resonance spectra (^{1}H NMR) were recorded on Bruker DPX 400 MHz or 600 MHz nuclear magnetic resonance spectrometers. Chemical shifts for ^{1}H NMR spectra are reported as δ in units of parts per million (ppm) relative to tetramethylsilane (δ 0.0) using the residual solvent signal as an internal standard or tetramethylsilane itself: chloroform-*d* (δ 7.26, singlet), dimethylsulfoxide-d_6 (δ 2.50, quintet), methanol-d_4 (δ 3.30, quintet), and deuterium oxide-d_2 (δ 4.80, singlet). Multiplicities are given as: br (broad), v br (very broad), s (singlet), d (doublet), t (triplet), q (quartet), quint. (quintet), sext. (sextet), sept. (septet), dd (doublet of doublets), ddd (doublet of doublets of doublets), dt (doublet of triplets), ddt (doublet of doublets of triplets), dq (doublet of quartets) or m (multiplet). Coupling constants

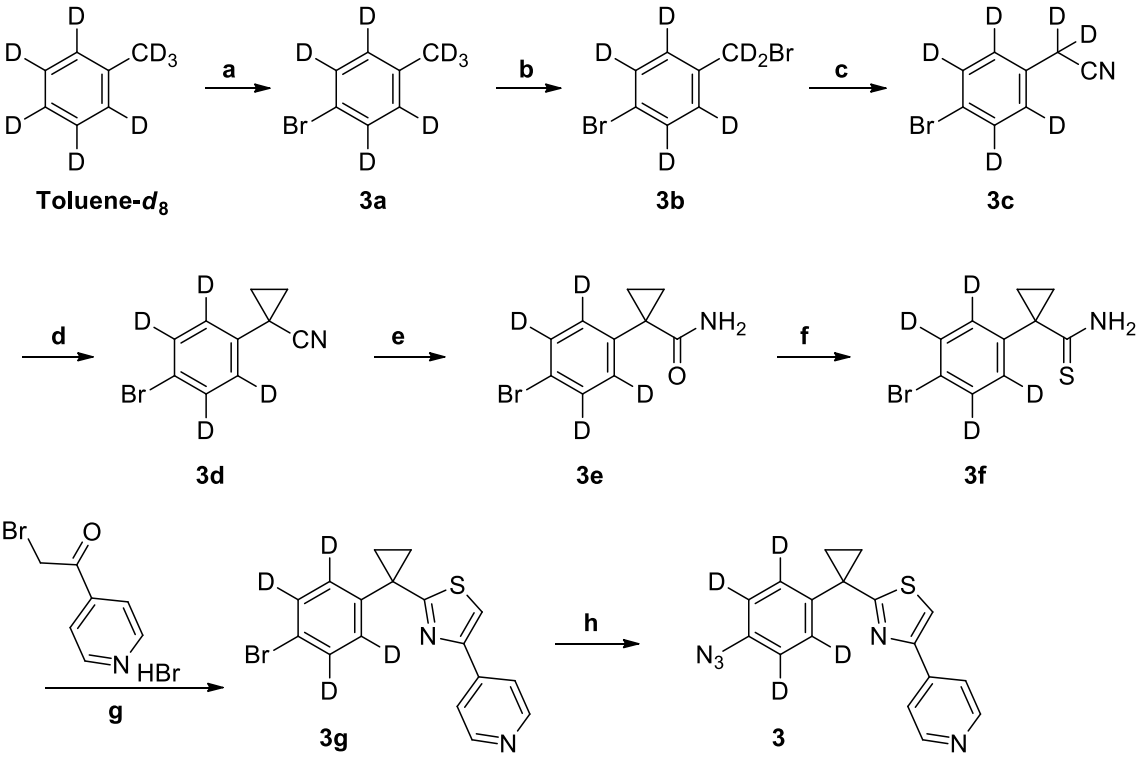
are reported as a *J* value in Hertz (Hz). The number of protons (n) for a given resonance is indicated by nH.

Liquid chromatography mass spectral analyses were obtained using a Waters MicroMassZQ mass spectrometer, with an electron spray ionization (ESI) probe, connected to a Waters 2795 HT Separation Module Alliance HT HPLC system running MassLynx (V4.0). The system used a Waters 996 Photodiode Array Detector set to 254 nm for peak detection, and a Symmetry® C18 (3.5 micron) 2.1 x 50 mm column for separation (mobile phase for positive mode: solvent A: water with 0.1% formic acid, solvent B: acetonitrile; mobile phase for negative mode: solvent A: water with 0.1% morpholine, solvent B: acetonitrile). Values are reported in units of mass to charge (*m/z*).

1.2 Synthesis and Characterization of Compounds 2 and 3

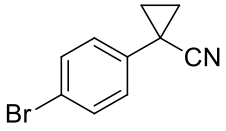


Scheme 1. Reagents and conditions: (a) 1-bromo-2-chloroethane, tetrabutylammonium bromide (TBAB), 75% KOH, KOH, toluene, 50 °C, 16 h, 81%; (b) 85% H_2SO_4, NaBr (cat.), 70 °C, 3 h, 71%; (c) *Lawesson*'s reagent, THF, 70 °C, 2 h, 72%; (d) EtOH, reflux, 3 h, 82%; (e) NaN_3, EtOH:H_2O (7:3), CuI, *trans*-N,N'-dimethyl-cyclohexane-1,2-diamine, Na-ascorbate, 90 °C, 16 h, 40%.



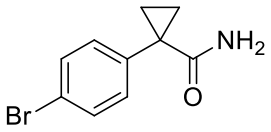
Scheme 2. Reagents and conditions: (a) Zeolite NaY, CH_2Cl_2, Bromine, 16 h; (b) NBS, CCl_4, h□, reflux, 16 h; (c) KCN, 18-crown-6, DMF:H_2O (9:1), 50 °C, 16 h, 50% (3 steps); (d) 1-bromo-2-chloroethane, tetrabutylammonium bromide (TBAB), 75% KOH, KOH, toluene-d_8, 50 °C, 16 h, 89%; (e) 85% H_2SO_4 in D_2O, NaBr (cat.), 70 °C, 3 h, 93%; (f) *Lawesson*'s reagent, THF, 70 °C, 2 h, 83%; (g) EtOH, reflux, 3 h, 40%; (h) NaN_3, EtOH:H_2O (7:3), CuI, *trans*-N,N'-dimethyl-cyclohexane-1,2-diamine, Na-ascorbate, 90 °C, 16 h, 64%.

General Synthesis Procedures



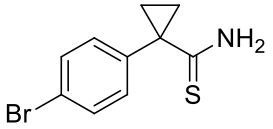
2a

1-(4-Bromophenyl)cyclopropanecarbonitrile **(2a).**[1,2] To a solution of 2-(4-bromophenyl)acetonitrile (1.95 g, 9.95 mmol, 1.0 equiv), 1-bromo-2-chloroethane (2.85 g, 1.65 ml, 19.89 mmol, 2.0 equiv), tetrabutylammonium bromide (650 mg, 1.99 mmol, 0.2 equiv) and potassium hydroxide (2.5 g, 49.73 mmol, 5.0 equiv) in 5 mL toluene was added saturated aqueous potassium hydroxide (3.7 g, 49.73 mmol, 5.0 equiv). The mixture was allowed to stir at 50 °C for 16 h. It was then allowed to cool to room temperature and poured into 50 mL of water. This suspension was extracted with three 25 mL portions of methylene chloride, and the combined organic layers were then washed with three 50 mL portions of 1.2 N HCl (aq.), three 50 mL portions of water, and 50 mL of saturated sodium chloride solution. The organic layer was then dried over sodium sulfate, filtered, and the solvent was removed in rotary evaporator under reduced pressure. The resulting crude material was then purified by column chromatography on silica gel using petroleum ether: ethyl acetate (10:1) in 81% isolated yield (1.8 g, 8.10 mmol) as white solid.
^{1}H NMR (400.13 MHz , $CDCl_3$): δ 7.46 (d, J = 8.23 Hz, 2H), 7.15 (d, J = 8.41 Hz, 2H), 1.71-1.75 (m, 2H), 1.35-1.39 (m, 2H). ^{13}C NMR (100.61 MHz, $CDCl_3$): δ 134.84, 131.54, 127.02, 121.58, 121.03, 17.97 (m), 13.05. Calcd mass for $C_{10}H_8BrN$: 220.98; LRMS (ESI) m/z [$M^+ + H^+$] = 195.16/197.14 (-CN) (bromine pattern).



2b

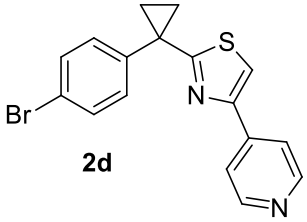
1-(4-Bromophenyl)cyclopropanecarboxamide **(2b).**[3] A solution of compound **2a** (1.75 g, 7.88 mmol) in 5 mL acetic acid was added to an iced cooled slurry of 85% Sulfuric acid (10 mL) and sodium bromide (50 mg). The reaction mixture was heated at 70 °C for 3 h. After the completion of reaction (adjudged by TLC) reaction mixture was cooled at room temperature and quenched with ice and extracted with ethyl acetate (2 x 100 mL), the organic layers were combined, washed with saturated sodium bicarbonate solution (30 mL) and then saturated brine (25 mL). The organic layer was then dried over sodium sulfate, filtered, and the solvent was removed in rotary evaporator. The resultant residues were then washed with petroleum ether to give compound **2b** in 71% yields (1.35 g, 5.62 mmol) as off-white solid.
^{1}H NMR (400.13 MHz , $CDCl_3$): δ 7.50 (d, J = 8.28 Hz, 2H), 7.31 (d, J = 8.03 Hz, 2H), 6.52 (br s, 1H), 5.33 (br s, 1H), 1.60-1.63 (m, 2H), 1.05-1.07 (m, 2H). ^{13}C NMR (100.61 MHz, $CDCl_3$): δ 176.54, 138.77, 132.53, 132.13, 132.07, 121.98, 29.46, 16.02. Calcd mass for $C_{10}H_{10}BrNO$: 238.99; LRMS (ESI) m/z [$M^+ + H^+$] = 239.94/241.96 (bromine pattern).



2c

1-(4-Bromophenyl)cyclopropanecarbothioamide **(2c).**[4] To a solution of compound **2b** (1.3 g, 5.41 mmol, 1.0 equiv) in anhydrous THF (10 mL) was added *Lawesson*'s reagent (2.4 g, 5.96 mmol, 1.1 equiv) at room temperature and reaction mixture was heated at 70 °C for 2 h. After the completion of reaction, mixture was cooled to room temperature and 10 g alumina was added to the reaction and filtered through a pad of celite and eluted with chloroform. The resulting solution was concentrated in rotary evaporator under reduced pressure, purified by column chromatography on silica gel using petroleum ether:EtOAc (2:1) in a 72% isolated yield (1 g, 3.90 mmol) as a pale yellow solid.

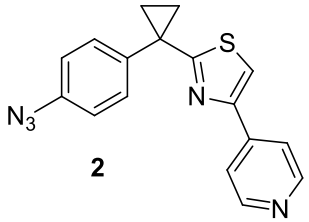
^{1}H NMR (400.13 MHz , $CDCl_3$): δ 7.63 (br s, 1H), 7.52 (d, *J* = 8.53 Hz, 2 H), 7.29 (d, *J* = 8.53 Hz, 2H), 6.51 (br s, 1H), 2.04-2.07 (m, 2 H), 1.29-1.32 (m, 2 H). ^{13}C NMR (100.61 MHz, $CDCl_3$+CD_3OD): δ 211.16, 138.11, 132.26, 132.02, 131.94, 121.98, 36.59, 21.25. Calcd mass for $C_{10}H_{10}BrNS$: 254.97; LRMS (ESI) m/z [$M^+ + H^+$] = 255.96/257.98 (bromine pattern).



2d

2-(1-(4-bromophenyl)cyclopropyl)-4-(pyridin-4-yl)thiazole **(2d).**[5] To a stirred solution of compound **2c** (300 mg, 1.17 mmol, 1.0 equiv) in anhydrous Ethanol (3 mL) was added 4-bromoacetylpyridinehydrogenbromide (395 mg, 1.41 mmol, 1.2 equiv). The reaction mixture was heated under reflux for 3 h. After the completion of reaction (adjudged by TLC), excess of solvent was evaporated under reduced pressure. Resulting crude reaction mixture was re-dissolved in ethyl acetate, 20 mL water added and extracted with ethyl acetate (2 x 100 mL). The organic layers were combined, washed with water (30 mL), saturated brine (25 mL) and dried over sodium sulfate. Following removal of solvent in rotary evaporator under vacuum and the crude thiazole was then purified by column chromatography on silica gel using petroleum ether:EtOAc (3:1) in a 82% isolated yield (340 mg, 0.959 mmol) as reddish-brown solid.

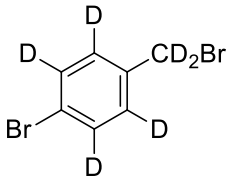
^{1}H NMR (400.13 MHz , $CDCl_3$): δ 8.63 (d, *J* = 4.59 Hz, 2H), 7.73 (d, *J* = 4.59 Hz, 2H), 7.52 (d, *J* = 6.79 Hz, 2H), 7.46 (s, 1H), 7.39 (d, *J* = 6.79 Hz, 2 H), 1.85-1.88 (m, 2H), 1.43-1.46 (m, 2H). ^{13}C NMR (100.61 MHz, $CDCl_3$): δ 176.87, 152.10, 150.13, 141.54, 140.91, 132.28, 131.94, 131.89, 121.97, 120.58, 120.44, 115.57, 115.48, 29.47, 18.95. Calcd mass for $C_{17}H_{13}BrN_2S$: 356; LRMS (ESI) m/z [$M^+ + H^+$] = 357.11/359.09 (bromine pattern).



2

2-(1-(4-azidophenyl)cyclopropyl)-4-(pyridin-4-yl)thiazole **(2).**[6,7] Compound **2d** (100 mg, 0.2799 mmol, 1.0 equiv) was coupled with sodium azide (182 mg, 2.799 mmol, 10.0 equiv) in 5 mL Ethanol/H_2O (7:3) under the action of 20 mol% CuI, 30 mol% *trans*-N,N'-dimethyl-cyclohexane-1,2-diamine, and 20 mol% sodium ascorbate at 90 °C for 16 h. After the completion of reaction (adjudged by TLC), mixture was cooled to room temperature, quenched with 20 mL water and extracted with ethyl acetate (2 x 100 mL). The organic layers were combined, washed with water (30 mL), saturated brine (25 mL) and dried over sodium sulfate. Following removal of solvent in rotary evaporator under vacuum and crude azide derivative was then purified by column chromatography on silica gel using petroleum ether:EtOAc (3:1) in a 40% isolated yield (36 mg, 0.1127 mmol) as off-white solid.

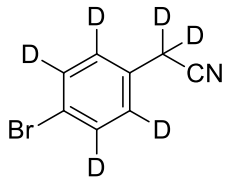
^{1}H NMR (400.13 MHz , $CDCl_3$): δ 8.63 (d, J = 3.15 Hz, 2H), 7.73 (d, J = 4.19 Hz, 2 H), 7.50 (d, J = 8.07 Hz, 2H), 7.44 (s, 1H), 7.05 (d, J = 8.06 Hz, 2H), 1.85-1.88 (m, 2H), 1.43-1.46 (m, 2H).
^{13}C NMR (100.61 MHz, $CDCl_3$): δ 177.39, 152.14, 150.27, 141.41, 139.67, 138.63, 132.06, 120.51, 120.37, 119.28, 115.38, 115.30, 29.38, 19.03. Calcd mass for $C_{17}H_{13}N_5S$: 319.09; LRMS (ESI) m/z [$M^+ + H^+$] = 320.19.



3b

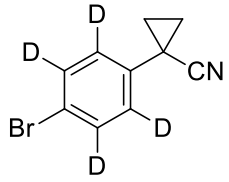
Compound **3b.**[8-10] Preheated catalyst NaY zeolite (720 mg) was placed in a round bottom flask with 50 mL CH_2Cl_2. A solution of Toluene-d_8 (1.0 g, 9.98 mmol, 1.0 equiv) in 5 mL CH_2Cl_2 was added to the mixture and allowed to stir at room temperature for 30 min. A solution of bromine (1.75 g, 10.98 mmol, 1.1 equiv) in 10 mL dichloromethane was then added dropwise to above mention solution, covered with aluminum foil and stirred at room temperature for 16 h. It was then allowed to settled, filtered and washed with CH_2Cl_2 and washing were combined with filtrate. The combined solution was evaporated on rotary evaporator at 25 °C to give pale yellow liquid in 84% (1.5 g, 8.42 mmol). The crude bromo-mixture was used for next step without further purification. Accordingly, a suspension of crude bromotoluene-d_7 (1.5 g, 8.42 mmol) and *N*-bromosuccinimide (1.65 g, 9.27 mmol) in 50 mL of carbon tetrachloride was heated to refluxed for overnight with a 40 W flood lamp shining on the reaction mixture. The resulting suspension was dissolved in CH_2Cl_2 and washed successively with 100 mL each of saturated aqueous sodium bicarbonate, water and brine. The organic layer was separated and dried over sodium sulfate. Removal of solvent by rotary evaporation gave 2.0 g of yellow oil containing *p*-bromobenzyl bromide-d_6 and over brominated product. The mixture was not separated for next step.

^{13}C NMR (100.61 MHz, $CDCl_3$): δ 136.31, 131.41 (t, J_{CD} = 25.6 Hz), 130.11 (t, J_{CD} = 24.83 Hz), 122.11, 32.08 (quint., J_{CD} = 23.84 Hz). Calcd mass for $C_7D_6Br_2$: 253.92; LRMS (ESI) m/z [M^+] = 175.01/177.00 (-Br) (bromine pattern).



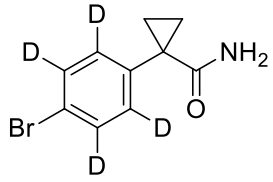
3c

Compound **3c.**[11] To a stirred solution of crude *p*-bromobenzyl bromide-d_6 (2.0 g, 7.81 mmol) in 10 mL DMF/D_2O (9:1) was added KCN (1.52 g, 23.44 mmol) and 18-crown-6 (2.06 g, 7.81 mmol). The reaction mixture was stirred vigorously at 90 °C for 16 h. After the completion of reaction, mixture was cooled, quenched with 20 mL water and extracted with ethyl acetate (2 x 100 mL). The organic layers were washed with 10 mL of water and dried over anhydrous sodium sulfate. The solvent was removed by evaporation and resulting crude material was purified by column chromatography on silica gel using petroleum ether:EtOAc (10:1) in a 50% isolated yield over three steps (1 g, 4.95 mmol) as off-white solid.
^{13}C NMR (100.61 MHz, $CDCl_3$): δ 131.76 (t, J_{CD} = 25.62 Hz), 126.10 (t, J_{CD} = 24.82 Hz), 128.59, 121.80, 117.26, 22.75 (quint., J_{CD} = 20.51 Hz). Calcd mass for C_8D_6BrN: 201.01; LRMS (ESI) m/z [M^+] = 175.01/177.00 (-CN) (bromine pattern).



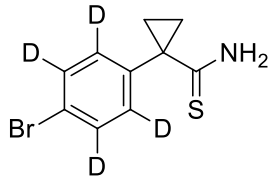
3d

Compound **3d.** The indicated compound was synthesized by using the same method described for compound **2a** and purified by column chromatography on silica gel using petroleum ether:EtOAc (10:1) in a 89% isolated yield (750 mg, 3.317 mmol) as off-white solid.
^{1}H NMR (400.13 MHz , $CDCl_3$): 1.70-1.75 (m, 2H), 1.33-1.39 (m, 2H). ^{13}C NMR (100.61 MHz, $CDCl_3$): δ 134.97, 131.53 (t, J_{CD} = 25.59 Hz), 126.95 (t, J_{CD} = 24.19 Hz), 121.95, 121.26, 18.22 (m), 13.35. Calcd mass for $C_{10}H_4D_4BrN$: 225.01; LRMS (ESI) m/z [M^+ - H^+] = 198.62/200.93 (-CN) (bromine pattern).



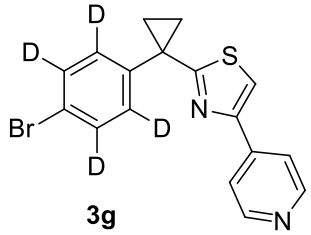
3e

Compound **3e.** The indicated compound was synthesized by using the same method described for compound **2b** and isolated in a 93% isolated yield (350 mg, 1.433 mmol) as off-white solid.
^{1}H NMR (400.13 MHz , $CDCl_3$ + CD_3OD): 6.20 (br s, 1H), 5.30 (br s, 1H), 1.59-1.61 (m, 2H), 1.04-1.06 (m, 2H). ^{13}C NMR (100.61 MHz, $CDCl_3$ + CD_3OD): δ 176.57, 138.41, 131.98 (t, J_{CD} = 24.25 Hz), 131.58 (t, J_{CD} = 25.57 Hz), 121.64, 29.21, 15.83. Calcd mass for $C_{10}H_6D_4BrNO$: 243.02; LRMS (ESI) m/z [M^+] = 243.93/245.98 (bromine pattern).



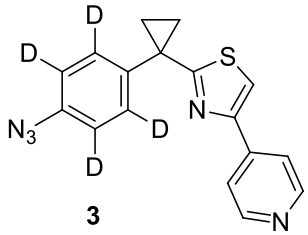
3f

Compound **3f.** The indicated compound was synthesized by using the same method described for compound **2c** isolated in a 83% isolated yield (220 mg, 0.8455 mmol) as yellowish solid.
^{1}H NMR (400.13 MHz , $CDCl_3$): δ 7.44 (br s, 1H), 6.49 (br s, 1H), 2.05-2.08 (m, 2 H), 1.30-1.33 (m, 2 H). ^{13}C NMR (100.61 MHz, $CDCl_3$): δ 212.06, 138.23, 132.17 (t, J_{CD} = 24.09 Hz), 132.08 (t, J_{CD} = 24.92 Hz), 121.29, 37.04, 22.20. Calcd mass for $C_{10}H_6D_4BrNS$: 259.00; LRMS (ESI) m/z [M^+] = 259.98/261.97 (bromine pattern).



3g

Compound **3g.** The indicated compound was synthesized by using the same method described for compound **2d** and purified by column chromatography on silica gel using petroleum ether:EtOAc (3:1) in a 40% isolated yield (110 mg, 0.3044 mmol) as reddish-brown solid.
^{1}H NMR (400.13 MHz , $CDCl_3$): δ 8.62 (d, J = 4.70 Hz, 2H), 7.73 (d, J = 5.45 Hz, 2H), 7.45 (s, 1H), 1.85-1.88 (m, 2H), 1.42-1.45 (m, 2H). ^{13}C NMR (100.61 MHz, $CDCl_3$): δ 176.85, 152.13, 150.19, 141.46, 140.76, 131.83 (t, J_{CD} = 24.11 Hz), 131.50 (t, J_{CD} = 25.67 Hz), 121.72, 120.52, 120.43, 115.48, 115.44, 29.38, 18.94. Calcd mass for $C_{17}H_9D_4BrN_2S$: 360.02; LRMS (ESI) m/z [M^+] = 360.90/362.89 (bromine pattern).



3

Compound **3.** The indicated compound was synthesized by using the same method described for compound **2** and purified by column chromatography on silica gel using hexanes:EtOAc (3:1) in a 64% isolated yield (40 mg, 0.124 mmol) as a off-white solid
^{1}H NMR (400.13 MHz , $CDCl_3$): δ 8.63 (br s, 2H), 7.74 (d, J = 3.87 Hz, 2 H), 7.45 (s, 1H), 1.85-1.88 (m, 2H), 1.43-1.46 (m, 2H). ^{13}C NMR (100.61 MHz, $CDCl_3$): δ 177.43, 152.15, 150.22, 141.48, 139.58, 138.47, 131.64 (t, J_{CD} = 24.18 Hz), 120.56, 120.50, 120.44, 118.93 (t, J_{CD} = 24.16 Hz), 115.42, 115.36, 29.32, 19.04. Calcd mass for $C_{17}H_9D_4N_5S$: 323.11; LRMS (ESI) m/z [M^+ + H^+]= 296.05 (-N_2).

1.3 Cloning, Expression, and Purification of Proteins

The human kinesin-5 ATPase domain (residues 1-368, Pubmed accession: NP_004514 from Open Biosystems) was cloned into a pET28a vector containing a PreScission protease cleavable N-terminal His-tag. This adds a 'GP' sequence at the N-terminus of the native protein sequence after removal of the His tag by PreScission proteolysis. The L214A mutant was generated from this plasmid using QuickChange site-directed mutagenesis kit (Stratagene). Protein expression in *Escherichia coli* BL21(DE3) Rosetta (Novagen) cells was induced with 0.5 mM isopropyl-1-thio-β-d-galactopyranoside, and cells were harvested after 20 h of growth at 18°C. Cells were resuspended in buffer containing 50 mM phosphate (pH 8), 250 mM KCl, 7.5 mM imidazole, 0.1% Tween-20, 0.5 mM $MgCl_2$, 0.5 mM ATP, and HALT protease inhibitor cocktail (Pierce), then lysed by sonication. The lysate was clarified by centrifugation and applied to Talon resin (Clontech). Resin was further washed with the lysis buffer before protein was removed from the column by overnight cleavage at 4°C with PreScission protease. Protein was concentrated and dialyzed into buffer containing 50 mM Pipes (pH 6.8), 50 mM KCl, 0.2 mM $MgCl_2$, 0.1 mM ATP, 1 mM EGTA, and 1 mM Tris-(2-carboxyethyl)phosphine (TCEP) HCl. Wildtype kinesin-5 was further purified by size exclusion chromatography (Superdex 200; Amersham Pharmacia Biotech). Full-length *Xenopus laevis* kinesin-5 used in the microtubule gliding assays was prepared as described previously.[12] Bovine tubulin was purified and labeled with X-Rhodamine according to published protocols.[13]

1.4 Steady-State ATP Hydrolysis and Microtubule Motility Assays

For steady-state ATP hydrolysis assays and crosslinking experiments, microtubules were polymerized from recycled tubulin in BRB80 buffer (80mM Pipes (pH 6.8), 1 mM $MgCl_2$, 1mM EGTA) containing 10% DMSO, 1 mM dithiothreitol, and 1 mM GTP at 37°C for 25 min. Microtubules were pelleted over a 40% glycerol cushion and resuspended in BRB80 with 20 μM paclitaxel.

ATP hydrolysis experiments used an assay that coupled ATP hydrolysis to oxidation of NADH.[14] Reactions were performed in BRB80 (reactions with microtubules) or 200 mM potassium phosphate (pH 8, reactions without microtubules) with 1 mM dithiothreitol, 0.1 mg/ml bovine serum albumin (Fisher Scientific), 2.5% DMSO, 1 mM MgATP, 2.5 mM reduced β-Nicotinamide adenine dinucleotide (disodium salt, Sigma), 200 U/ml L-lactic dehydrogenase (from rabbit muscle, Sigma), 1000 U/ml pyruvate kinase (from rabbit muscle, Sigma), 5 mM Phospho(enol)pyruvic acid (monopotassium salt, Sigma), and 400 nM microtubules. Kinesin-5 ATPase domain was added to begin reactions, which were conducted in 50 μl volumes in 384-well plates. Time courses of fluorescence decrease upon NADH oxidation were obtained using a FlexStation microplate reader (λ_{ex} = 340 nm, 440 nm emission filter).

For microtubule gliding assays, X-Rhodamine labeled microtubules were polymerized from GMPPCP seeds in BRB80 and stabilized with 10 μM paclitaxel. Microtubule gliding assays were performed on an inverted microscope (Axiovert; Carl Zeiss)[15] and conducted as described previously.[16]

1.5 Photo-crosslinking Reactions

Compounds **2** and **3** (40x final, in DMSO) were combined with kinesin-5 ATPase domain (1.5 μM) in 50 mM HEPES (pH 7, no microtubules) or in BRB80 with 3.3 μM microtubules (polymerized as in the ATP hydrolysis assays). Samples were irradiated at 254 nm using a Spectroline ENF 260C UV lamp for 30 min at 4°C (no microtubules) or room temperature (with microtubules). Reactions were stopped by the addition of dithiothreitol (12.5 mM final) and gel loading buffer. For competition experiments, **2** was pre-mixed with **1**[17] or S-Trityl-L-cysteine (Sigma) in DMSO to obtain a 40x solution of each compound.

1.6 Sample Preparation for Mass Spectrometry

Crosslinked samples, combined with gel loading buffer and dithiothreitol, were briefly heated (10 min, 70°C), and then treated with iodoacetamide (125 mM) for 30 min to alkylate all reduced cysteines. Proteins were separated and immobilized on Bis-Tris gels (Invitrogen), followed by fixation in a 50% methanol/7% acetic acid solution. Protein bands were visualized by GelCode Blue stain (Pierce). Kinesin-5 bands were excised from the gel, sliced, and destained in 50 mM ammonium bicarbonate/50% acetonitrile for 30 min. Gel slices were then dehydrated in acetonitrile (HPLC-grade, Pierce) for 10 min. The dried gel slices were rehydrated in 25 mM ammonium bicarbonate with 125 ng trypsin (Promega) for protein digestion at 37°C overnight. The resulting peptides were enriched with StageTips.[18] The peptides eluted from the StageTips were dried down by SpeedVac and then resuspended in 0.5% acetic acid for analysis by LC-MS/MS.

1.7 Mass Spectrometry

Mass spectrometry was performed on an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific), using a home-built micro electrospray source with a liquid junction. First, peptide samples in 0.5% acetic acid were pressure loaded onto a self-packed PicoFrit column (New Objective) with integrated emitter tip (360-μm o.d., 75-μm i.d., 15-μm tip), packed with 6 cm of reverse-phase C_{18} material (Alltima C_{18} 5-μm beads from Alltech Associates), rinsed for 10 min with 0.1 M acetic acid and subsequently gradient eluted with a linear gradient from 0 to 100% B in 30-50 min (A = 0.1 M acetic acid, B = 70% acetonitrile in 0.1M acetic acid, flow rate 200 nL/min) into the mass spectrometer. The instrument was operated in a data dependent mode cycling through a full scan (300–2,000 *m*/*z*, single μscan) followed by 7 CID MS/MS scans on the 7 most abundant ions from the immediate preceding full scan. The cations were isolated with a 2-Da mass window and set on a dynamic exclusion list for 60 seconds after they were first selected for MS/MS. The raw data were analyzed by Quant module of MaxQuant[19,20] (version 1.1.16) to find peptide pairs with a 4 Da mass difference.

2. Data

2.1 Supporting Table

Table S1. Kinetic parameters of microtubule-stimulated (MT) and basal (no MT) activities of kinesin-5 wildtype (WT) and L214A mutant in a steady-state ATP hydrolysis assay

Kinesin-5	IC_{50} (MT)	IC_{50} (no MT)	Specific Activity (MT)	Specific Activity (no MT)	Km ATP (no MT)
WT	1.2 µM ± .3	30 µM ± 7	9 sec^{-1} ± 4	1.9 sec^{-1} ± .1	4.7 µM ± .7
L214A	110 nM ± 60	2.4 µM ± .4	3 sec^{-1} ± 1	0.5 sec^{-1} ± .1	15 µM ± 7

2.2 Supporting Figures

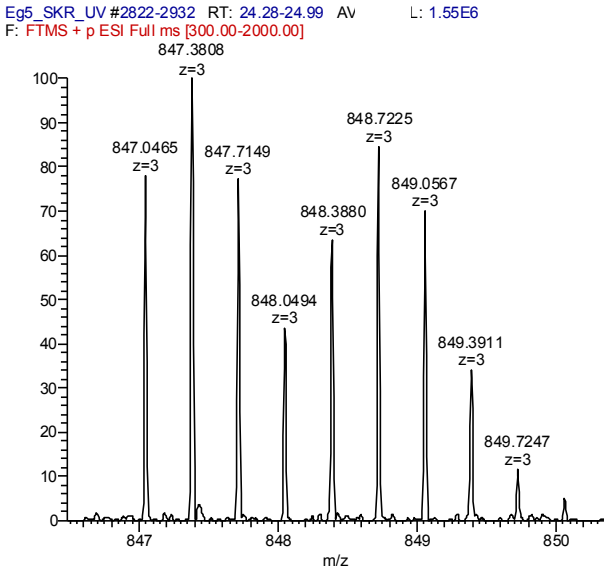


Figure S1. Mass spectrum of peptide-inhibitor adduct after crosslinking of kinesin-5 with **2** and **3** in the absence of microtubules. The 'signature' peaks, with the expected isotopic distribution and mass differences indicate crosslinking occurs without microtubules.

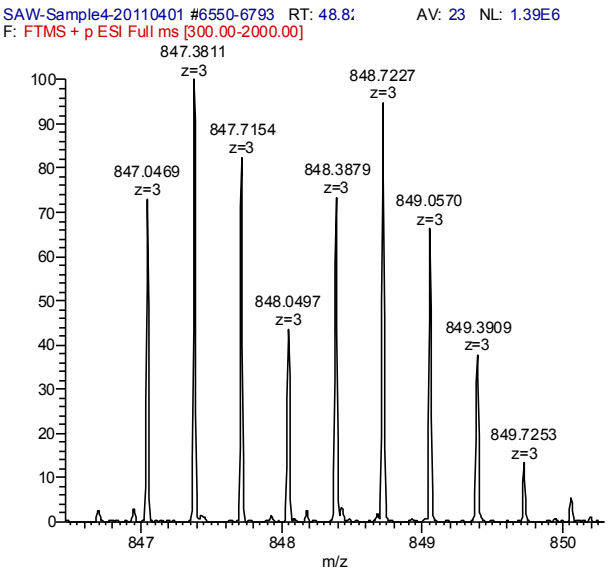


Figure S2. Mass spectrum of peptide-inhibitor adduct after crosslinking of kinesin-5 L214A with **2** and **3** (no microtubules). The 'signature' peaks, with the expected isotopic distribution and mass differences indicate crosslinking occurs to the mutant protein.

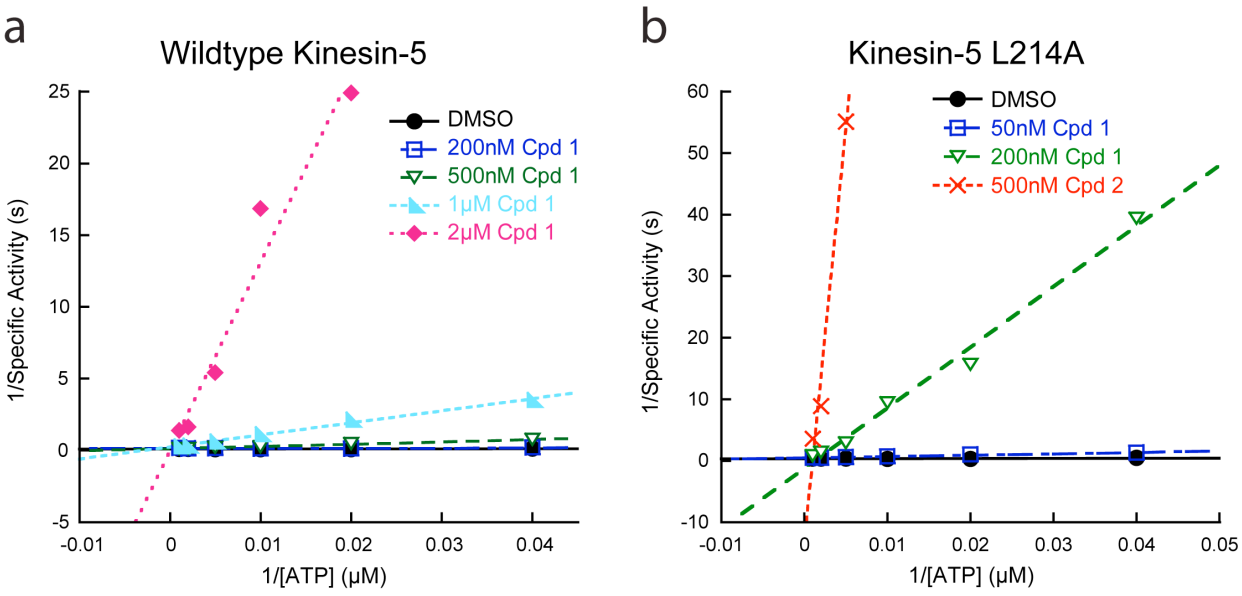


Figure S3. Lineweaver-Burk plots of kinesin-5 wildtype (a) and L214A mutant (b) microtubule-stimulated ATPase activity at 25μM, 50μM, 100μM, 200μM, 500μM, and 1mM ATP. Individual linear regressions were calculated for each inhibitor concentration. Data values are average of 3.

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