Electronic Supplementary Material (ESI) for MedChemComm.

Development of inverse electron demand Diels-Alder ligation and TR-FRET assays for the determination of ligand-protein target occupancy in live cells

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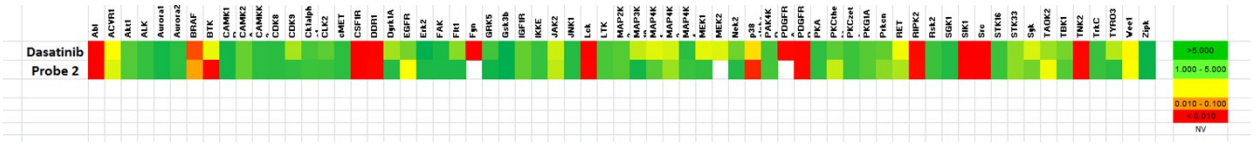

B

	c-Src K_i [μM]	p38-α K_i [μM]
Dasatinib (1)	**0.0006**	**0.047**
***Trans*-cyclooctene probe (2)**	**0.0011**	**0.011**

C

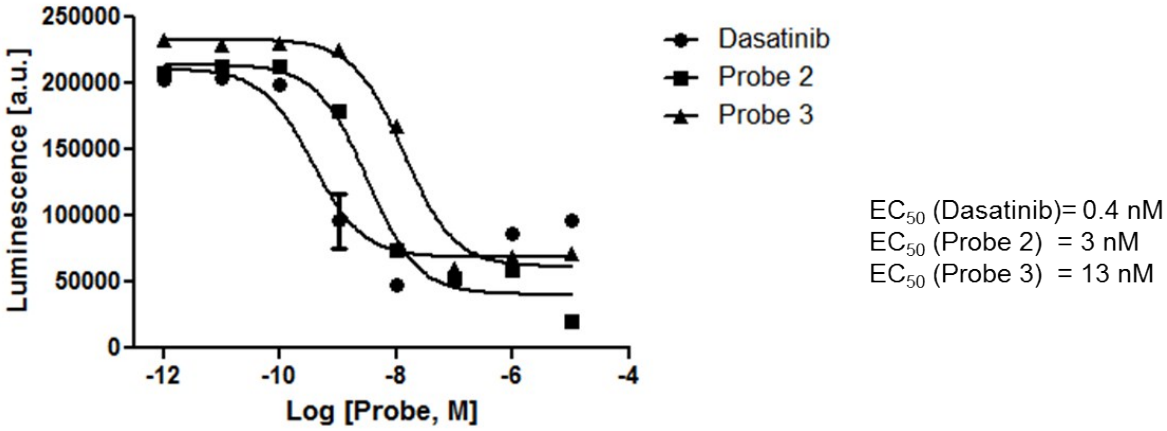

Figure S1. Biochemical TR-FRET binding assay-based kinome profiling of Dasatinib (**1**) and *trans*-cyclooctene probe (**2**). Cell proliferation assay profiling of Dasatinib (**1**), probe **2** and probe **3**. A. Heat map representation of kinome profiling against selected kinases for Dasatinib and probe **2**. Evaluation of fluorescent probe **3** was prevented by experimental constraints posed by reporter interference. B. Inhibition constants (K_i) determined for Dasatinib and probe **2** against c-Src and p38-α in TR-FRET binding assay. C. EC_{50} values for Dasatinib, probe **2** and probe **3** determined in K562 cell proliferation assay. Results are representative of six independent experiments.

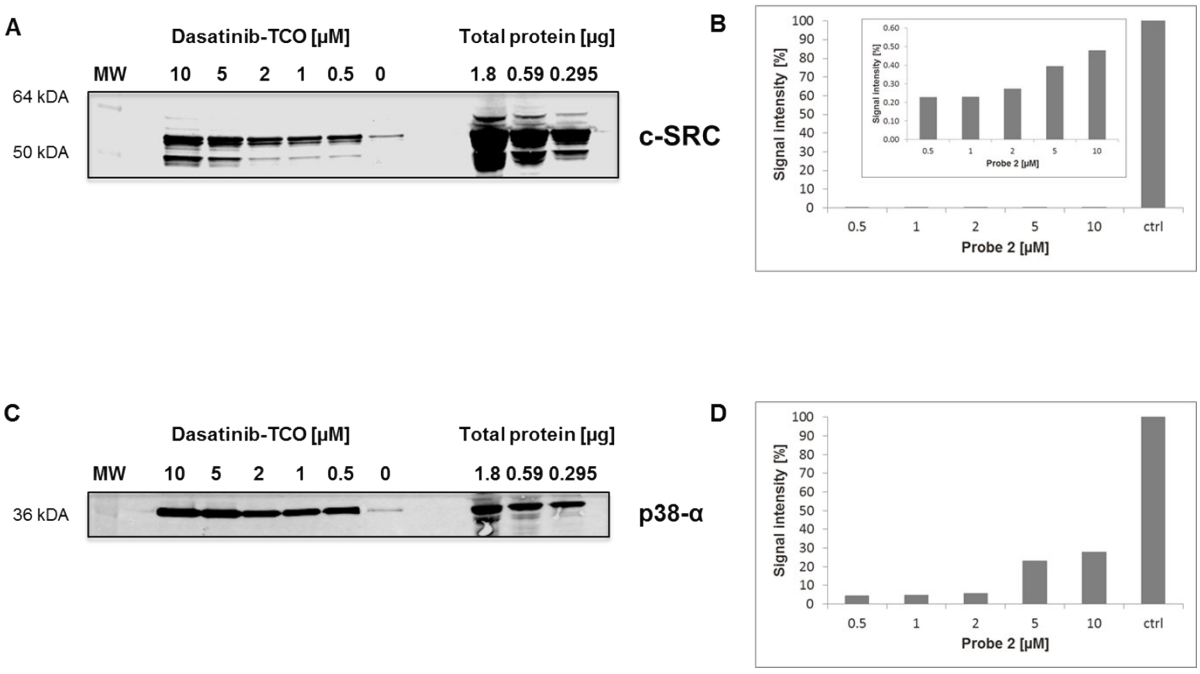

Figure S2. Determination of capture efficiency of c-Src and p38-α with the use of *trans*-cyclooctene probe **2**. K562 cells were incubated with probe **2** at the concentration 0.5 μM, 1 μM, 2 μM, 5 μM or 10 μM. After 2 h, cells were harvested, lysates (20 μg of total protein was used per sample) were mixed with tetrazine-conjugated beads. After 45 min incubation followed by elution in LDS buffer and SDS-PAGE electrophoresis, immunoblot-based analysis was conducted. Probe **2** enabled capture of ~0.23-0.48% of total c-Src and 5-27% of total p38-α. All quantifications of signal intensities from samples treated with probe **2** were normalized to control sample. A. Immunoblot analysis-based detection of the amount of c-Src capture with different concentrations of probe **2**. B. Quantification of the amount of c-Src capture with different concentrations of probe **2** based on the immunoblot shown in A. Control value (ctrl) was calculated as an average of signal intensities of protein band corresponding to c-Src derived from known amounts of total proteome. C. Immunoblot analysis-based detection of the amount of p38-α capture with different concentration of probe **2**. D. Quantification of the amount of p38-α capture with different concentrations of probe **2** based on the immunoblot shown in C. Control value (ctrl) was calculated as an average of signal intensities of protein band corresponding to p38-α derived from known amounts of total proteome. Results are representative of a single experiment.

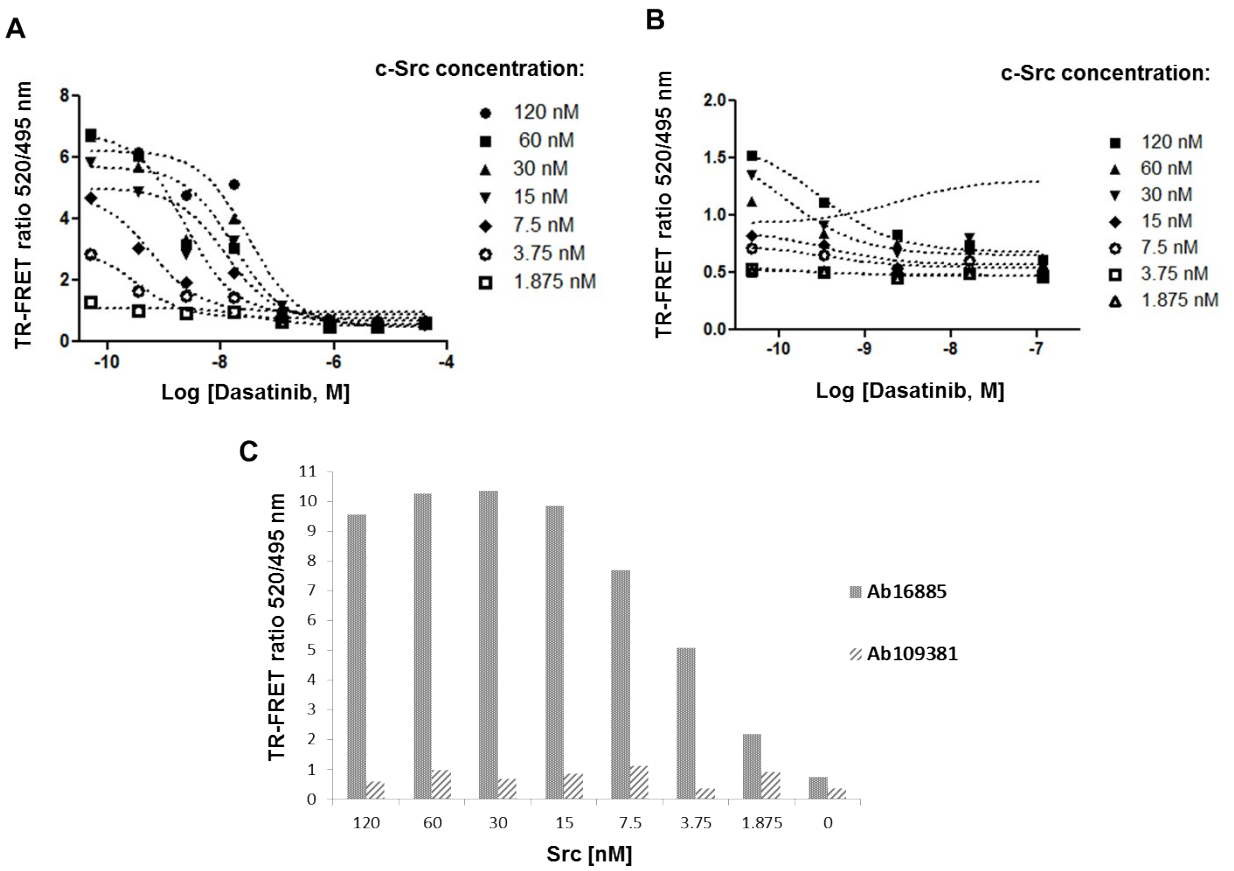

Figure S3. Antibody determination for detection of c-Src in TR-FRET assay based on the use of BODIPY-FL probe **3**. Ab16885 (2 nM in kinase buffer A) or Ab109381 (2 nM in kinase buffer A) was incubated for 120 min in the presence of probe **3** (30 nM), combination of increasing concentrations of Dasatinib and increasing concentrations of recombinant c-Src. A. TR-FRET signal ratio detected in the presence of Ab16885 for different concentrations of c-Src (0-120 nM) and Dasatinib (40 pM – 40 μM). B. TR-FRET signal ratio detected in the presence of Ab109381 for different concentrations of c-Src (0-120 nM) and Dasatinib (40 pM - 0.1 μM). C. Fold difference between maximum and minimum TR-FRET signal ratio observed in the presence of Ab16885 or Ab109381 for different (0-120 nM) concentrations of c-Src. ~20 fold difference between maximum and minimum TR-FRET signal ratio was observed for Ab16885, but only ~3 fold difference between maximum and minimum TR-FRET signal ratio was observed for Ab109381. Ab16885 was selected for further studies. Results are representative of a single experiment.

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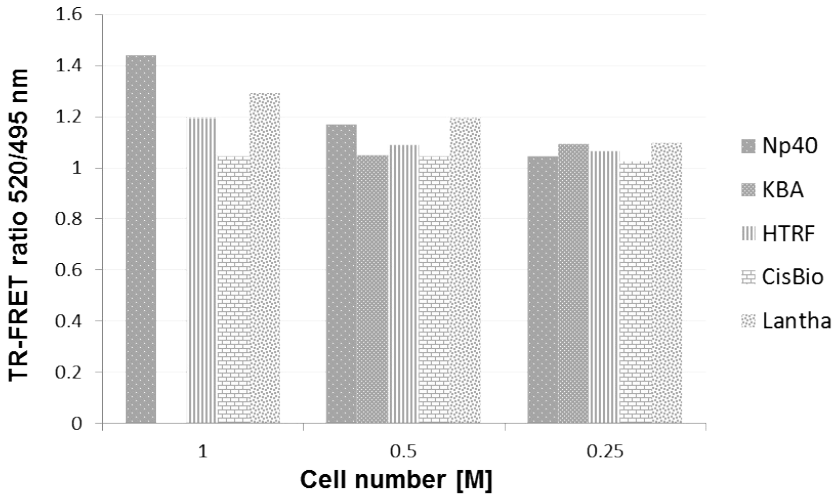

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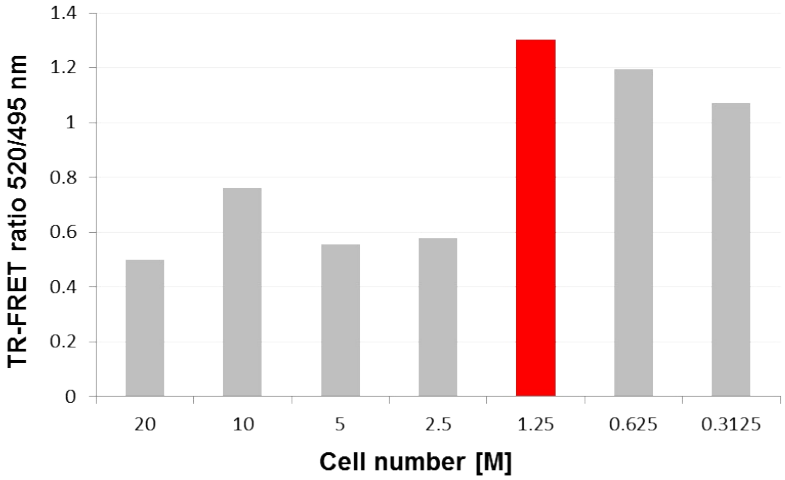


Figure S4. Determination of buffer solution and cell number for detection of most optimal TR-FRET signal. A. Five buffers in combination with three different cell concentrations (0.25 M, 0.5 M and 1 M) were evaluated in the presence of probe **3** (30 nM) and antibody Ab16885 (2 nM). The measurements were conducted after 180 min of incubation. The measurement conducted in NP40 buffer produced the highest TR-FRET signal ratio and was selected for further studies. Evaluated buffers: 1. Kinase buffer A (50 mM HEPES, 10 mM MgCl2, 1 mM EGTA, 0.01% Triton), 2. NP40 buffer (0.1% NP40, 5mM EDTA, 50 mM $MgCl_2$), 3. Lantha Buffer (A12891), 4. HTRF Buffer (64KL3FDF), 5. CisBio Buffer (64KL1FDF). B. Probe **3** and antibody Ab16885 (2 nM) were incubated in NP40 buffer in the presence of increasing concentrations of K562 cells (0.31–20 M/100 µL). After 180 min incubation, measurement was conducted in 384 Proxy plate. It was determined that sample containing 62500 cells (derived from 1.25 million /100 µL sample) produced the highest TR-FRET signal ratio and this condition was selected for further studies. Results are representative of a single experiment.

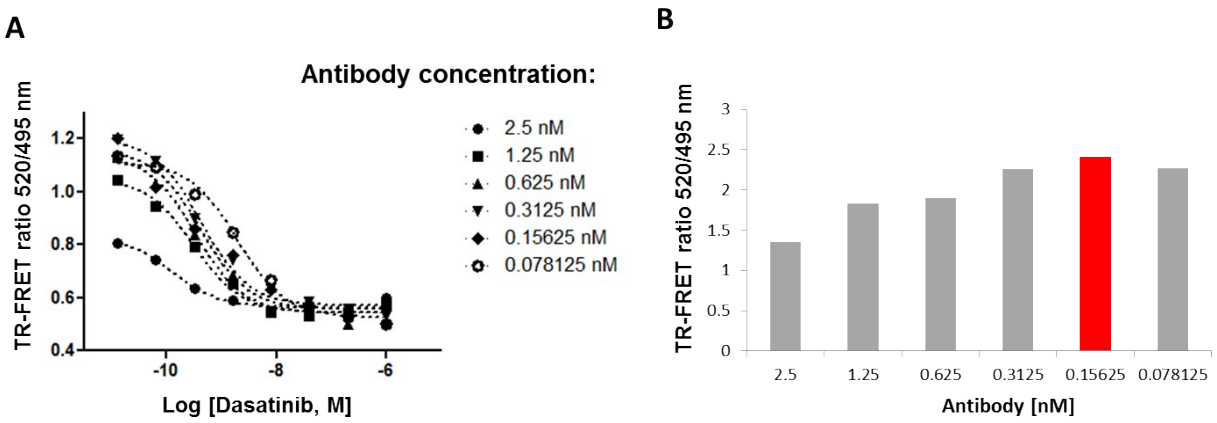


Figure S5. Determination of optimal antibody concentration. Increasing concentrations of Ab16885 (0.078 nM, 0.156 nM, 0.312 nM, 0.635 nM, 1.25 nM, 2.5 nM) were mixed with probe **3** (30 nM) and increasing concentrations of Dasatinib (50 pM – 1 μM) in K562 cell lysates (62500 cells per well were used). After 240 min incubation at RT, the measurement was conducted. A. TR-FRET signal ratio detected for different concentrations of Ab16885. B. Plot representing fold difference between maximum and minimum TR-FRET signal ratio observed for given concentration of Ab16885. The most optimal TR-FRET signal ratio was observed at 0.15 nM concentration of Ab16885. Results are representative of a single experiment.

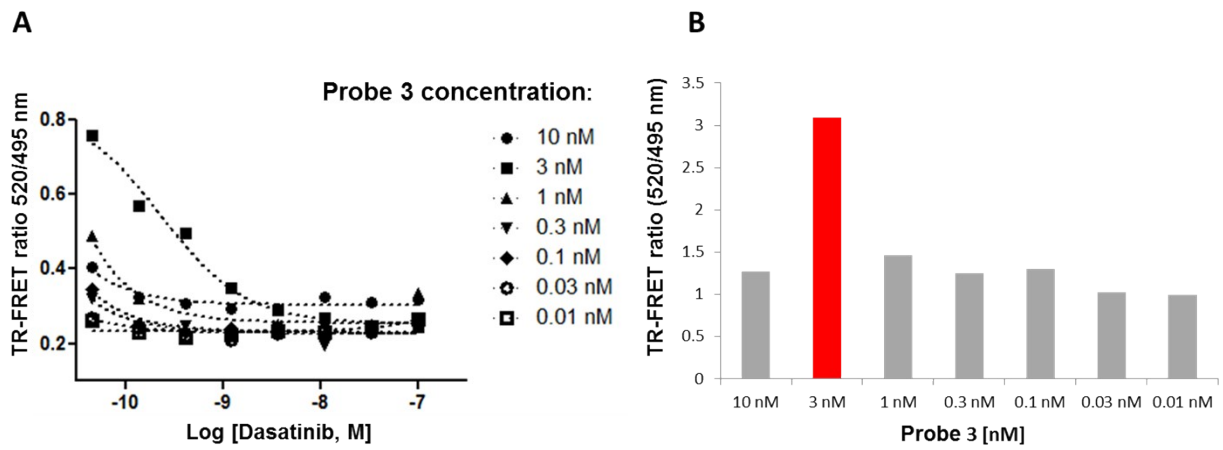


Figure S6. Determination of optimal probe concentration. Increasing concentrations of probe **3** (0.01 nM, 0.03 nM, 0.1 nM, 0.3 nM, 1 nM, 3 nM, 10 nM) were mixed with Ab16885 (0.15 nM) and increasing concentrations of Dasatinib (50 pM – 100 nM) in K562 cell lysates (62500 cells per well were used). The measurement was conducted after 210 min incubation at RT. A. TR-FRET signal ration detected for different concentrations of probe **3**. B. Plot representing fold difference between maximum and minimum TR-FRET signal ratio observed for given concentration of probe **3**. Probe **3** at concentration 3 nM was used in further studies. Results are representative of a single experiment.

General Information

Recombinant human c-Src and p38-α proteins were purchased from Abcam (Cat. No. ab79635, ab82188). Antibodies used for immunoblotting were purchased from Cell Signaling and LI-COR (Cat. No. CS #2109, CS#9218S, LI-COR 926-32211). Antibodies used for TR-FRET assay were purchased from Abcam (Ab31828, Ab170099, Ab16885, Ab109381). Tb-labeled secondary antibodies (LanthaScreen PV3774, PV3766) were purchased from Thermo-Fisher Scientific. Invitrogen PVDF membranes were used for immunoblots and scanned using Odyssey Imager. Solvents and chemicals were purchased from commercial sources and used directly without further purification. ^{1}H NMR spectra were recorded with Agilent 400-MR, Varian Inova 500 MHz, Varian VNMRS 500 MHz, Bruker Avance III 500 MHz. Chemical shifts were reported in ppm using either TMS or deuterated solvents as internal standards (TMS, 0.00; $CDCl_3$, 7.26; CD_3OD, 3.34; DMSO-d_6, 2.50). Multiplicity was reported as follows: s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, m = multiplet, brs = broad.

IED-DA ligation-based pull-down assay

30 million of K562 cells were plated in 5 mL of full media (IMDM + 10% FBS) and incubated with Dasatinib at the indicated concentration (1 µL of 5000x DMSO stock solution was added). After 1 h, cells were treated with probe **2** (1 µL of 10 mM DMSO stock solution). After 2 h cells were harvested, washed twice with 5 mL of PBS, and 250 µL of lysis buffer was added (lysis buffer: PBS, 0.02 % Tween, 100 mM NaCl, protease and phosphatase inhibitors, pH=7.4). Cells were subjected to sonication on ice for 20 s and subsequent centrifugation for 10 min at 14000 xg (4 °C). Supernatants were removed and Bradford analysis was performed to estimate total protein concentration. Lysates (20 µg of total protein was used per sample) were mixed with tetrazine-conjugated beads (60 µL). After 45 min incubation at 4 °C, beads were washed with ice cold lysis buffer (3x750 µL). Protein from enriched samples was eluted from beads with 50 µL of 2× LDS sample buffer (Invitrogen): 141 mM Tris base, 106 mM Tris HCl, 2% LDS, 10% glycerol, 0:51 mM EDTA, 0:22 mM SERVA Blue G, 0.175 mM Phenol Red pH 8.5 and kept at 80 °C for 10 minutes before being subjected to electrophoresis (15 µL per sample was loaded, 4-12% Bis-Tris SDS-PAGE and MOPS running buffer were used). Following immunoblot analysis was performed using iBlot transfer (Invitrogen), and c-Src and p38-α were visualized using commercial antibodies (CS #2109, CS#9218S, LI-COR 926-32211). Immunoblots were visualized on Odyssey Imager. Analysis of immunoblots was performed using Image Studio software.

Cellular TR-FRET assay

1.25 M cells in 100 µL/well of media were plated in 96-well plates and treated with Dasatinib at the indicated concentration (1 µL of 100x DMSO stock solution was added). After 1 h incubation, cells were treated with probe **3** (1 µL of 0.3 µM stock solution was added for the final concentration 3 nM). After 1 h, cells were harvested, washed three times with 200 µL of cold PBS +1% FBS and 100 µL of lysis buffer was added (0.1% NP40, 5 mM EDTA, 50 mM $MgCl_2$, protease inhibitors, pH=7.4). Cells were incubated at RT for 30 min and 5 µL of lysate was transferred to 384-well white Proxi plates (Promega). 10 µL of kinase buffer A containing c-Src antibody (0.225 nM) (Ab16885, final concentration 0.15 nM) and 0.1 µg of Tb-labeled secondary antibody

(LanthaScreen Tb-labeled Anti-rabbit, PV3774) was added. The mixture was incubated at RT. The measurements were conducted on Envision plate reader (Perkin-Elmer) over 24 hours.

Kinome profiling

Plates were stamped with 5 µL of kinase buffer (Life Technologies #PR4940D) containing recombinant kinase (2.5 -10 nM final concentration), Eu- or Tb-labeled antibodies (His or GST; 0.5-2 nM final concentration) and fluorescently-tagged probe (3-200 nM final concentration). Appropriate probes were diluted in kinase buffer and 120 µL of compound was added to the plate using Biomex FX. Plates were incubated at RT for 2 h and the measurements were conducted using Envision plate reader (Perkin-Elmer). The K_i values were calculated using the Assay Explorer software (Accelrys). Example for BTK kinase: BTK (Invitrogen, PV3363) was added to 5 µL kinase buffer (Life Technologies, #PR4940D) to a final concentration of 10 nM, supplemented with 2 nM of Tb-labeled anti-His antibody and 200 nM Oregon Green labeled probe. Afterwards, 120 µL of diluted compound in kinase buffer was added and the plate was incubated at RT for 2 h. The measurements were conducted using Envision plate reader (Perkin-Elmer) and the K_i values were calculated using the Assay Explorer software.

Cell proliferation assay

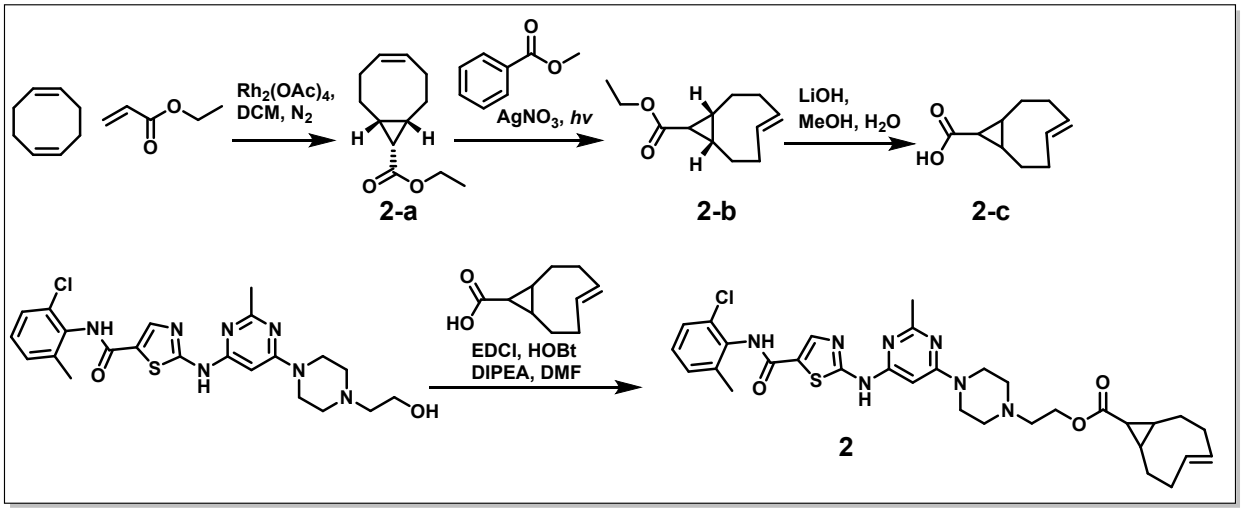
Dasatinib and all probes were profiled in K562 cell line according to reported Promega procedure: https://www.promega.com/resources/protocols/technical-bulletins/0/celltiter-glo-luminescent-cell-viability-assay-protocol/

Synthetic procedures:

Compound 1.

N-(2-chloro-6-methylphenyl)-2-((6-(4-(2-hydroxyethyl)piperazin-1-yl)-2-methylpyrimidin-4-yl)amino)thiazole-5-carboxamide (Dasatinib) (1) was purchased from Ark Pharm Inc. (Arlington Heights IL) and used as received.

Synthesis of Probe 2



Synthesis of (1R,8S,9r,Z)-ethyl bicyclo[6.1.0]non-4-ene-9-carboxylate (2-a)

A solution of ethyl acrylate (20 g, 200 mmol) in 300 mL dichloromethane was added dropwise via syringe pump at 0°C under a nitrogen atmosphere to a solution of (1Z,5Z)-cycloocta-1,5-diene (184 g, 1698 mmol) and tetrakis(acetato)dirhodium(II) (2 g, 4.53 mmol) in 300 mL dichloromethane. The resulting mixture was allowed to warm up and it was stirred for two days at room temperature. The mixture was filtered and concentrated under reduced pressure and the residue was purified by silica gel column chromatography using a gradient of petroleum ether: ethyl acetate 400:1 to 100:1 to afford the title compound (9 g, 46.3 mmol, 39.8 % yield) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.17-1.19 (t, J=4.4 Hz, 1H), 1.27-1.43 (t, J=7.2 Hz, 3H), 1.43-1.59 (m, 4H), 2.04- 2.12 (m, 2H), 2.15-2.23 (m, 2H), 2.26-2.34 (m, 2H), 4.07-4.12 (q, J=7.2 Hz, 2H), 5.60-5.67 (m, 2H).

Synthesis of (1R,8S,E)-ethyl bicyclo[6.1.0]non-4-ene-9-carboxylate (2-b)

A Biotage / ISCO MPLC continuous flow apparatus was used for the photoisomerization reaction using a procedure previously reported,[(1)] with the following modification: two 120 g (Biotage SNAP cartridges) were used to house the silica gel and silver nitrate impregnated silica gel. One of the SNAP cartridges was filled with unmodified silica gel. The other SNAP cartridge contained a bed of unmodified silica gel that was topped with 100 g of silica gel which was impregnated with silver nitrite (20 g, 118 mmol). (1R,8S,9r,Z)-ethyl bicyclo[6.1.0]non-4-ene-9-carboxylate (**2-a**) (3.0 g, 15.4 mmol) and methyl benzoate (4.2 g, 30.9 mmol) were placed in a quartz flask and dissolved in a mixture solvent of ethyl ether (1 L) and n-hexane (1 L). The solution was equilibrated through the continuous flow system at a 100 mL/min flow rate and simultaneously degassed with nitrogen for 15 minutes. The solution in the quartz flask was then irradiated (254nm, 20W, Hg lamp, XPA-2) under continuous flow conditions (100 mL/min) for 48 hrs. The SNAP cartridges were flushed with 1 L of 1/1 ethyl ether/hexanes and then dried with compressed air. To the dried silica gel was sequentially added 28% ammonium hydroxide aqueous (500 mL) and dichloromethane (500 mL) and the resulting biphasic mixture was filtered. The filter cake was washed with additional dichloromethane (200 mL) and ammonium hydroxide (200 mL). The filtrate was transferred to a separatory funnel and partitioned. The aqueous layer was extracted with dichloromethane (300 mL). The organic layers were combined, washed twice with water (500 mL), dried with sodium sulfate, filtered and concentrated at 20°C to give a residue, which was purified by column chromatography on silica gel, eluted with petroleum ether/ethyl acetate = 20/1) to afford the title compound (0.7 g, yield 23%) as colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.57-0.67 (m, 1 H), 0.87-0.95 (m, 2 H), 1.15-1.19 (m, 1 H), 1.23-1.32 (m, 4 H), 1.91-2.03 (m, 2 H), 2.21-2.42 (m, 4 H), 4.07-4.13 (q, J=7.3 Hz, 2 H), 5.12-5.19 (m, 1 H), 5.85-5.93 (m, 1 H).

Synthesis of (E)-bicyclo[6.1.0]non-4-ene-9-carboxylic acid (2-c)

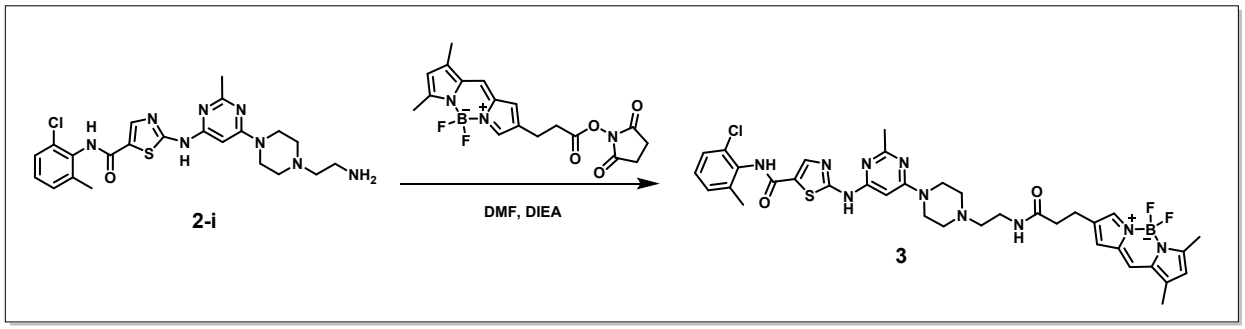
To a solution of (1R,8S,E)-ethyl bicyclo[6.1.0]non-4-ene-9-carboxylate (2-b) (0.7 g, 5.2 mmol) in methanol (20 mL) and water (8 mL) was added lithium hydroxide (0.6 g, 25.7 mmol). The mixture was stirred at 45°C for 3 hours. The reaction mixture was concentrated under reduced pressure to give a residue. The residue was diluted with water (50 mL) and washed with methyl tert-butyl ether (3 × 30 mL). The aqueous phase was adjusted to pH = 4 with 0.5 M hydrochloride solution and then extracted with methyl tert-butyl ether (3 × 30 mL). The combined organic phase was

washed with brine, dried over sodium sulfate, filtered and concentrated under reduced pressure to afford (E)-bicyclo[6.1.0]non-4-ene-9-carboxylic acid (2-c) (0.48 g, yield 80%) as white solid. ^{1}H NMR (400 MHz, CD_3OD) δ 0.66-0.76 (m, 1 H), 0.90-1.03 (m, 2 H), 1.10-1.18 (m, 1 H), 1.22-1.30 (m, 1 H), 1.91-2.03 (m, 2 H), 2.20-2.31 (m, 3 H), 2.36-2.42 (m, 1 H), 5.14- 5.22 (m, 1 H), 5.83-5.91 (m, 1 H).

Synthesis of (E)-2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethyl bicyclo[6.1.0]non-4-ene-9-carboxylate (2)

To a solution of Dasatinib (200 mg, 0.410 mmol) in anhydrous DMF (3 mL) was added (E)-bicyclo[6.1.0]non-4-ene-9-carboxylic acid (**2-c**) (82 mg, 0.492 mmol), EDCI (118 mg, 0.615 mmol), HOBT (94 mg, 0.615 mmol) and DIPEA (0.143 ml, 0.820 mmol). The resulting mixture was stirred at 25 °C for 8 hrs. The reaction mixture was directly purified by prep-HPLC using a gradient of acetonitrile (5-75%) in ammonium acetate (10 mM) to obtain (E)-2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethyl bicyclo[6.1.0]non-4-ene-9-carboxylate (**2**) (56.8 mg, yield 21%) as white solid. LCMS (ESI$^+$): observed m/z = 636 (M+H)$^+$; ^{1}H NMR (400 MHz, DMSO): δ 11.42 (s, 1H), 9.83 (s, 1H), 8.19 (s, 1H), 7.38-7.36 (m, 1H), 7.27-7.21 (m, 2H), 6.03 (s, 1H), 5.82-5.76 (m, 1H), 5.13-5.07 (m, 1H), 4.11-4.08 (t, 2H), 3.48 (s, 4H), 2.57-2.52 (m, 6H), 2.38 (s, 3H), 2.21-2.16 (m, 7H), 1.92-1.86 (m, 2H), 1.21-0.95 (m, 4H), 0.74-0.66 (m, 1H).

Synthesis of probe 3



Synthesis of 2-(3-((2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethyl)amino)-3-oxopropyl)-5,5-difluoro-7,9-dimethyl-5H-dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-4-ium-5-uide (3)

Compound **2-i** (8 mg, 0.016 mmol) and BODIPY-FL, SE (5 mg, 0.013 mmol, Invitrogen) were mixed in dimethylformamide (1 mL) with DIEA (N,N-diisopropylethylamine) (0.011 mL, 0.066 mmol). The reaction was stirred at room temperature overnight. The crude reaction mix was purified by reverse phase HPLC using a Gilson system and a C18 25 x 100 mm column, eluting with 5-85% acetonitrile in water containing 0.1% v/v trifluoroacetic acid. The product fractions were lyophilized to give the title compound as the TFA salt (7.8 mg, 47.4 % yield). MS (ESI+) 761 (M+H)+, 783 (M+Na)+; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.61 (s, 1H), 9.87 (s, 1H), 8.22 (d, J = 5.9 Hz, 2H), 7.66 (s, 1H), 7.37 (dd, J = 7.6, 1.9 Hz, 1H), 7.30 – 7.18 (m, 2H), 7.07 (d, J = 4.0 Hz, 1H), 6.36 – 6.25 (m, 2H), 6.12 (s, 1H), 4.32 (s, 2H), 3.13 (dt, J = 46.0, 7.1 Hz, 6H), 2.85 (s, 2H),

2.70 (s, 2H), 2.53 (dd, *J* = 8.8, 6.8 Hz, 2H), 2.43 (d, *J* = 7.8 Hz, 6H), 2.21 (d, *J* = 8.3 Hz, 6H), 1.23 – 1.11 (m, 2H).

References:

1. Royzen, M., Yap, G. P. A., Fox, J. M., "A Photochemical Synthesis of Functionalized trans-Cyclooctenes Driven by Metal Complexation", J. Am. Chem. Soc. **2008**, 130, 3760-3761.