Conformation-selective inhibitors reveal differences in the activation and phosphate-binding loops of the tyrosine kinases Abl and Src.

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Contents

I. SUPPORTING FIGURES

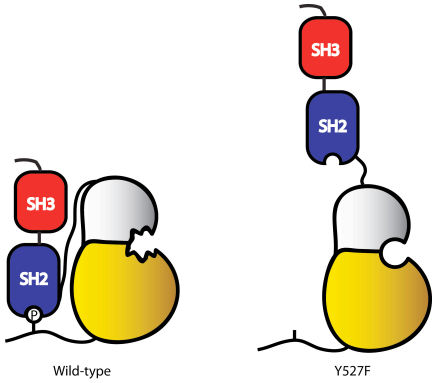


Figure S1. Phosphorylation of Src at position Tyr527 facilitates a binding interaction between the tail of the catalytic domain and the SH2 domain, causing a decrease in catalytic activity (left). Mutating this position to Phe releases this interaction and restores activity (right).

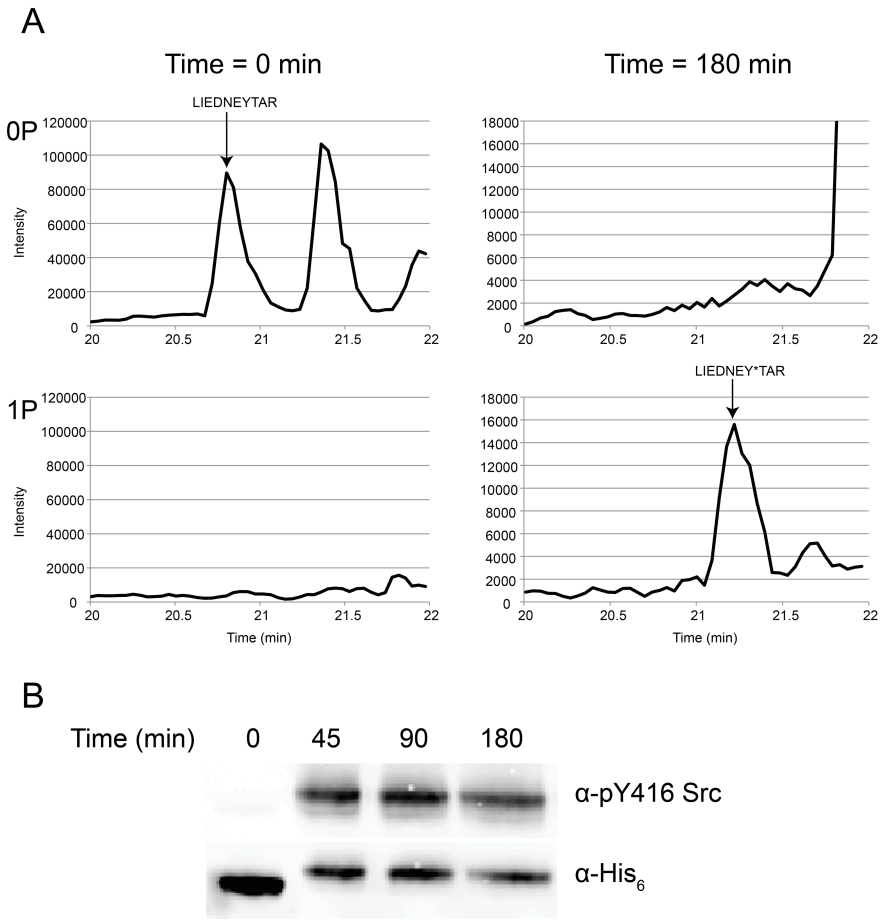


Figure S2. A. Extracted ion chromatographs from LC/MS analyses of tryptic digests of Src either alone (left) or after incubation with ATP for 3 h (right). Only the monophosphorylated peak is observed after 3 h, indicating quantitative phosphorylation. B. Timecourse analysis by immunoblot of Src autophosphorylation.

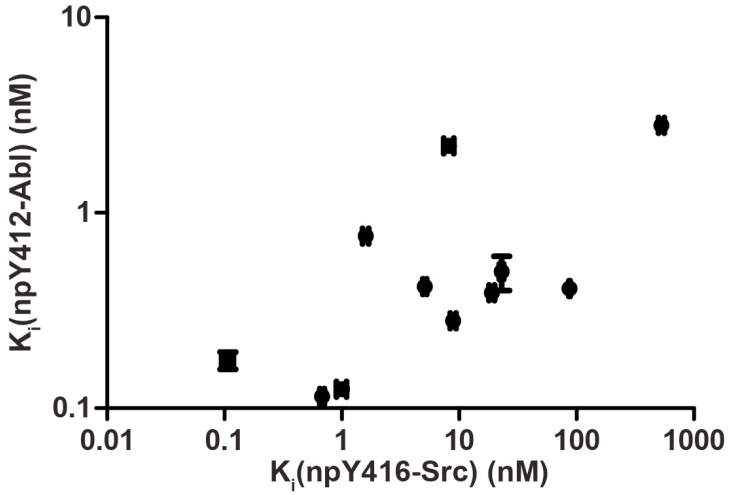


Figure S3. A plot of the K_is of inhibitors **1**-**11** for npY412-Abl and npY416-Src. The K_is of **1**-**11** for npY412-Abl are plotted on the y-axis, while the K_is of **1**-**11** for npY416-Src are plotted on the x-axis.

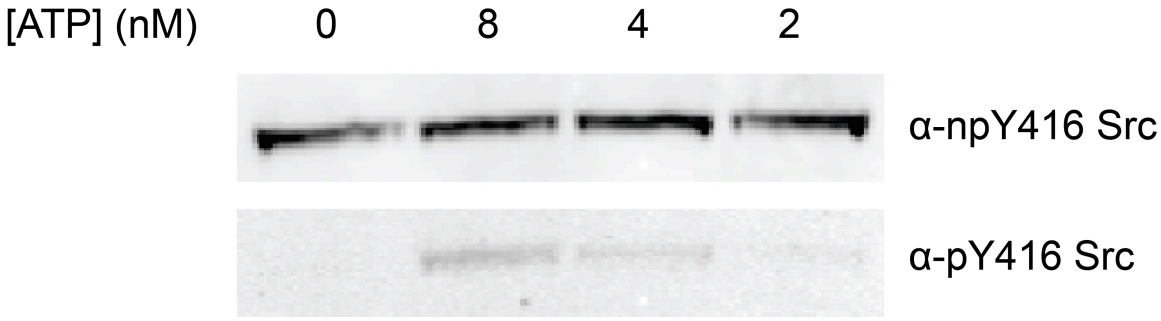


Figure S4. Autophosphorylation of Src (10 nM) at low concentrations of ATP. The concentration of γ^{32}P ATP used in the activity assays was ~2 nM, and the concentration of Src was less than 2 nM. A higher Src concentration (10 nM) was used for immunoblot analysis to allow the detection of any activation loop phosphorylation.

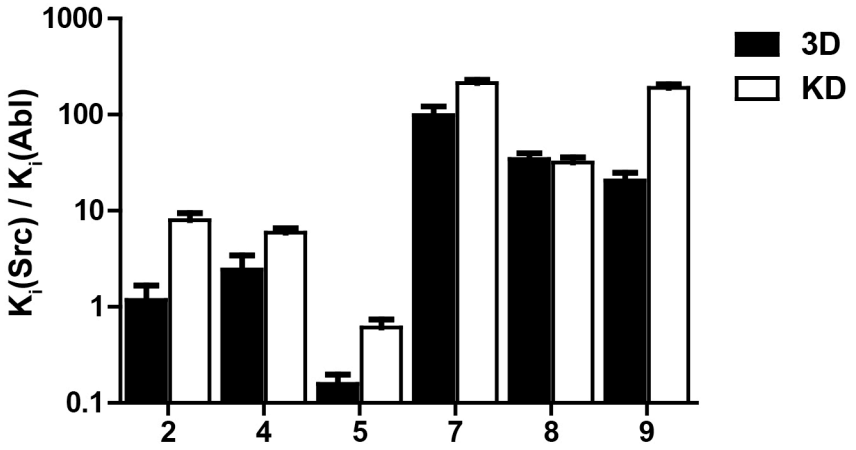


Figure S5. Fold differences in K_i between 3-domain (3D) and kinase domain (KD) versions of Abl and Src for several inhibitors.

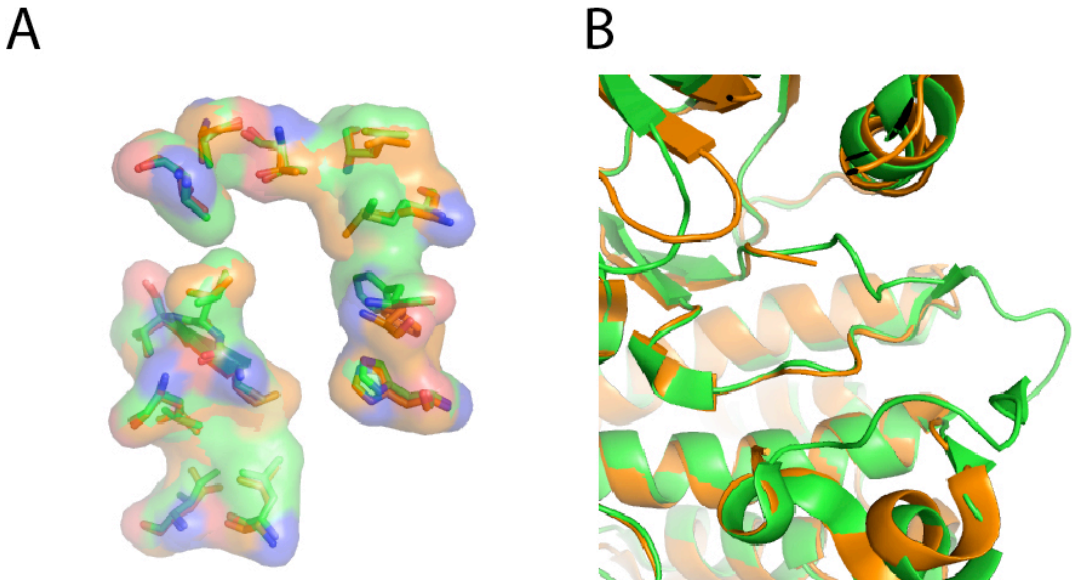


Figure S5. Comparison of pY412-Abl (PDB ID: 2GQG) and pY416-Src (1YOM). (A) Superimposed hydrophobic spines of activated Abl and Src. (B) Superimposed structures of activated Abl (green) and Src (orange), with focus on the activation loop. Most of the activation loop is missing in the Src structure.

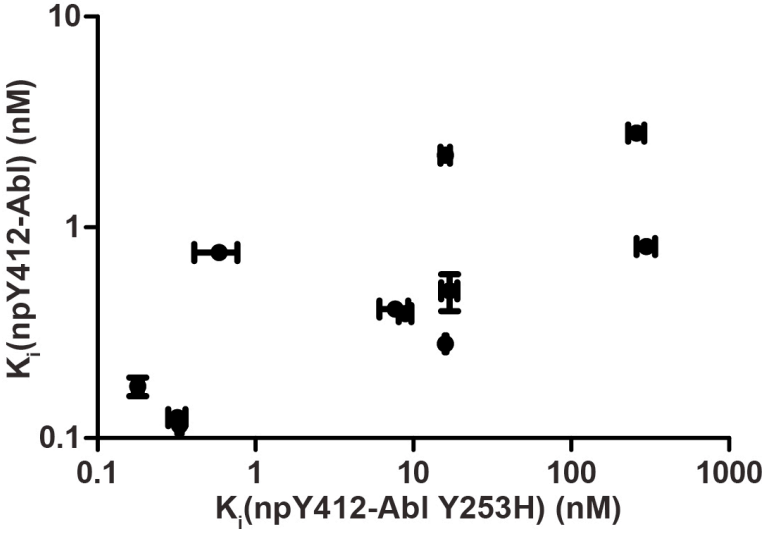


Figure S6. A plot of the K_is of inhibitors **1-11** for npY412-Abl wt and npY412-Abl Y253H. The K_is of **1-11** for npY412-Abl wt are plotted on the y-axis, while the K_is of **1-11** for npY412-Abl Y253H are plotted on the x-axis.

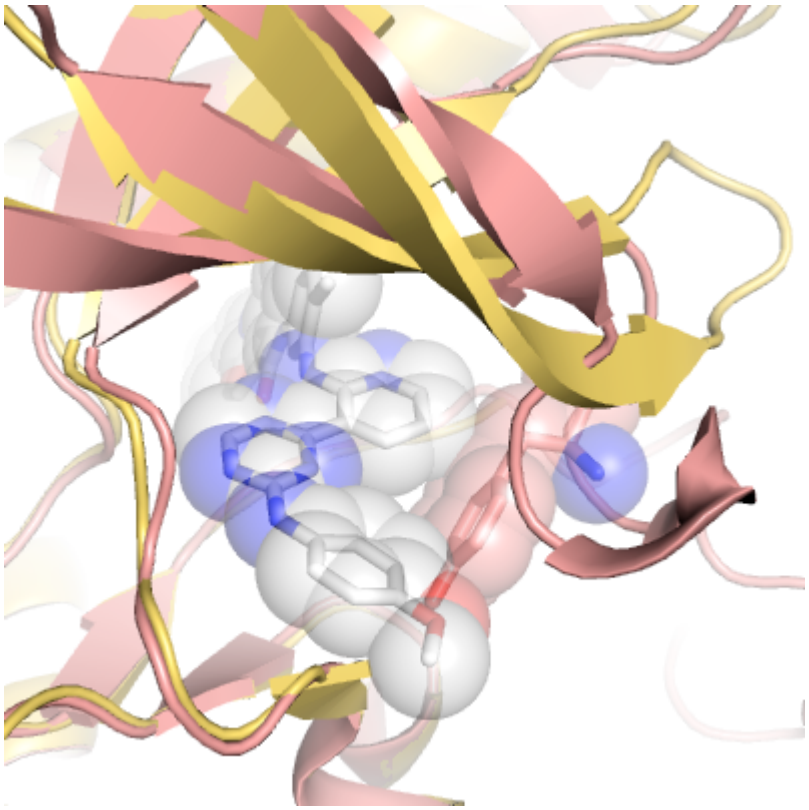


Figure S7. Superimposition of Abl bound to imatinib (salmon, inhibitor hidden) (PDB ID: 1IEP) and Src bound to a dual Src/Abl inhibitor (yellow, inhibitor in white) (PDB ID: 3G6G). There is insufficient space for the inhibitor to bind to Abl with a kinked p-loop due to steric hindrance by Tyr253 (salmon, stick and sphere form).

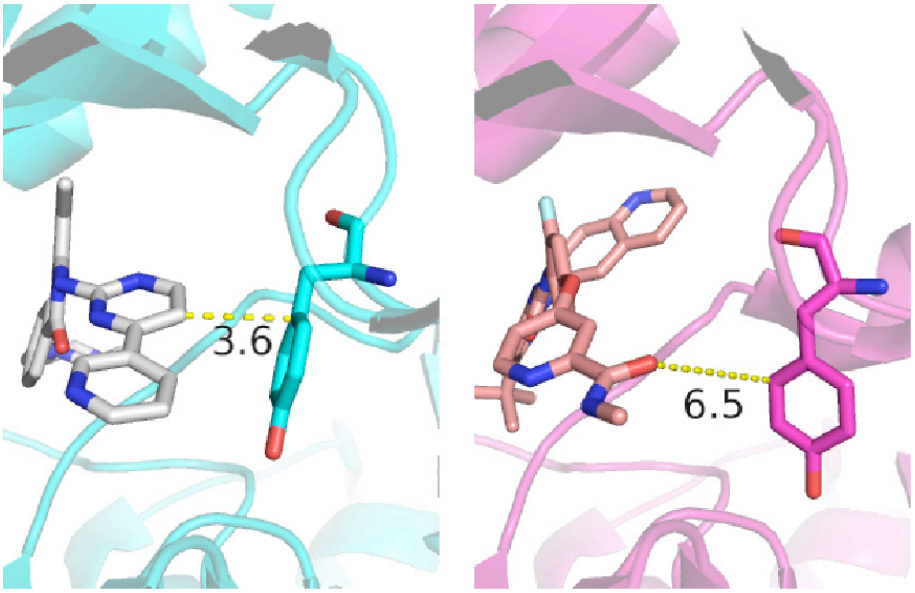


Figure S8. Exploring p-loop interactions with inhibitors. Tyr253 in Abl is closer to imatinib (left, cartoon in cyan, ligand in white) (PDB ID: 1IEP) than **1** (right, cartoon in magenta, ligand in salmon) (PDB ID: 3QRI).

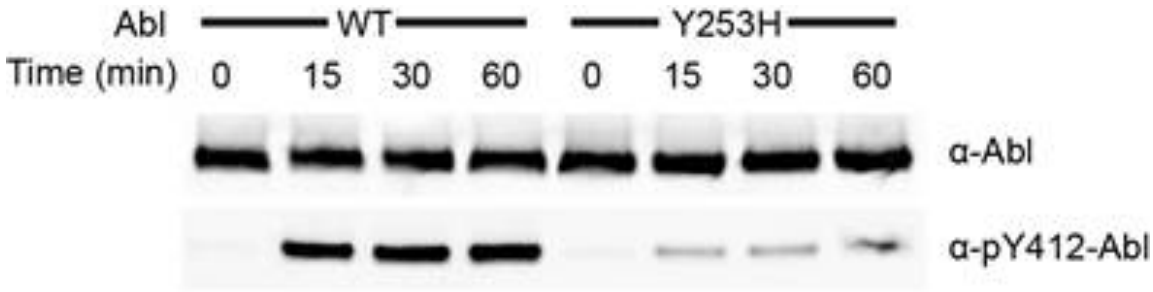


Figure S9. Activation loop phosphorylation of Abl wt and Abl Y253H (both at 15 µM) by Hck (700 nM) and ATP (500 µM) at 37 °C. The top and bottom immunoblots were performed separately using the same samples at the same volumes.

II. SUPPORTING TABLES

	K_i (nM)		
	2	**3**	**6**
npY416-Src (Y527F)	5.2 ± 0.1	2.8 ± 0.1	13 ± 1
npY416-Src (wt)	29.8 ± 0.9	3.1 ± 0.1	12.5 ± 1.3

Table S1. K_is of npY416-Src (Y527F) and npY416-Src (wt) against type II inhibitors shows that the two constructs have similar inhibition profiles.

	K_i (nM)

	2	3	4
npY416-Hck	1.5 ± 0.1	4.2 ± 0.3	10 ± 1
pY416-Hck	4.8 ± 0.3	7.7 ± 0.2	20 ± 0.3

Table S2. K_is of npY416-Hck and pY416-Hck against three ligands shows little difference between phosphoforms, similar to that observed for Src.

III. SYNTHESIS

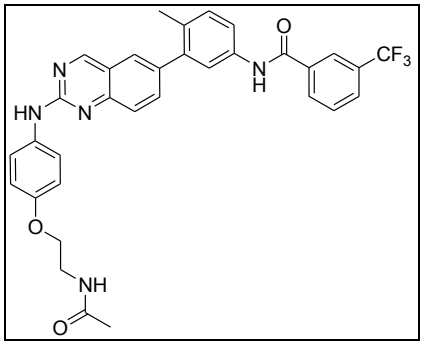
A. General Information

Unless otherwise noted, all reagents were obtained from commercial suppliers and used without further purification. NMR spectra were obtained on a Bruker AV-300 or -301 instrument at room temperature. Chemical shifts are reported in ppm and coupling constants in Hz. Mass spectra were obtained on a Bruker Esquire Ion Trap instrument.

General HPLC Purification Conditions: Samples were injected on a preparatory reverse-phase C18 column (250 x 21 mm) run over 60 minutes at 8 mL/min (Acetonitrile/Water–0.05% TFA gradient: 1:99 to 100:0). Purified products were detected by UV at the detection frequency of 254 nm detection.

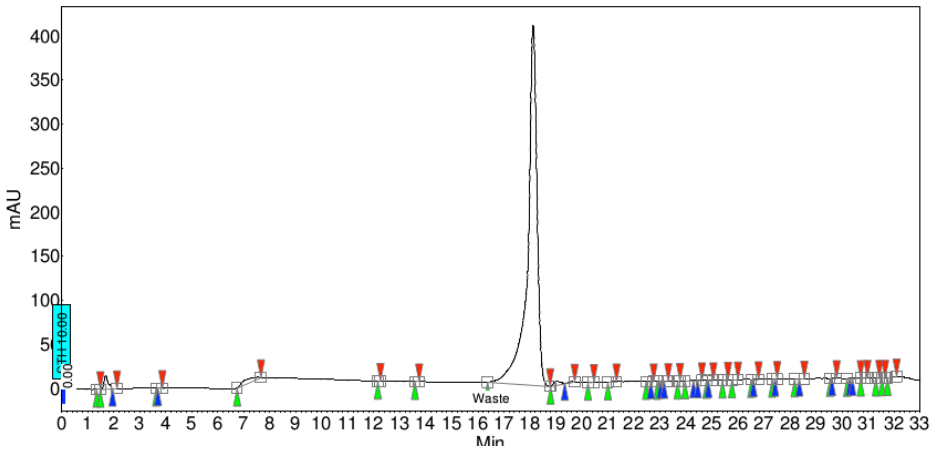
B. Synthesis of Compounds 5 and 8 – 10

N-(3-(2-(4-(2-aminoethoxy)phenylamino)quinazolin-6-yl)-4-methylphenyl)-3-(trifluoromethyl)benzamide,(*1*) 4-(2-chloropyridin-3-yl)pyrimidine,(*2*) (E)-1-(2-chloropyridin-3-yl)-3-(dimethylamino)prop-2-en-1-one,(*2*) and N-(3-amino-4-methylphenyl)-3-(trifluoromethyl)benzamide (*3*) were made as described.

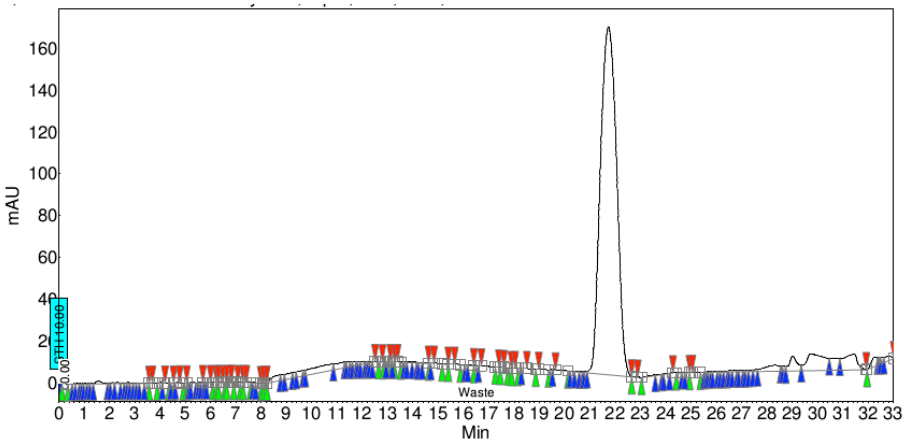


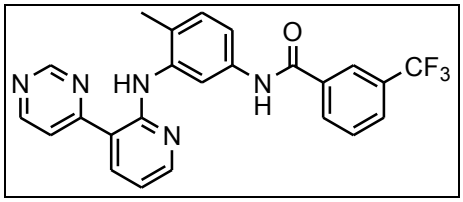
[5] N-(3-(2-(4-(2-aminoethoxy)phenylamino)quinazolin-6-yl)-4-methylphenyl)-3-(trifluoromethyl)benzamide (11.6 mg, 0.02 mmol) was stirred in a mixture of Ac_2O (416 µL) and NEt_3 (11.6 µL) for 2.5 h at room temperature. The reaction mixture was concentrated *in vacuo*, diluted with a mixture of acetonitrile/water and purified by reverse phase chromatography (HPLC) to obtain 10.2 mg of the desired product **5** (82% yield). ^{1}H NMR (300 MHz, Chloroform-d) δ 9.43 (s, 1H), 8.27 (s, 1H), 8.21 (d, *J* = 9.0 Hz, 1H), 8.03 – 7.90 (m, 2H), 7.81 – 7.71 (m, 2H), 7.64 – 7.48 (m, 4H), 7.37 – 7.29 (m, 3H), 7.16 – 7.08 (m, 2H), 5.80 (s, 1H), 4.13 (t, *J* = 6.0 Hz, 2H), 3.60 (t, *J* = 3.0 Hz, 2H), 2.34 – 2.28 (m, 3H), 2.00 - 1.99 (m, 3H). MS *m/z* ($C_{33}H_{28}F_3N_5O_3$) calc'd = 599.21, observed: M+1 = 600.5

Analytical Condition A - CH_3CN/H_2O–0.1% CF_3CO_2H



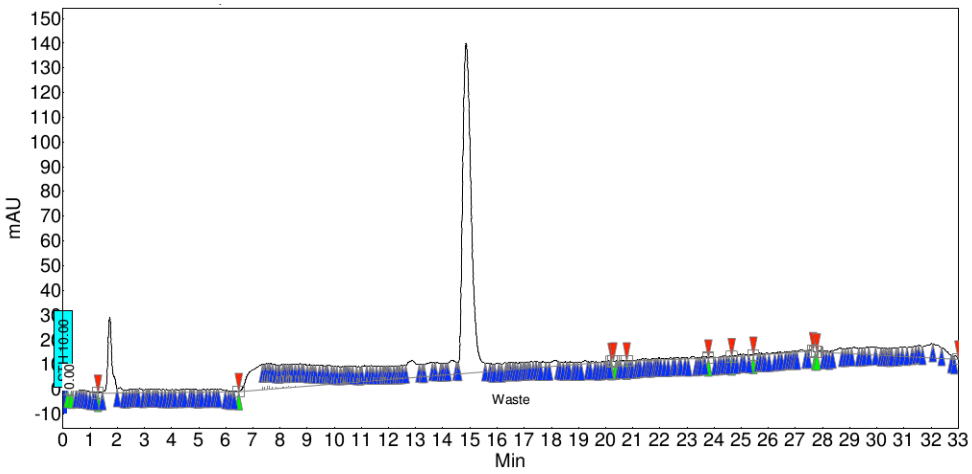
Analytical Condition B - CH_3OH/H_2O–0.1% CF_3CO_2H





[8] 4-(2-chloropyridin-3-yl)pyrimidine (14 mg, 0.073 mmol) and N-(3-amino-4-methylphenyl)-3-(trifluoromethyl)benzamide (50 mg, 0.17 mmol) were dissolved in DMSO (30 µL) and a drop of NEt_3-TFA salt was added to the reaction mixture. The reaction was stirred for 4 d at 95°C. The crude material was purified using reverse phase liquid chromatography to obtain 6.3 mg of the desired product **8** (20% yield). ^{1}H NMR (300 MHz, $CDCl_3$-d_1) δ 9.37 (s, 1H), 9.06 (s, 1H), 9.02 (d, J = 3 Hz, 1H), 8.51 (d, J = 6 Hz, 1H), 8.28 (s, 1H), 8.20-8.16 (m, 2H), 7.95 (d, J = 6 Hz, 1H), 7.80-7.75 (m, 3H), 7.64-7.59 (m, 1H), 7.37 (d, J = 6 Hz, 1H), 7.09-7.04 (m, 1H), 2.37 (s, 3H). MS *m/z* ($C_{24}H_{18}F_3N_5O$) calc'd = 449.15, observed: ($M+H^+$) = 450.5

Analytical Condition A - CH_3CN/H_2O–0.1% CF_3CO_2H



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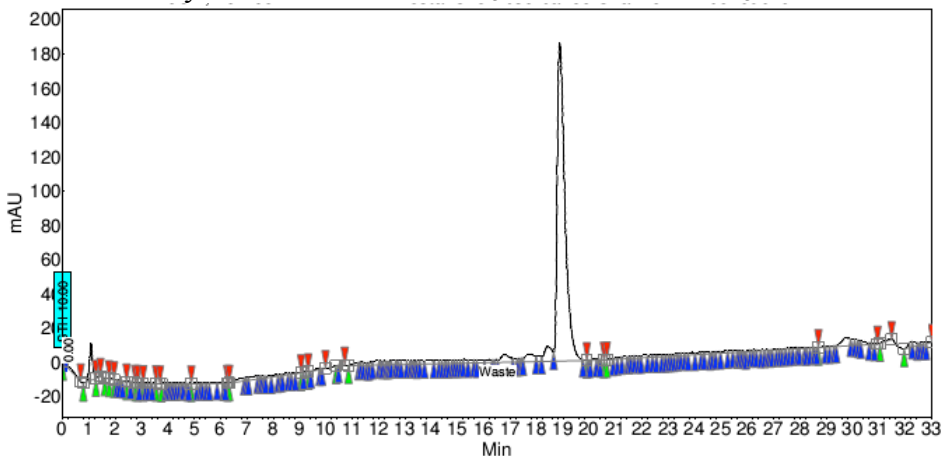
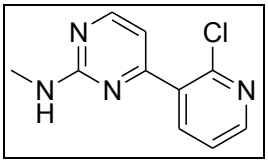
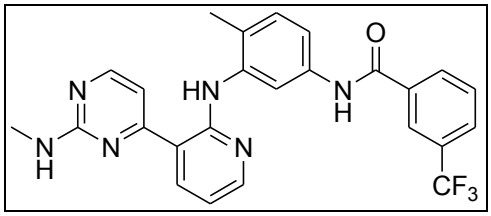


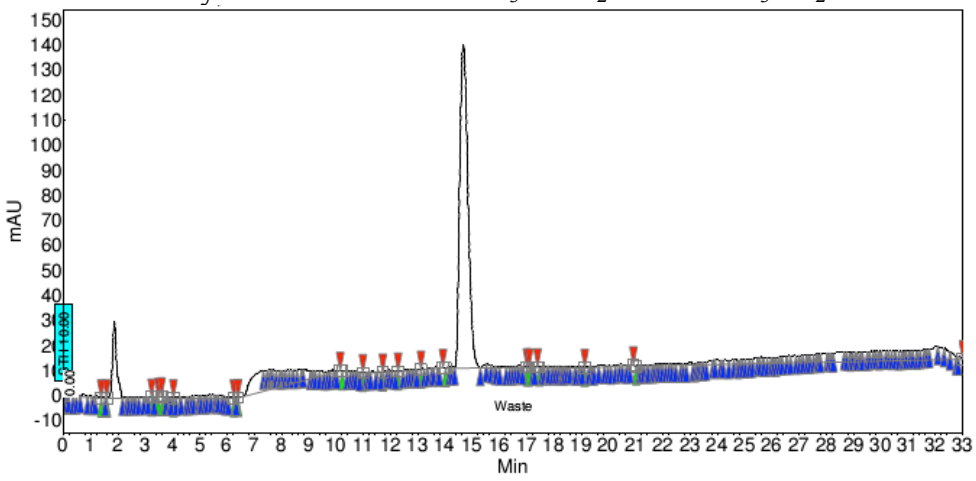


[4-(2-chloropyridin-3-yl)-N-methylpyrimidin-2-amine] N-methylguanidine (126 mg, 1.15 mmol) and sodium methoxide (50 mg, 0.93 mmol) were suspended in methanol (0.2 mL) at room temperature for 30 min. Then (E)-1-(2-chloropyridin-3-yl)-3-(dimethylamino)prop-2-en-1-one (76 mg, 0.36 mmol) in methanol (1 mL) was added to the reaction dropwise. The reaction was refluxed at 50 °C for 23 h. The crude material was purified by column chromatography (50% ethyl acetate in hexanes) to obtain 51 mg of the desired product (64% yield). ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.32 (dd, J = 3 Hz, J = 6 Hz, 1H), 8.23 (d, J = 6 Hz, 1H), 7.84 (broad s, 1H), 7.39-7.35 (m, 1H), 7.10-7.09 (m, 1H), 6.68 (d, J = 6 Hz, 1H), 2.63 (d, J = 6 Hz, 3H).



[9] 4-(2-chloropyridin-3-yl)-N-methylpyrimidin-2-amine (20 mg, 0.09 mmol) and N-(3-amino-4-methylphenyl)-3-(trifluoromethyl)benzamide (63 mg, 0.21 mmol) were dissolved in DMSO (75 µL), and a drop of NEt_3-TFA salt was added to the reaction mixture. The reaction was stirred for 4 d at 95 °C. The crude material was purified using reverse phase liquid chromatography to obtain 16 mg of the desired product **9** (37% yield). ^{1}H NMR (300 MHz, CD_3OD-d_4) δ 8.82 (d, J = 6 Hz, 1H), 8.50 (d, J = 6 Hz, 1H), 8.27 (s, 1H), 8.23 (d, J = 6 Hz, 1H), 7.98-7.91 (m, 3H), 7.78-7.73 (m, 1H), 7.65 (d, J = 9 Hz, 1H), 7.53 (d, J = 6 Hz, 1H), 7.39 (d, J = 6 Hz, 1H), 7.15 (t, J = 9 Hz, 1H), 2.98 (s, 3H), 2.34 (s, 3H). MS m/z ($C_{25}H_{21}F_3N_6O$) calc'd = 478.17, observed: ($M+H^+$) = 479.4

Analytical Condition A - CH_3CN/H_2O–0.1% CF_3CO_2H



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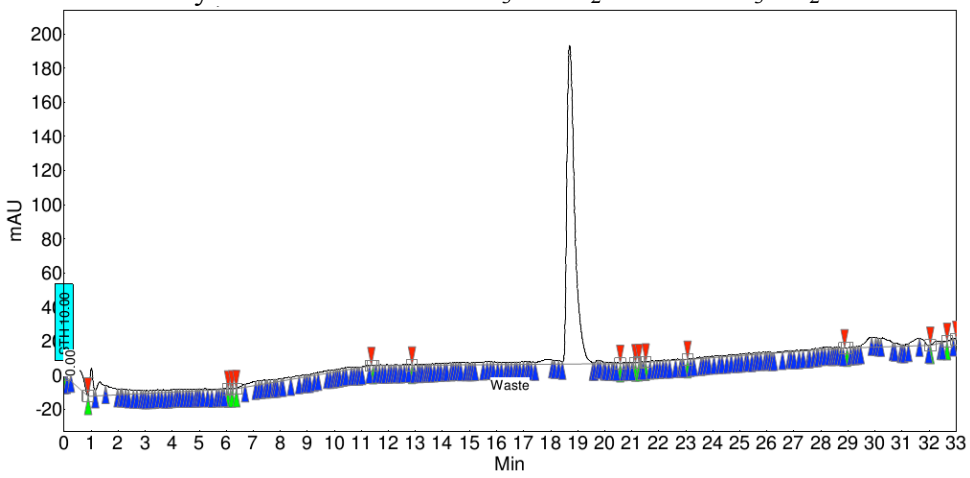
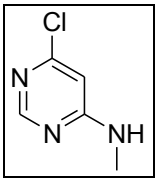
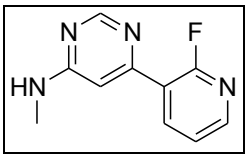
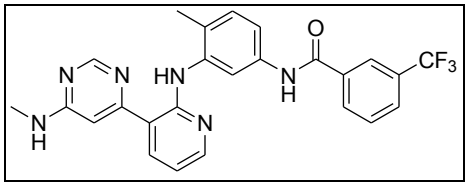




[6-chloro-N-methylpyrimidin-4-amine] 4,6-dichloropyrimide (600 mg, 4 mmol), methylamine-HCl salt (570 mg, 8.4 mmol) and NEt_3 (480 mg, 4.8 mmol) were suspended in 2-propanol (4 mL) and refluxed at 80 °C for 5 h. The reaction was taken up in ethyl acetate, and the organic layer was washed with saturated K_2CO_3. The organic layer was collected, dried over Na_2SO_4 and concentrated *in vacuo*. The crude material was purified by column chromatography (30-50% ethyl acetate in hexanes) to obtain 390 mg of the desired product (68% yield). ^{1}H NMR (300 MHz, $CDCl_3$-d_1) δ 8.37 (s, 1H), 6.37 (S, 1H), 2.98 (d, J = 6 Hz, 3H). MS *m/z* ($C_5H_6ClN_3$) calc'd = 143.03, observed: ($M+H^+$) = 144.0

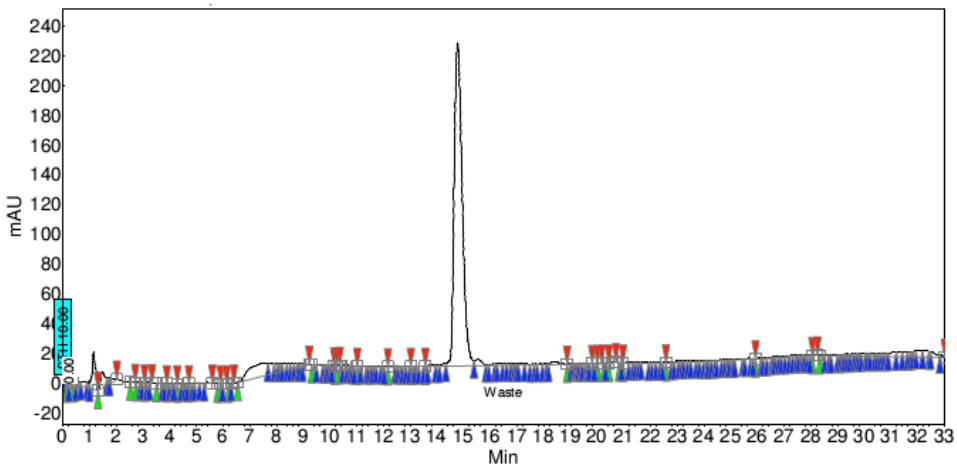


[6-(2-fluoropyridin-3-yl)-N-methylpyrimidin-4-amine] 6-chloro-N-methylpyrimidin-4-amine (170 mg, 1.2 mmol), 2-fluorophenylboronic acid (84 mg, 0.62 mmol), tetrakis(triphenylphosphine)palladium (36 mg, 0.031 mmol) and K_2CO_3 (170 mg, 1.2 mmol) were dissolved in 1:1 acetonitrile:H_2O (1.2 mL) in a round bottom flask. The flask was saturated with N_2 for 10 minutes, and the reaction was refluxed for 2 h at 80 °C. The reaction was then diluted with ethyl acetate, and the organic layer washed with H_2O (3X). The ethyl acetate layer was collected, dried over Na_2SO_4 and concentrated *in vacuo*. The crude material was purified by column chromatography (60-80% ethyl acetate in hexanes) to obtain 52 mg of the desired product (41% yield). ^{1}H NMR (300 MHz, CD_3OD-d_4) δ 8.53 -8.46 (m, 2H), 8.30 (s, 1H), 7.51-7.46 (m, 1H), 6.97 (s, 1H), 2.98 (s, 3H). MS *m/z* ($C_{10}H_9FN_4$) calc'd = 204.08, observed: ($M+H^+$) = 205.1

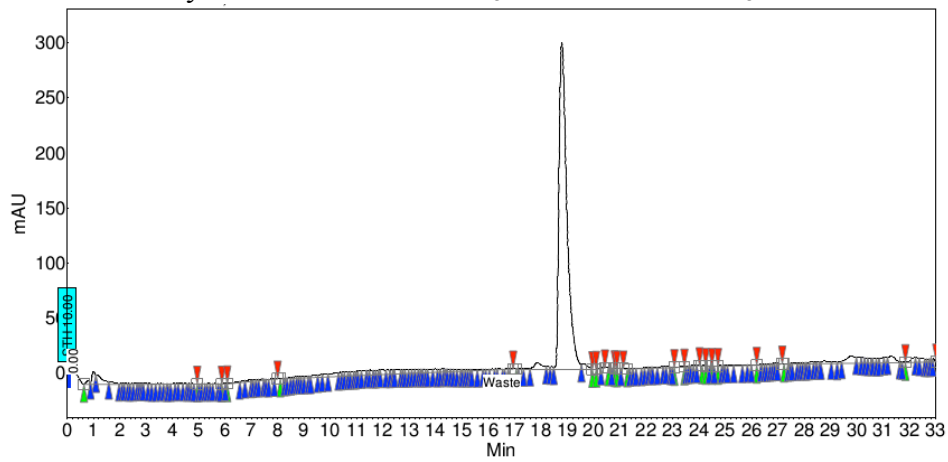


[10] 6-(2-fluoropyridin-3-yl)-N-methylpyrimidin-4-amine (20 mg, 0.099 mmol) and N-(3-amino-4-methylphenyl)-3-(trifluoromethyl)benzamide (70 mg, 0.24 mmol) were dissolved in DMSO (80 µL), and a drop of NEt_3-TFA salt was added to the reaction mixture. The reaction was stirred for 3 days at 95 °C. The crude material was purified using reverse phase liquid chromatography to obtain 7 mg of the desired product **10** (14% yield). ^{1}H NMR (300 MHz, CD_3OD-d_4) δ 8.84 (broad s, 1H), 8.40 (broad s, 1H), 8.27 (s, 1H), 8.22 (d, J = 6 Hz, 1H), 8.03 (m, 1H), 7.94-7.91 (m, 2H), 7.78 -7.73 (m, 1H), 7.65-7.51 (m, 3H), 7.46-7.43 (m, 1H), 7.12-7.06 (m, 2H), 3.05 (s, 3H), 2.34 (s, 3H). MS *m/z* ($C_{25}H_{21}F_3N_6O$) calc'd = 478.17, observed: ($M+H^+$) = 479.3

Analytical Condition A - CH_3CN/H_2O–0.1% CF_3CO_2H



Analytical Condition B - CH_3OH/H_2O–0.1% CF_3CO_2H



IV. Supplemental Methods

A. Activation of Abl Y253H

Abl Y253H (15 µM) was incubated with Hck Y527F (7.4 µM) in buffer (50 mM HEPES [pH 7.5], 67 mM NaCl, 60 mM $MgCl_2$, 1 mM EGTA) with ATP (500 µM) for 6 h at 30 °C and used at 80 pM in activity assays. Due to the high concentration of Hck, a negative control with no Abl was incubated simultaneously and used as the background correction factor in the activity assays. However, since the dilution factor was so large (>5 orders of magnitude) and the substrate used was Abl-specific, the background was no higher than that observed in other assays.

IV. Supplemental References

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3. Seeliger, M. A., Ranjitkar, P., Kasap, C., Shan, Y., Shaw, D. E., Shah, N. P., Kuriyan, J., and Maly, D. J. (2009) Equally potent inhibition of c-Src and Abl by compounds that recognize inactive kinase conformations, *Cancer Res. 69*, 2384-2392.