Supporting information

Optimized Near-IR fluorescent agents for *in vivo* imaging of Btk expression

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Materials and Instrumentation

Materials

All reagents were obtained from commercial sources and used without further purification. Dry THF, MeOH, DCM, and DMF were obtained from Sigma-Aldrich (St. Louis, MO).

^{1}H and ^{13}C NMR spectra were recorded at 23°C on a Bruker 400 MHz spectrometer. Recorded shifts are reported in parts per million (δ) and calibrated using residual undeuterated solvent. Data are represented as follows: Chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, m = multiplet, br = broad), coupling constant (*J*, Hz) and integration. LC-ESI-MS analysis and HPLC-purifications were performed on a Waters (Milford, MA) LC-MS system.

Synthesis and Characterization of CIDs

<u>(*S*,*E*)-4-((4-(3-(4-amino-3-(4-phenoxyphenyl)-1*H*-pyrazolo[3,4-*d*]pyrimidin-1-yl)piperidin-1-yl)-4-oxobut-2-en-1-yl)carbamoyl)-2-(7-(dimethylamino)-3-(dimethyliminio)-5,5-dimethyl-3,5-dihydrodibenzo[*b*,*e*]silin-10-yl)benzoate (compound **13**; Ibrutinib-SiR-COOH)</u>
100 µL of compound **6** solution in DMF (0.1 M), was mixed with HBTU (4 mg, 0.01 mmol), compound **12** (5 mg, 0.01 mmol) and TEA (3 µL, 0.02 mmol). The reaction mixture was stirred at ambient temperature. After 2 hr, the reaction mixture was directly loaded onto a C18 reverse phase column for purification (Water : ACN w/ 0.1% Formic acid = 95 : 5 to 0 : 100) to give compound **13** (3.5 mg, 37.8 % yield) as a deep blue solid. ^{1}H NMR (400 MHz, $(CD_3)_2SO$) δ 9.11-8.94 (m, 1H), 8.32 (s, 0.5H), 8.29-7.95(m, 3H), 7.73 (d, *J* = 13.4 Hz, 1H), 7.66 (d, *J* = 8.1 Hz, 2H), 7.50-7.38 (m, 2H), 7.24-7.08 (m, 5H), 7.03 (s, 2H), 6.71-6.52 (m, 6H), 4.77-4.60 (m, 1H), 4.59-4.45 (m, 0.5H), 4.23 (t, *J* = 14.9 Hz, 1H), 4.12-3.93 (m, 2.5H), 3.66 (t, *J* = 11.7 Hz, 0.5H), 3.28-3.04 (m, 1.5H), 2.91 (s, 12H), 2.32-2.18 (m, 1H), 2.19-2.04 (m, 1H), 1.95-1.80 (m, 1H), 1.67-1.46 (m, 1H), 0.65 (s, 3H), 0.53 (s, 3H).; ^{13}C NMR (101 MHz, $(CD_3)_2)SO$) δ 169.2, 164.8, 164.3, 158.1, 157.1, 156.3, 155.6, 154.7, 153.9, 149.2, 143.3, 141.0, 139.6, 135.9, 130.4, 130.1, 130.0, 128.3, 127.9, 127.6, 125.4, 123.7, 122.9, 121.2, 119.0, 118.9, 116.4, 113.6, 97.4, 91.3, 79.2, 52.9, 52.0, 49.2, 45.7, 45.1, 41.5, 40.5, 29.5, 24.8, 23.4, 0.1, -1.4.; LRMS (ESI) *m*/*z* calcd for $C_{53}H_{53}N_9O_5Si$ $[M-H]^-$ 922.39, found 922.27.

<u>2-(2-((*tert*-butoxycarbonyl)amino)ethoxy)ethyl methanesulfonate (compound **14**)</u>
To a solution of 2-[2-(Boc-amino)ethoxy]ethanol (100 mg, 0.49 mmol) and TEA (102 µL, 0.73 mmol) in DCM (2.5 mL), MsCl (57 µL, 0.73 mmol) was added in DCM (1 mL) dropwise. The reaction mixture was stirred at ambient temperature. After reaction completion, the mixture was diluted with water and organic material was extracted with EA 3 times. The combined organic layer was washed with $NaHCO_3$ (sat), dried over Na_2SO_4 and concentrated in vacuo. Without further purification, crude product was used for the next reaction.

<u>*tert*-butyl (2-(2-(4-nitrophenoxy)ethoxy)ethyl)carbamate (compound **15**)</u>
A solution of compound **14** (2.4 g, 8.5 mmol), potassium carbonate (1.756 g, 12.7 mmol) and 4-nitrophenol (1.414 g, 10.2 mmol) in DMF (25 mL) was stirred at 60°C overnight. The reaction mixture was concentrated in vacuo. The resulting reaction mixture was diluted with EA, washed with water, dried over Na_2SO_4 and concentrated in vacuo. The reaction mixture was then purified with silica gel column chromatography (EA : Hex = 0 :100 to EA only) to give compound **15** (2.6 g, 94.1% yield) as a yellow solid. ^{1}H NMR (400MHz, $CDCL_3$) δ 8.15 - 8.09 (m, 2H), 6.97-6.91 (m, 2H), 4.21-4.11 (m, 2H0, 3.85-3.75 (m, 2H), 3.56 (t, *J* = 5.26 Hz, 2H), 3.28 (t, *J* = 5.26 Hz, 2H), 1.38 (s, 9H).; ^{13}C NMR (101 MHz, $CDCL_3$) δ 163.8, 156.0, 141.6, 125.8, 114.6, 79.4, 70.5, 69.0, 68.1, 40.4, 28.4.; LRMS (ESI) *m*/*z* calcd for $C_{15}H_{22}N_2O_6$ $[M-H+CH_3CO_2H]^-$ 371.37, found 371.21.

<u>*tert*-butyl (2-(2-(4-aminophenoxy)ethoxy)ethyl)carbamate (compound **16**)</u>
A solution of compound **15** (1.0 g, 3.1 mmol) in EtOH was purged with nitrogen for 15 minutes. Pd/C was added to the reaction mixture and purged again with hydrogen for 15 minutes. The reaction mixture was stirred at ambient temperature under hydrogen atmosphere overnight. The next day, Pd/C was removed by filtration through celite pad and concentrated in vacuo. The reaction mixture was purified with a C18 reverse phase column (Water : ACN w/ 0.1% Formic acid = 95 : 5 to 0 : 100) to give compound **16** (894 mg, 98.4 % yield) as a brown solid. ^{1}H NMR (400MHz, $CDCL_3$) δ 6.82 - 6.71 (d, *J* = 8.2 Hz, 2H), 6.69-6.58 (d, *J* = 8.5 Hz, 2H), 5.01 (br s, 1H), 4.11-3.97 (m, 2H), 3.84-3.71 (m, 2H), 3.65-3.53 (m, 2H), 3.46-3.18 (m, 4H), 1.44 (s, 9H).;

^{13}C NMR (101 MHz, $CDCL_3$) δ 156.1, 152.0, 140.3, 116.6, 116.1, 79.3, 70.5, 69.8, 68.3, 40.5, 28.5.; LRMS (ESI) *m*/*z* calcd for $C_{15}H_{24}N_2O_4$ $[M+H]^+$ 297.37, found 297.17.

2-chloro-5-fluoro-*N*-(3-nitrophenyl)pyrimidin-4-amine (compound **17**)

A solution of 2,4-dichloro-5-fluoropyrimidine (1.0 g, 5.99 mmol), 3-nitroaniline (0.40 g, 2.90 mmol) and TEA (404 uL, 2.90 mmol) in n-butanol (10.0 mL) was heated at 120°C. After reaction completion, the mixture was cooled down to room temperature. Solid precipitate was collected by filtration through Buchner funnel and washed with hexane. The resulting solid was purified with a C18 reverse phase column (Water : ACN w/ 0.1% Formic acid = 95 : 5 to 0 : 100) to give compound **17** (425 mg, 54.6% yield) as a yellow solid. ^1H NMR (400MHz, $(CD_3)_2SO$) δ 10.37 (s, 1H), 8.71 (t, *J* = 2.1 Hz, 1H), 8.39 (d, *J* = 3.3 Hz, 1H), 8.14 (q, *J* = 3.2 Hz, 1H), 7.93 (q, *J* = 2.4 Hz, 1H) 7.64 (t, *J* = 8.2 Hz, 1H).; ^{13}C NMR (101 MHz, $(CD_3)_2SO$) δ 152.6, 151.2, 147.8, 146.5, 143.9, 142.4, 142.2, 139.3, 129.9, 126.8, 118.1, 115.1. LRMS (ESI) *m*/*z* calcd for $C_{10}H_6ClFN_4O_2$ $[M+H]^+$ 269.63, found 269.25.

tert-butyl (2-(2-(4-((5-fluoro-4-((3-nitrophenyl)amino)pyrimidin-2-yl)amino)phenoxy)ethoxy)ethyl)carbamate (compound **18**)

A solution of compound **17** (500 mg, 1.86 mmol) and compound **16** (607 mg, 2.05 mmol) and AcOH (10 drop) in EtOH was stirred at 90°C for overnight. The next day, the reaction mixture was directly loaded onto a C18 reverse phase column for purification (Water : ACN w/ 0.1% Formic acid = 95 : 5 to 0 : 100) to give compound **18** (417 mg, 42.4 % yield) as a yellow solid. ^1H NMR (400MHz, $CDCL_3$) δ 8.57 (s, 1H), 8.01-7.91 (m, 2H), 7.92-7.85 (m, 1H), 7.48 (t, *J* = 8.1 Hz, 1H), 7.42-7.33 (m, 2H), 6.89 (d, *J* = 8.1 Hz, 2H), 5.03 (br s, 1H), 4.15-4.06 (m, 2H), 3.86-3.77 (m, 2H), 3.62 (t, *J* = 5.2 Hz, 2H), 3.35 (t, *J* = 5.4 Hz, 2H), 1.44 (s, 9H).; ^{13}C NMR (101 MHz, $CDCL_3$) δ 156.2, 156.1, 155.7, 155.3, 155.2, 150.0, 149.9, 148.8, 141.7, 140.0, 139.3, 139.1, 139.0, 132.3, 129.8, 126.3, 126.2, 122.7, 122.6, 118.6, 118.5, 115.5, 115.4, 115.2, 79.4, 70.5, 69.7, 67.9, 29.8, 28.6.; LRMS (ESI) *m*/*z* calcd for $C_{25}H_{29}FN_6O_6$ $[M+H]^+$ 529.54, found 529.38.

tert-butyl (2-(2-(4-((4-((3-aminophenyl)amino)-5-fluoropyrimidin-2-yl)amino)phenoxy)ethoxy)ethyl)carbamate (compound **19**)

A solution of compound **18** (100 mg, 0.19 mmol) in EtOH was purged with nitrogen for 15 minutes. Pd/C was added to the reaction mixture and purged again with hydrogen for 15 minutes. The reaction mixture was stirred at ambient temperature under hydrogen atmosphere overnight. The next day, Pd/C was removed by filtration through a celite pad and concentrated in vacuo. The reaction mixture was purified with a C18 reverse phase column (Water : ACN w/ 0.1% Formic acid = 95 : 5 to 0 : 100) to give compound **19** (90 mg, 95.4 % yield) as a pale yellow oil. ^1H NMR (400MHz, $CDCL_3$) δ 7.91 (d, *J* = 3.3 Hz, 1H), 7.48-7.38 (m, 2H), 7.09 (t, *J* = 8.0 Hz, 1H), 6.97-6.87 (m, 2H), 6.83 (s, 1H), 6.77-6.70 (m, 1H), 6.67 (d, *J* = 3.5 Hz, 1H), 5.00 (br s, 1H), 4.16-4.07 (m, 2H), 3.85-3.77 (m, 2H), 3.61 (t, *J* = 5.2 Hz, 2H), 3.35 (q, *J* = 5.5 Hz, 2H), 1.45 (s, 9H).; ^{13}C NMR (101 MHz, $CDCL_3$) δ 156.3, 156.1, 154.6, 150.1, 150.0, 147.4, 142.1, 140.3, 140.1, 139.6, 139.1, 133.5, 129.7, 122.5, 114.9, 110.5, 110.4, 107.3, 79.4, 70.5, 69.6, 67.8, 29.8, 28.5.; LRMS (ESI) *m*/*z* calcd for $C_{25}H_{31}FN_6O_4$ $[M+H]^+$ 499.56, found 499.13.

tert-butyl (2-(2-(4-((4-((3-acrylamidophenyl)amino)-5-fluoropyrimidin-2-yl)amino)phenoxy)ethoxy)ethyl)carbamate (compound **20**)

To a solution of compound **19** (20 mg, 0.04 mmol) and TEA (6.7 µL, 0.05 mmol) in THF (0.5 mL) acryloyl chloride was added(3.9 µL, 0.05 mmol). After reaction completion, the mixture was diluted with water and organic material was extracted with EA 3 times. The combined organic layer was dried over Na_2SO_4 and concentrated in vacuo. The reaction mixture was purified with a C18 reverse phase column (Water : ACN w/ 0.1% Formic acid = 95 : 5 to 0 : 100) to give compound **20** (15 mg, 82.6 % yield) as a white solid. Without further characterization, crude product was used for the next reaction.

N-(3-((2-((4-(2-(2-aminoethoxy)ethoxy)phenyl)amino)-5-fluoropyrimidin-4-yl)amino)phenyl)acrylamide (compound **21**)

A solution of compound **20** (10 mg, 0.05 mmol) in DCM and TFA (v:v = 3:1) was stirred at ambient temperature. After 30 minute of stirring, the reaction mixture was concentrated in vacuo. After azeotroph with DCM and ACN three times, the crude product was concentrated in vacuo and then diluted with DMF to make a 0.1 M solution. Without further purification, the crude product was used for next reaction.

General Method for preparation of CID.

The amine version of the drug (compound **6** and compound **21**) in anhydrous DMF was mixed with triethylamine and the succinimidyl ester of the appropriate fluorophore. For Silicon rhodamine-COOH and Silicon rhodamine-Me, the fluorophore was added and activated with HBTU and TEA. The resulting solution was stirred at ambient temperature. After reaction completion, the crude reaction mixture was purified by preparative reverse phase HPLC. The identity and purity of the conjugates were confirmed by electrospray mass spectrometry and analytical HPLC. For LC-ESI-MS analyses, a Waters XTerra® C18, 5 µm, 125Å, 4.6 mm x 50 mm column (product #: 186000482) was used. (Total 2 min run with eluents 0.1% TFA (v/v) in water and MeCN; flow rate: 5 mL/min; gradient: 0-1.5 min, 5-100% MeCN; 1.5-1.99 min 100% MeCN; 1.99-2.00 min 100-5% MeCN). For preparative runs, an Atlantis® Prep T3 OBD™, 5 µm, 100Å, 19 mm x 50 mm column (product #: 186003696) was used (Total 10 min run with eluents 0.1% TFA (v/v) in water and MeCN; flow rate: 15 mL/min; gradient: 0-1.25 min, 5% MeCN; 1.25-9.00 min 5-100% MeCN; 9.00-9.98 min 100% MeCN; 9.98-9.99 min 100-5% MeCN; 9.99-10.00 min 5% MeCN).

Imaging drugs	Formula	Calcd. mass	Retention time (analysis mode)	Found Mass
Ibrutinib-Si-COOH	$C_{53}H_{53}N_9O_5Si$	$[M+H]^+$ 925.40	1.17 min	925.39
Ibrutinib-Si-Me	$C_{53}H_{56}N_9O_3Si^+$	$[M]^+$ 894.43	1.06 min	894.43
Ibrutinib-B650	$C_{54}H_{52}BF_2N_{11}O_5$	$[M+H]^+$ 998.44	1.21 min	998.44
Ibrutinib-Rhx	$C_{47}H_{39}N_9O_6$	$[M+H]^+$ 826.30	0.83 min	826.31
Ibrutinib-FITC	$C_{47}H_{36}N_8O_7S^{2-}$	$[M+H]^+$ 858.93	0.95 min	859.27
AVL-292-Si-COOH	$C_{50}H_{51}FN_8O_6Si$	$[M+H]^+$ 907.37	0.91 min	907.39
AVL-292-BFL	$C_{37}H_{38}BF_3N_8O_4$	$[M+H]^+$ 727.31	0.85 min	727.31

Biological Works

Cell Lines

The diffuse large B-cell lymphoma (DLBCL) cell lines DB and Toledo were generously provided by Dr. Anthony Letai (Dana Farber Cancer Institute, Boston, MA). The RC-K8 DLBCL cell line was a generous gift from Dr. Thomas Gilmore (Boston University, Boston, MA, USA). The Daudi Burkitt's lymphoma and Jurkat T-cell leukemia cell lines were from ATCC (Manassas, VA, USA). Lymphoma cell lines were cultured in RPMI 1640 media (supplemented with 10% fetal bovine serum, penicillin/streptomycin, and L-glutamine) at 37°C and 5% CO_2. To test the Btk inhibitor in adherent cells, we used HT1080 human fibrosarcoma cells, which have previously been shown to be ideal for intravital imaging studies[1]. HT1080 cells were from ATCC, grown in DMEM supplemented with 10% fetal bovine serum and 2% glutamine-penicillin-streptomycin at 37°C and 5% CO_2. HT1080-Btk-mCherry cells were prepared by viral transduction. Virus generated from pMSCVpuro-mCherry-Btk retroviral vector[2] was a generous gift from Dr. Hidde Ploegh (Massachusetts Institute of Technology, Cambridge, MA, USA). Viral supernatant was added directly to HT1080 cells for 48 hrs and Btk-mCherry-expressing cells were selected with RPMI media containing 2 ug/mL puromycin for 96 hrs. Following selection, HT1080-Btk-mCherry cells were cultured under the same conditions as the untransduced HT1080 cells.

Kinase Inhibition Assay

The Z'-LYTE Kinase Assay Kit - Tyrosine 1 Peptide (Invitrogen, Grand Island, NY, USA) was used according to the manufacturer's instructions and tested against purified BTK enzyme (Promega, Madison, WI, USA) and ATP. The assay was then performed in the presence of inhibitors Ibrutinib, Ibrutinib-SiR-COOH and AVL-292-SiR-COOH using 1 µg/mL BTK and 50 µM ATP, without pre-incubation of inhibitors with the enzyme. Concentrations of compounds were 4-fold serial dilutions ranging from 8000 nM to 0.031 nM. Negative control samples did not contain ATP, and positive controls contained the pre- phosphorylated peptide included in the assay kit. Kinase reaction buffer contained 0.67% DMSO. Experiments were performed in quadruplicate in 384-well plates. Fluorescence resulting from peptide phosphorylation was measured on a TECAN plate reader system (Zurich, Switzerland). Background fluorescence from the fluorescent probe was subtracted from the final measurements. Prism (GraphPad Software, La Jolla, CA, USA) was used to plot dose-response curves and to calculate IC_{50} values.

Live cell fluorescence microscopy of different CID

HT1080-Btk-mCherry cells were seeded into a 96-well plate at 5,000 cells per well and incubated for 48 hrs. Cells were incubated in growth media containing 500 nM of CID in 0.05% DMSO at 37°C for 2.5 hours. Cells were then washed three times with media for 5 minutes. Live cells were subsequently imaged on a DeltaVision imaging system (Applied Precision, GE Healthcare). Images were processed using Fiji software, an open-source version of ImageJ. For the fluorescence microscopy imaging experiment with Toledo and Jurkat cells, cells were incubated in growth media containing 500 nM of CID in 0.05 % DMSO at 37°C for 3 hrs. Cells were then washed with growth media for 3 hours. Cells were stained with Hoechst33342 (1 µg/mL) and with DiI (0.5 µg/mL). Live cells were subsequently imaged on DeltaVision.

Flow Cytometry

Jurkat and Toledo cells were incubated in growth media containing 200 nM of Ibrutinib-SiR-COOH at 37°C for 3 hrs. Cells were centrifuged and resuspended in growth media at time-points of 1, 2, and 3 hrs. After 3 hrs, $5x10^5$ cells were harvested for analysis by flow cytometry.

Cells collected for flow cytometry were centrifuged and resuspended in 200 µL PBS containing 0.5% BSA, and filtered through 35 µM Cell Strainer tubes (BD). Flow cytometry in the APC channel was performed on an LSRII flow cytometer (BD) and subsequent data analysis was done using FlowJo software (TreeStar, Ashland, OR, USA).

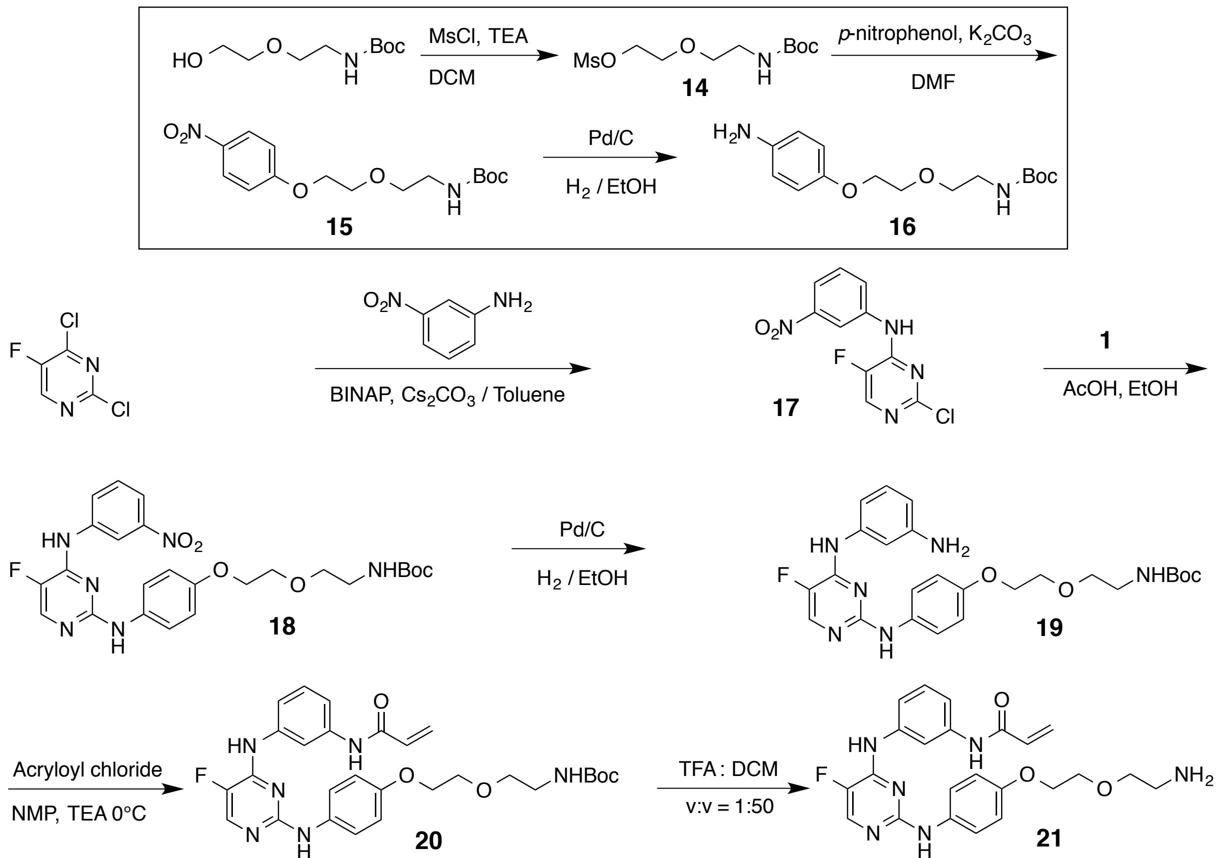
Gel Electrophoresis

To test the covalent binding of the CID to Btk, 0.1 ng (1 µL) of purified Btk was combined with 0.4 µL Ibrutinib-SiR-COOH (prepared in advance in 3-fold dilutions ranging from 10 µM to 0.5 nM, 33% DMSO in PBS) and 18.6 µL PBS, and incubated in the dark at room temperature for one hour. In the second experiment, the selectivity of the CID was examined in live cells. Toledo and Jurkat cells ($2.2x10^6$ cells per well in culture media) were incubated in growth media containing 5-fold dilutions of Ibrutinib-SiR-COOH ranging from 500 nM to 0.5 nM in 0.05 % DMSO at 37°C for three hours. In the third experiment, Toledo cells ($2.2x10^6$ cells per well in culture media) were incubated in growth media containing 5-fold dilutions of Ibrutinib ranging from 500 nM to 0.5 nM in 0.05 % DMSO at 37°C for one hour, then incubated again in growth media containing 500 nM of Ibrutinib-SiR-COOH in 0.05 % DMSO at 37°C for three hours. Cells were then washed once with ice cold PBS and lysed in 150 µL 1X RIPA buffer (Cell Signaling Technology, Beverly, MA, USA). To the purified enzyme samples or cell lysates, NuPAGE LDS sample buffer and NuPAGE reducing agent (Invitrogen) were added to a final concentration of 25% and 10%, respectively. Samples were heated to 70°C for 10-12 minutes in a Mastercycler thermal cycler (Eppendorf, Hamburg, Germany). 20 µL per lane was loaded onto a 12-well NuPAGE Novex 4-12% Bis-Tris gels (Invitrogen). Using 5 µL of Novex Sharp Pre-stained protein standard (Invitrogen) as a size marker, the gels were run in NuPAGE MES SDS running buffer at 200 V for 35 minutes in the XCell SureLock Mini-Electrophoresis system (Invitrogen). The gels were removed from the cassette and imaged using a Typhoon 9410 fluorescence scanner (GE Healthcare, Pittsburgh, PA, USA) using 630 nm excitation and a 670 nm emission filter. To show total protein loading, gels were silver-stained using the Pierce Silver Stain for Mass Spectrometry kit (Thermo Fisher Scientific, Rockford, IL, USA).

In vivo tumor imaging

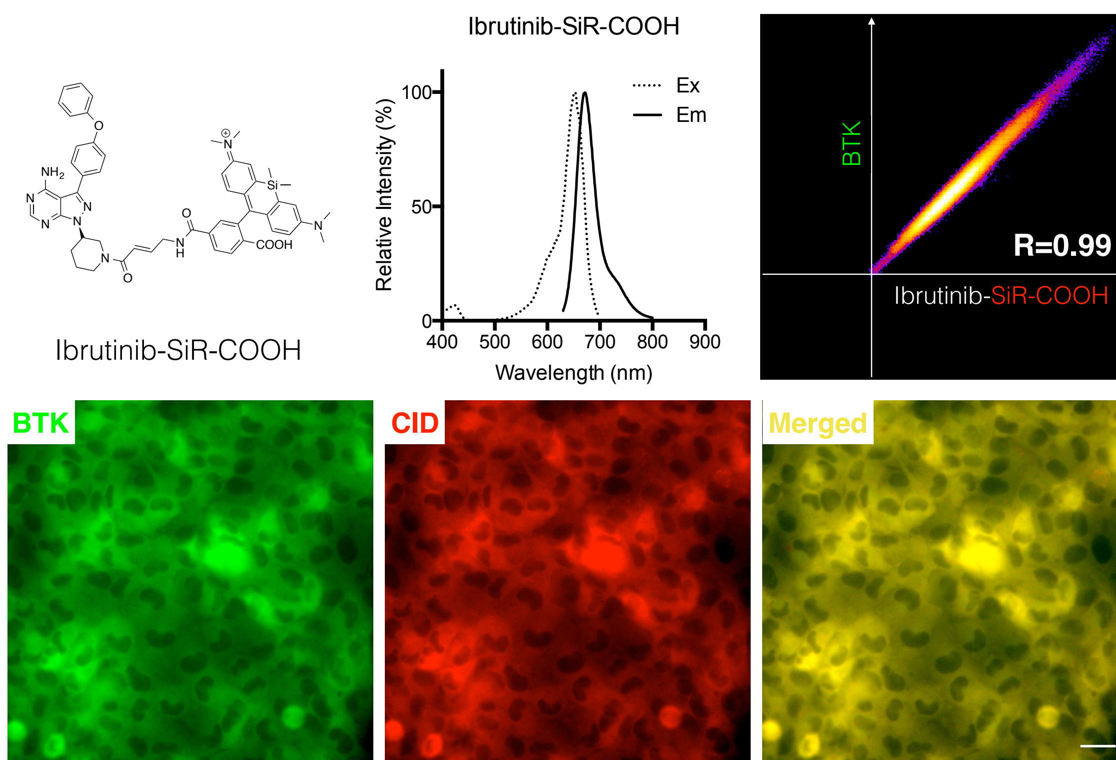
Nu/nu mice were implanted with $1x10^6$ HT1080-Btk-mCherry cells or mixed $1x10^6$ HT1080-Btk-mCherry/HT1080-H2B-GFP cells into a dorsal skinfold window chamber (APJ Trading Company, Ventura, CA, USA) according to established protocols[3,4] and according with guidelines from the Institutional Subcommittee on Research Animal Care. Tumors were allowed to grow and vascularize for 2 weeks. Mice were anesthetized with 2% isoflurane in 2 L/min oxygen. 75 nmol of CID in 150 µL solution containing DMAc and solutol (30 µL) in PBS (112.5 µL) was then injected via the tail vein. Time-lapse microscopy was performed for four hours using a customized Olympus FV1000 confocal/multiphoton microscope equipped with a 20x objective (both Olympus America, Chelmsford, MA, USA). Tumors were imaged before injection and then for 4 continuous hours immediately after injection and again 24 hrs post-injection. Images were processed using Fiji.

Supporting Figures.



Scheme S1. Synthesis of AVL-292-SiR-COOH.

a.



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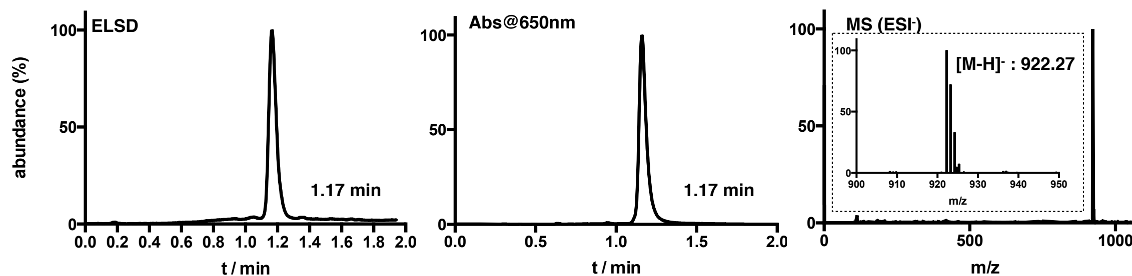


Figure S1. a. Ibrutinib-SiR-COOH chemical structure, photo physical properties and co-localization with Btk Scale bar: 20 µm. b. Purity of Ibrutinib-SiR-COOH was confirmed by Evaporation Light Scattering Detector (ELSD), photodiode array detector (at 650 nm) and mass spectrometry with high performance liquid chromatography (HPLC) chromatogram.

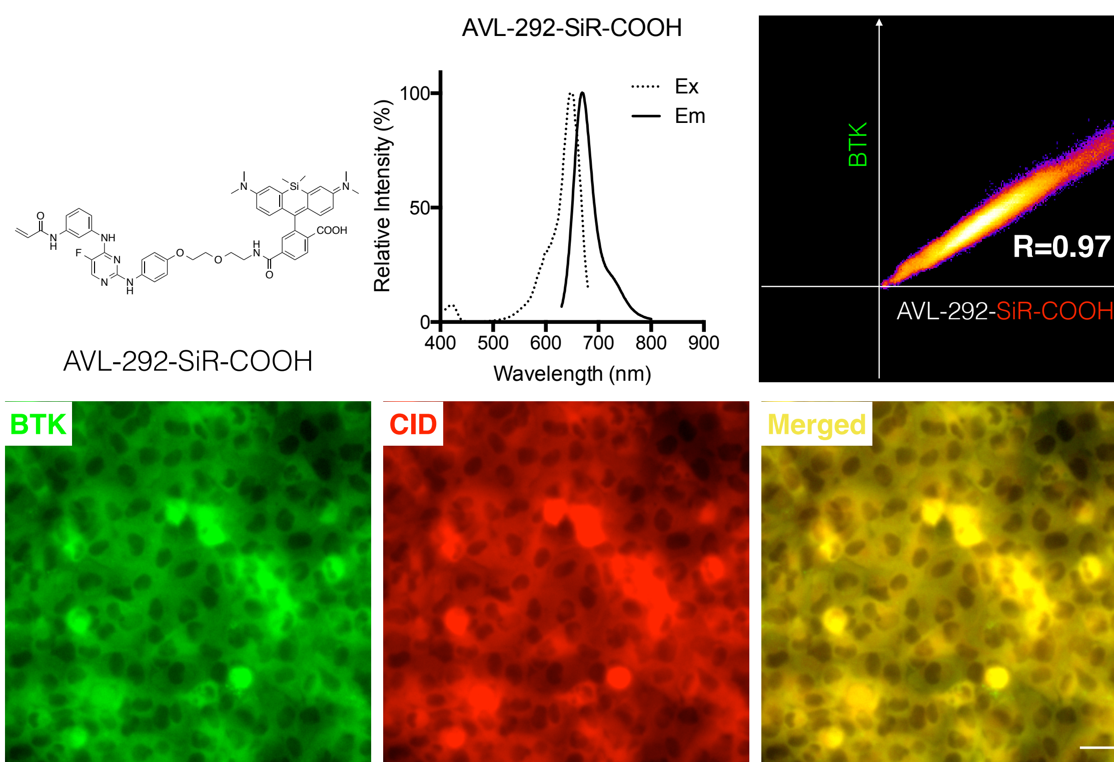
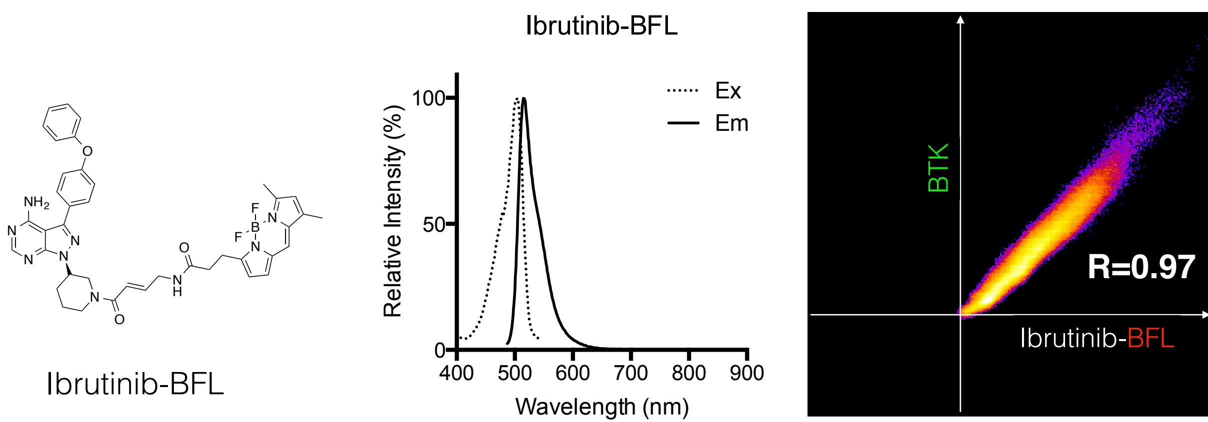


Figure S2. AVL-292-SiR-COOH chemical structure, photo physical properties and colocalization with Btk. Scale bar: 20 µm.



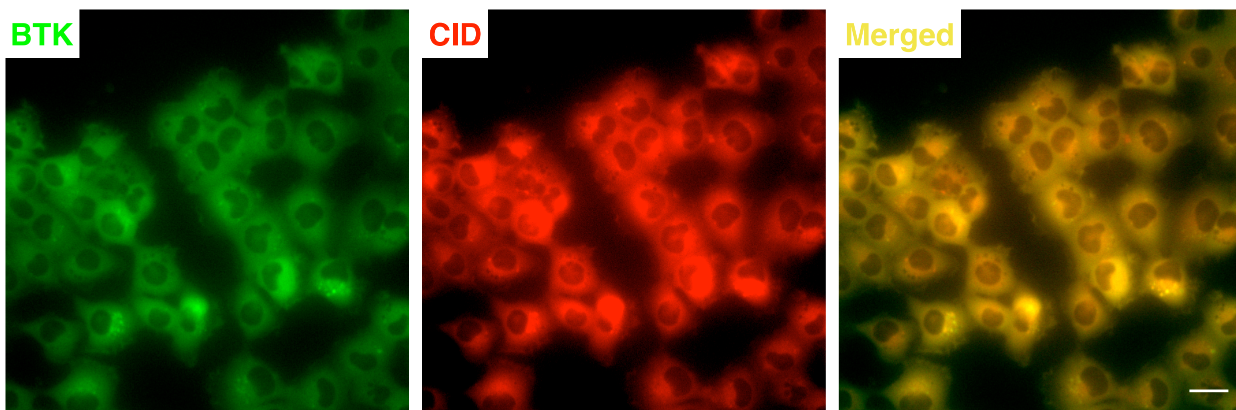


Figure S3. Ibrutinib-BFL chemical Structure, photo physical properties and colocalization with Btk. Scale bar: 20 μm.

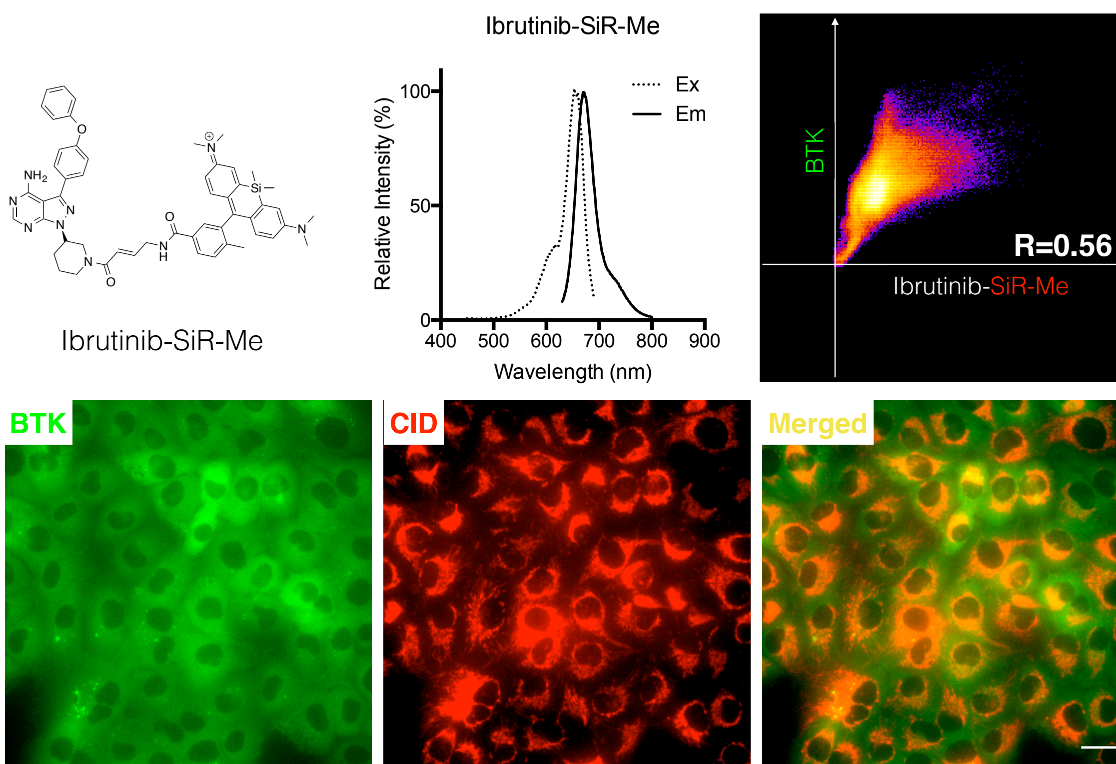


Figure S4. Ibrutinib-SiR-Me chemical Structure, photo physical properties and colocalization with Btk. Scale bar: 20 µm.

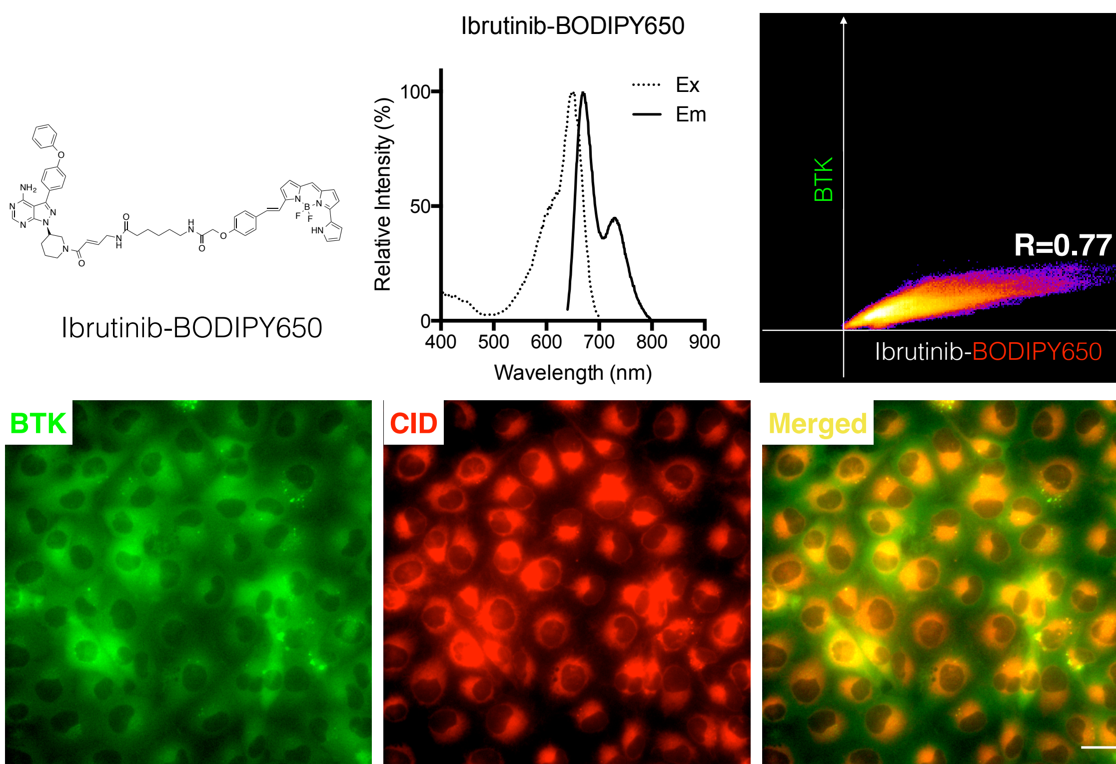


Figure S5. Ibrutinib-BODIPY650 chemical structure, photo physical properties and colocalization with Btk. Scale bar: 20 μm.

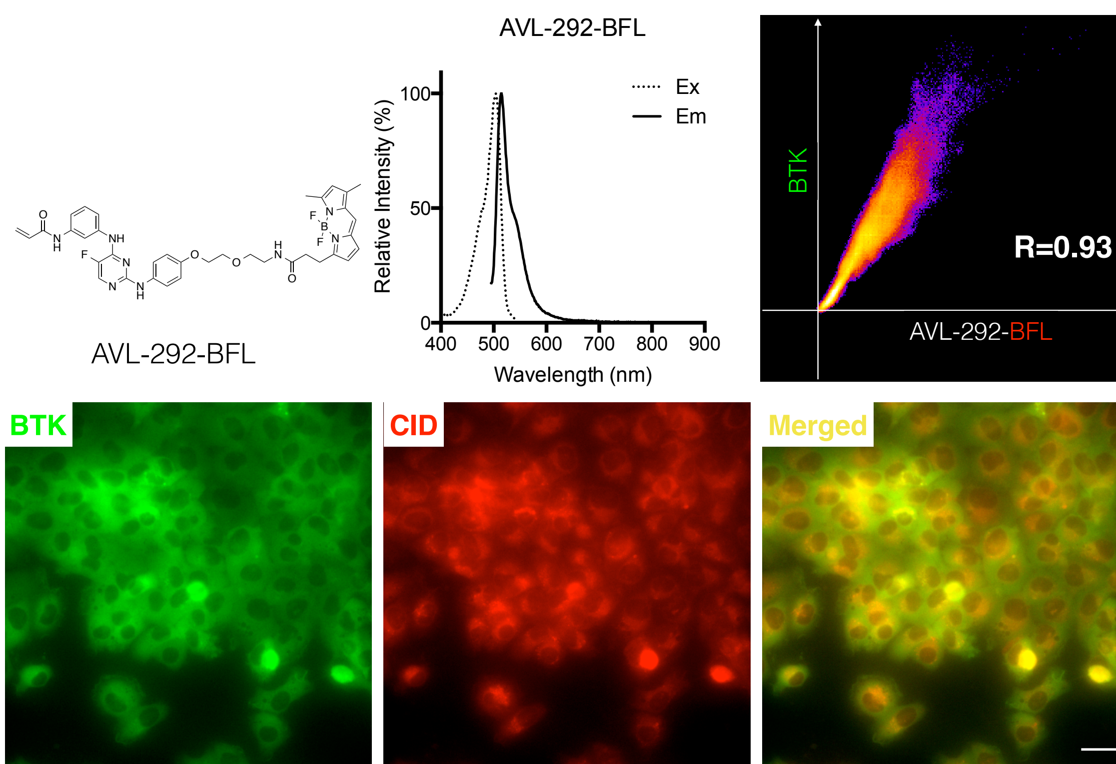


Figure S6. AVL-292-BFL chemical structure, photo physical properties and colocalization with Btk. Scale bar: 20 μm.

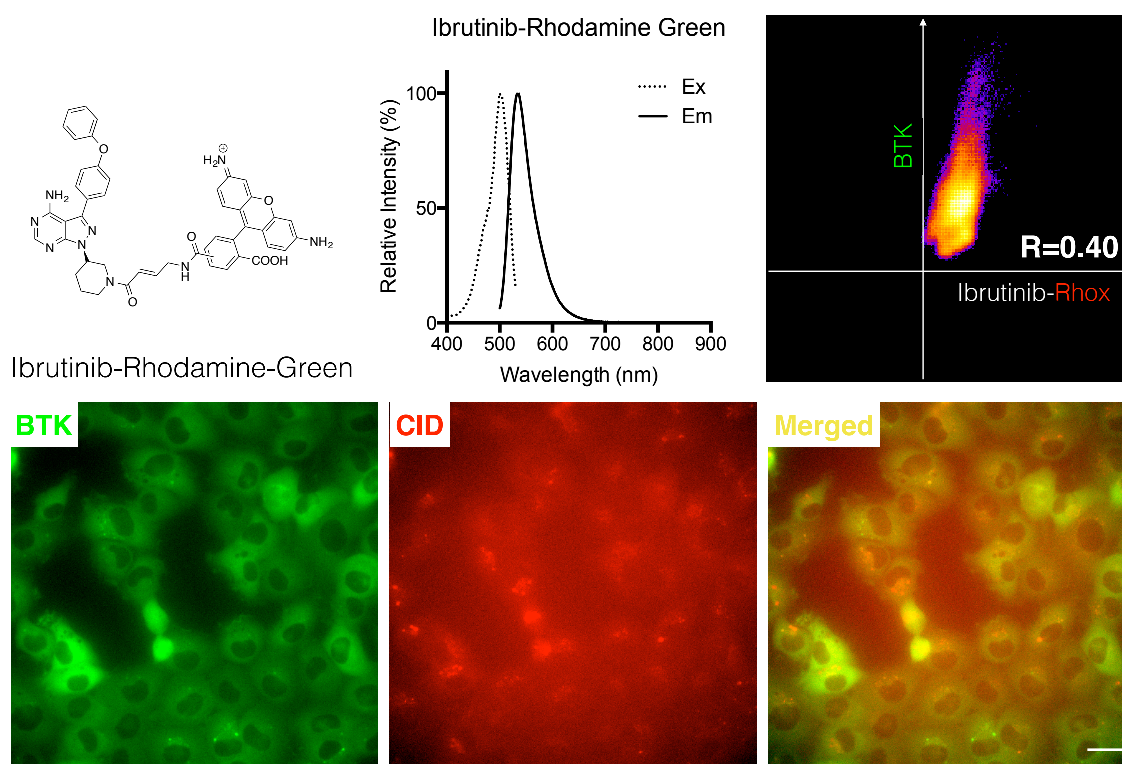


Figure S7. Ibrutinib-Rhodamine-Green chemical structure, photo physical properties and colocalization with Btk. Scale bar: 20 µm.

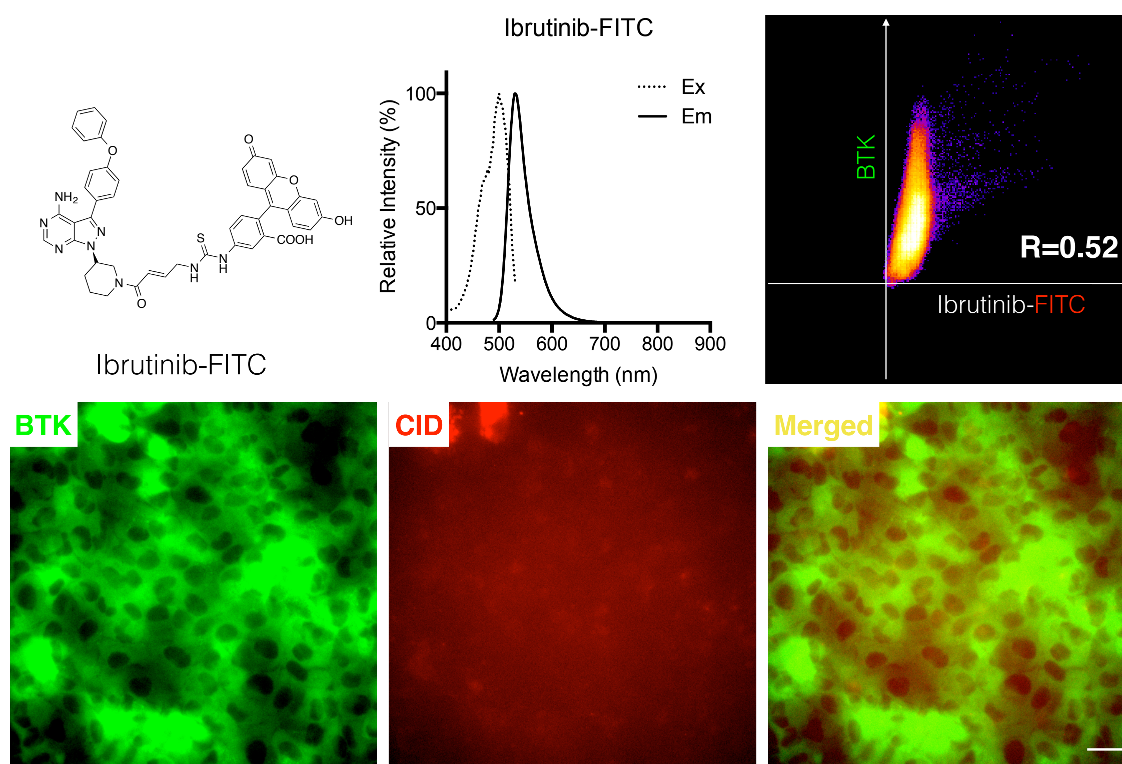
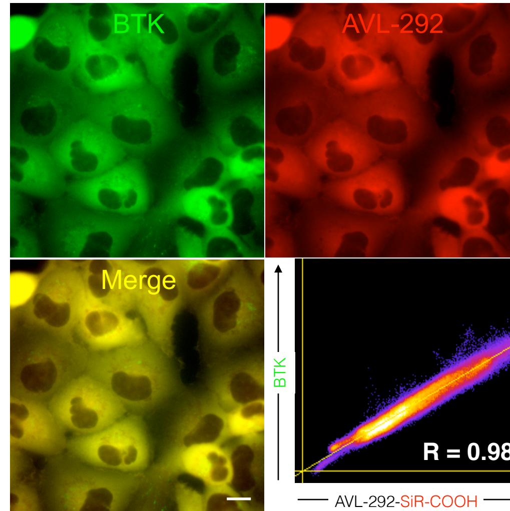


Figure S8. Ibrutinib-FITC chemical structure, photo physical properties and colocalization with Btk. Scale bar: 20 μm.

a.



b.

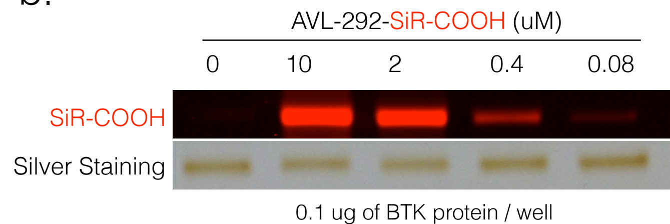
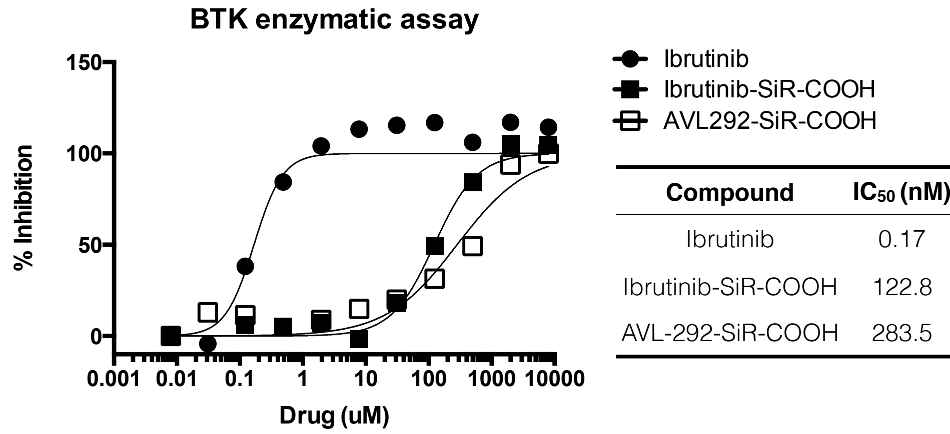


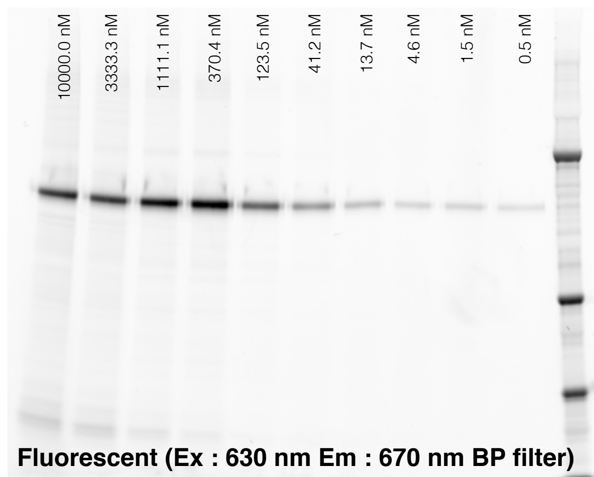
Figure S9. Covalent and selective binding of AVL-292-SiR-COOH for Btk. a. Imaging of adherent Btk-mCherry cells to determine co-localization with AVL-292-SiR-COOH. Co-localization between 500 nM AVL-292-SiR-COOH (red) and Btk-mCherry in stably transduced HT1080 cells (green), following a 3-hour incubation with AVL-292-SiR-COOH. Note the exquisite co-localization (Pearson's correlation coefficient is 0.98). Scale bar: 20 μm. b. Target binding. Denaturing gel electrophoresis of decreasing concentrations of AVL-292-COOH incubated with 0.1 ng purified Btk for one hour and imaged with a fluorescence gel scanner using 630 nm excitation / 670 nm emission. Note the dose dependent covalent binding of AVL-292-SiR-COOH.



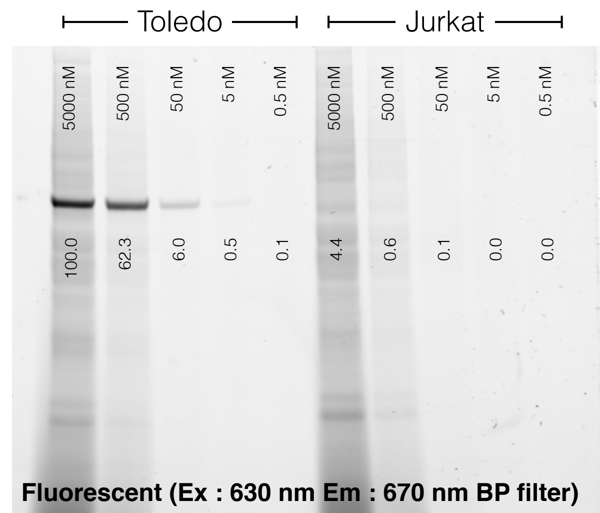
Compound	IC_{50} (nM)
Ibrutinib	0.17
Ibrutinib-SiR-COOH	122.8
AVL-292-SiR-COOH	283.5

Figure S10. Enzymatic assay of Ibrutinib and Ibrutinib fluorescent conjugates (Ibrutinib-SiR-COOH, and AVL-SiR-COOH) against purified BTK protein.

a.



b.



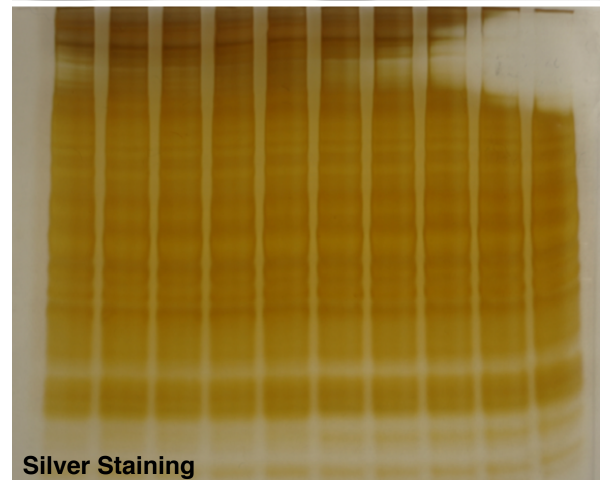


Figure S11. Covalent and selective binding of Ibrtuinib-SiR-COOH to Btk. a. Covalent target binding. Denaturing gel electrophoresis of decreasing concentrations of Ibrutinib-SiR-COOH incubated with 0.1 ng purified Btk for one hour and then imaged with 630 nm excitation / 670 nm emission fluorescence gel scanning. Note the dose dependent binding of Ibrutinib-SiR-COOH. Protein loading level was confirmed with silver staining. Molecular weight marker in the far right lane. b. Selectivity of Ibruinib-SiR-COOH for Btk protein in live cells. Denaturing gel electrophoresis of cell lysates following incubation of decreasing concentrations of Ibrutinib-SiR-COOH with Toledo (Btk+, left half of gel) or Jurkat (Btk-, right half of gel) cells at 37°C degrees for three hours. Total protein loading was confirmed with silver staining. Note the specificity of the probe.

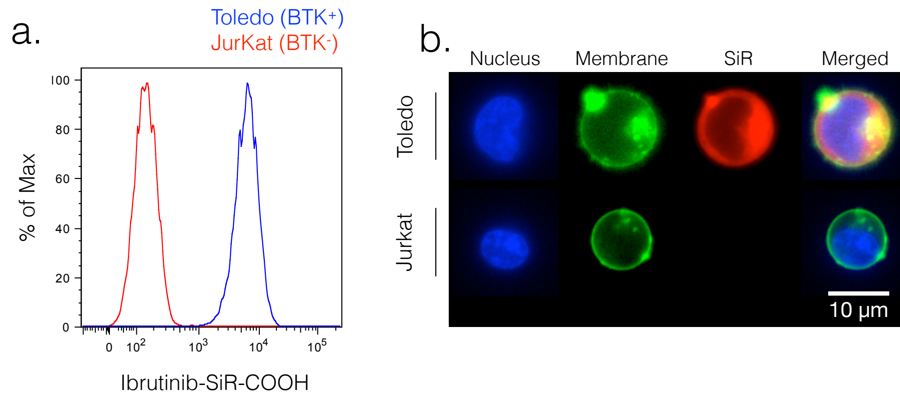


Figure S12. Flow cytometry analysis and fluorescence imaging of live Toledo and Jurkat cells using Ibrutinib-SiR-COOH. a. flow cytometry data of Toledo (BTK+) and Jurkat (BTK-) cells. b. fluorescence imaging of live Toledo and Jurkat cells using Ibrutinib-SiR-COOH (500 nM). Nucleus of the cells were stained with Hoechst 33342 and membrane was stained with DiI dye. scale bar: 10 μm.

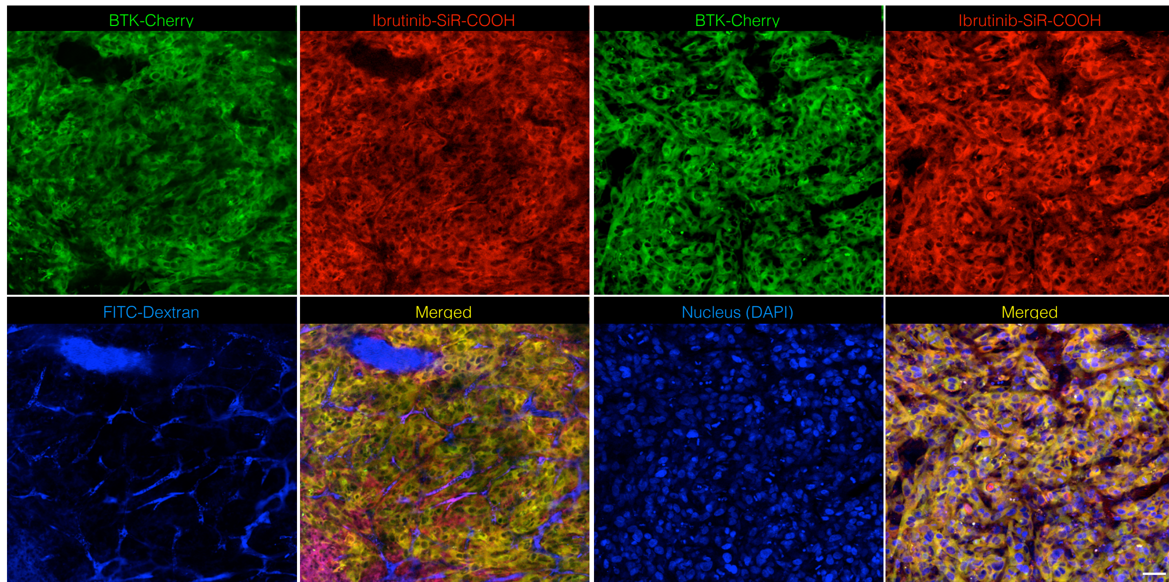


Figure S13. *In vivo* imaging of Btk co-localization with Ibrutinib-SiR-COOH. 75 nmol Ibrutinib-SiR-COOH was injected into the tail vein of nu/nu mice and serial imaging was performed in the window chamber. Imaging was performed using a customized Olympus FV1000 confocal/multiphoton microscope equipped with a 20x objective (both Olympus America, Chelmsford, MA, USA). Scale bar: 50 μm

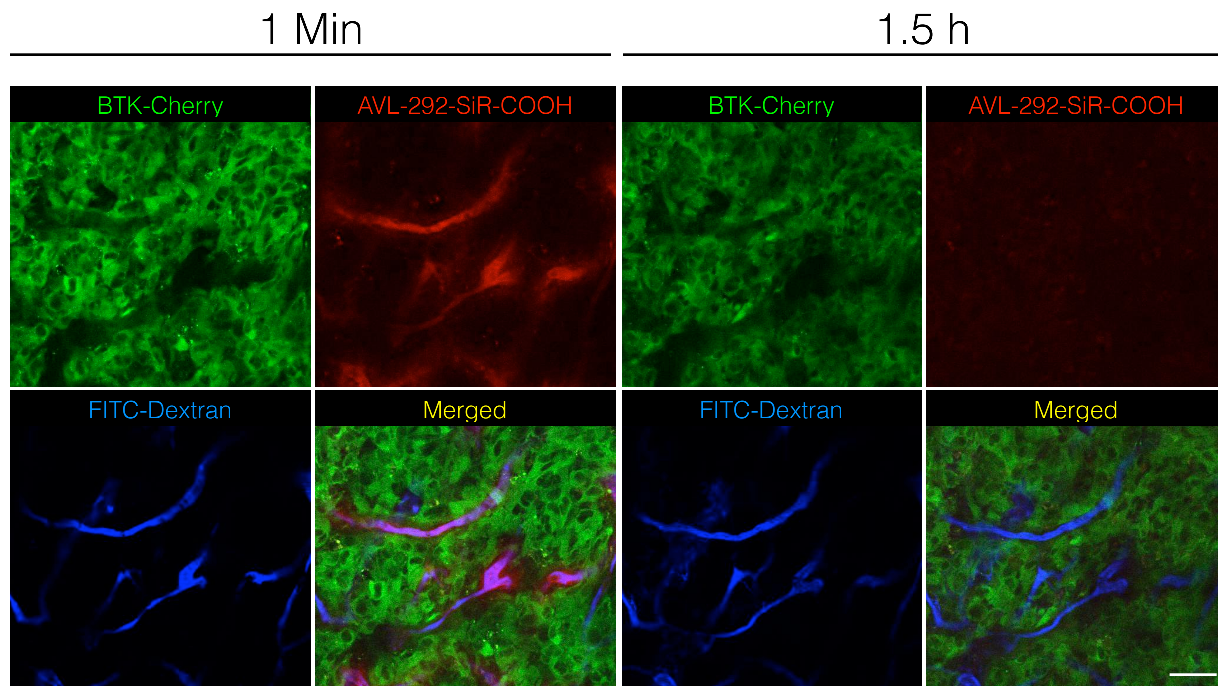
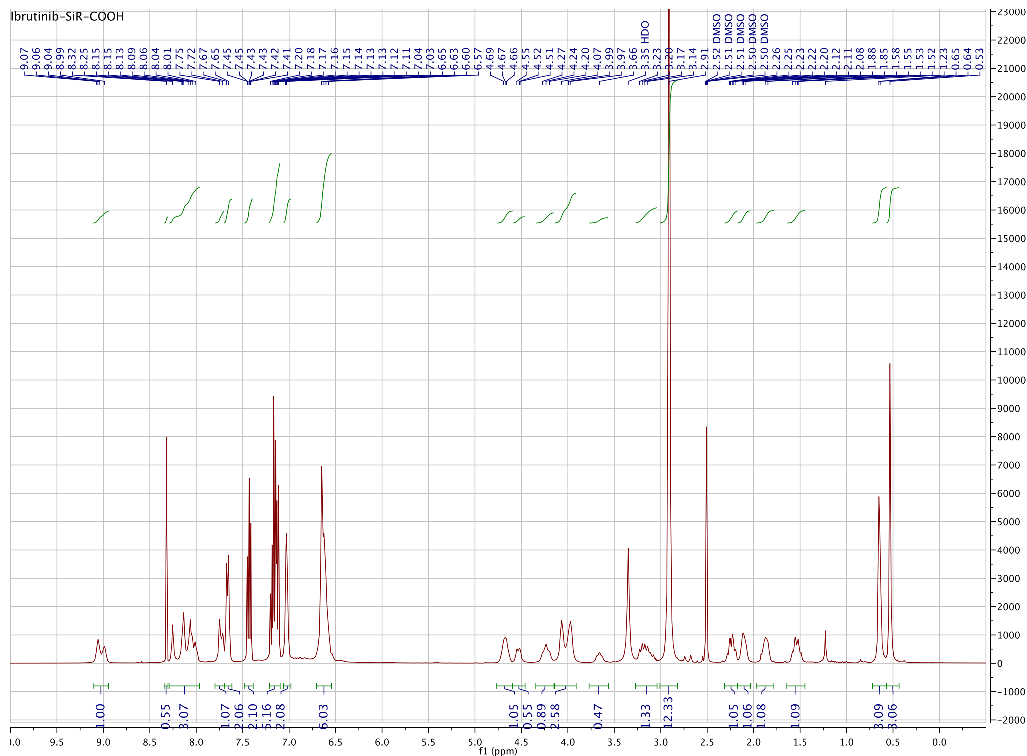
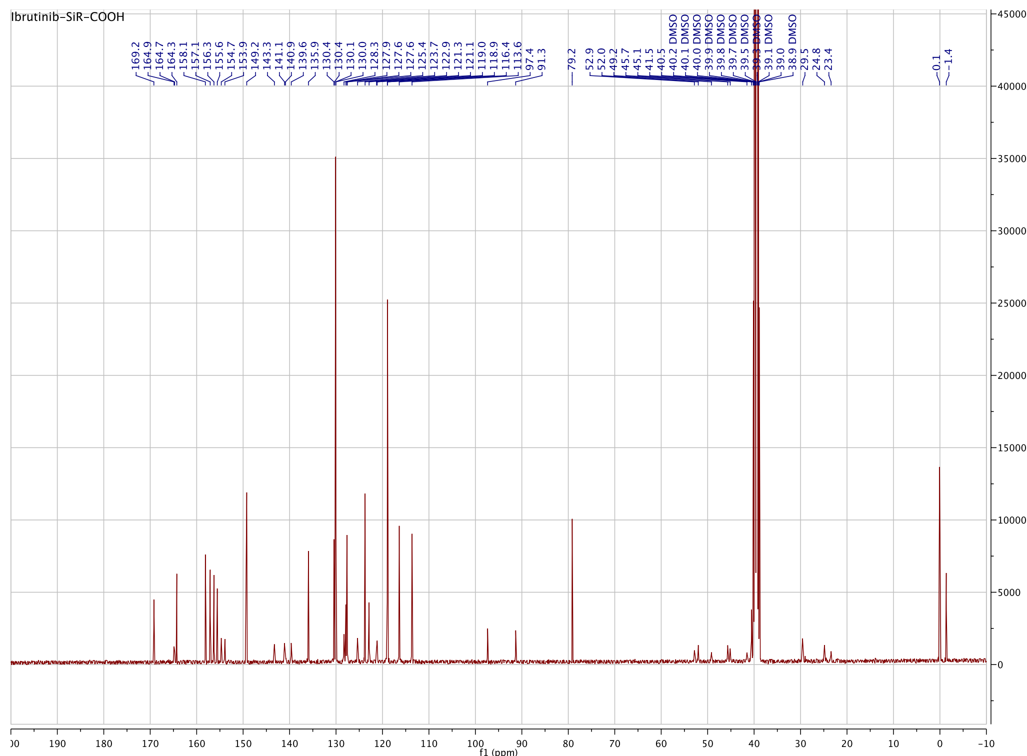


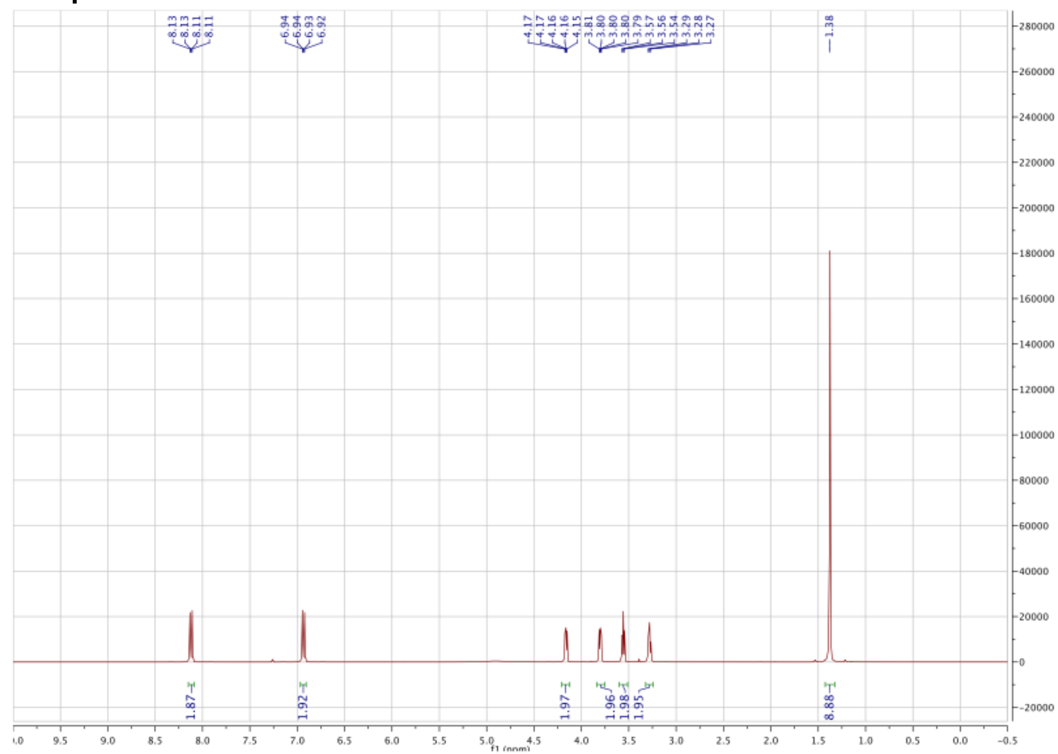
Figure S14. *In vivo* imaging of Btk colocalization with AVL-292-SiR-COOH. 75 nmol AVL-292-SiR-COOH was injected into the tail vein of nu/nu mice and serial imaging was performed in the window chamber. Imaging was performed using a customized Olympus FV1000 confocal/multiphoton microscope equipped with a 20x objective (both Olympus America, Chelmsford, MA, USA). Scale bar: 50 µm.

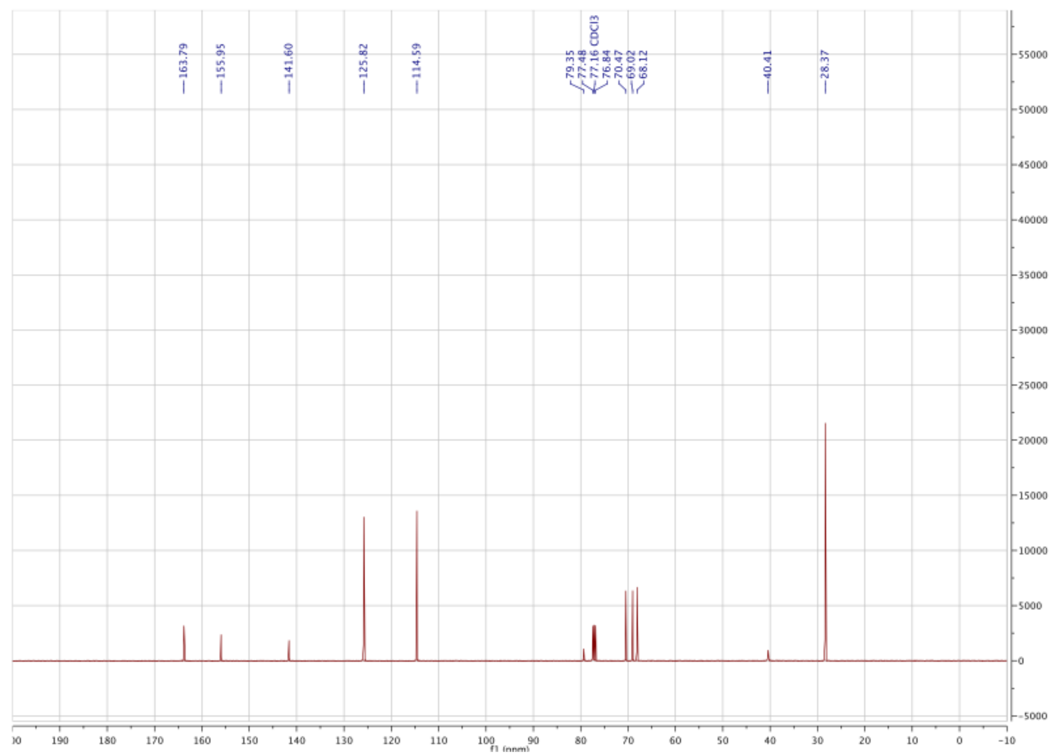
1H and 13C NMR spectra
compound 13 (Ibrutinib-SiR-COOH)



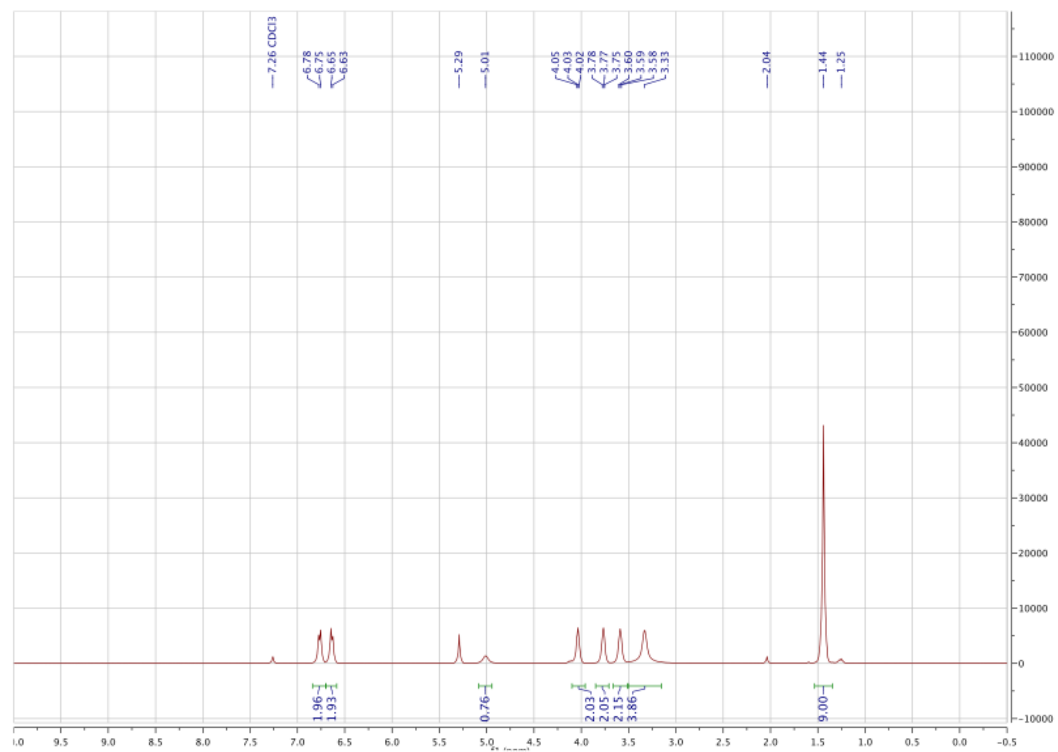


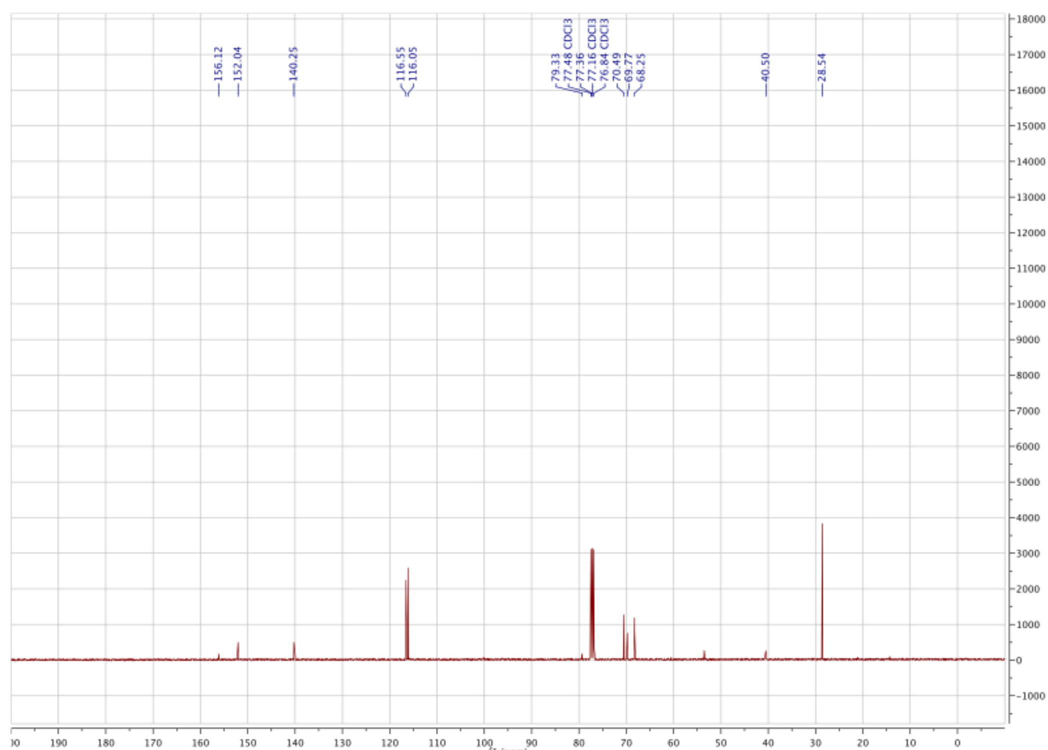
compound 15



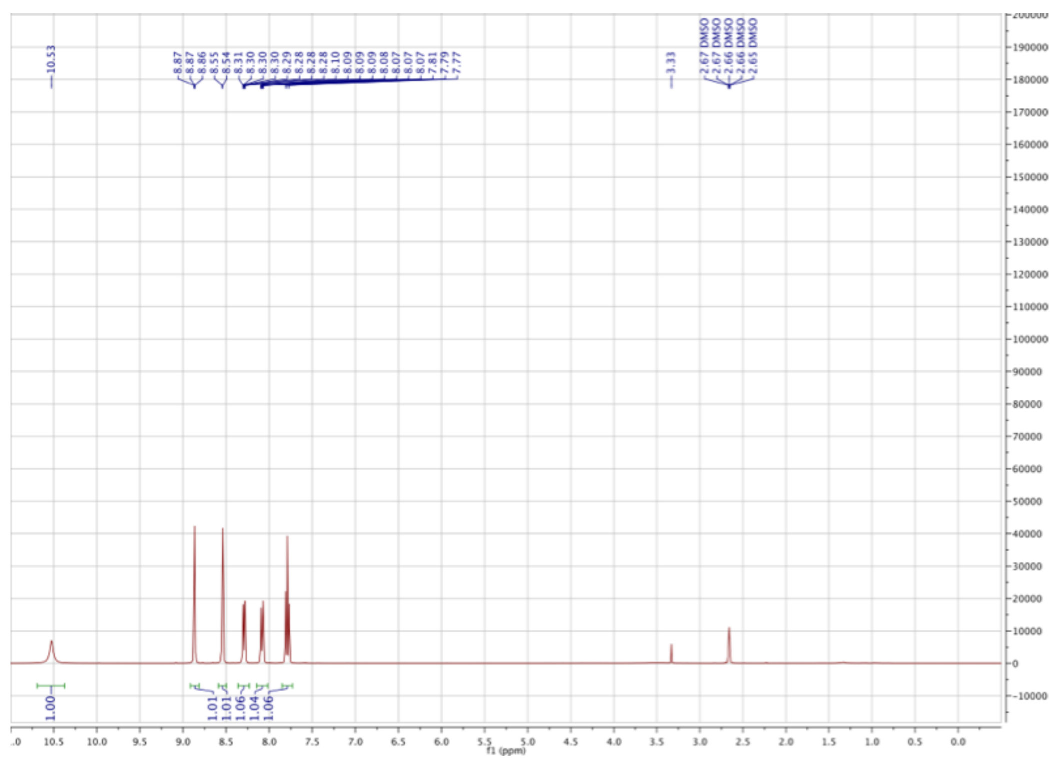


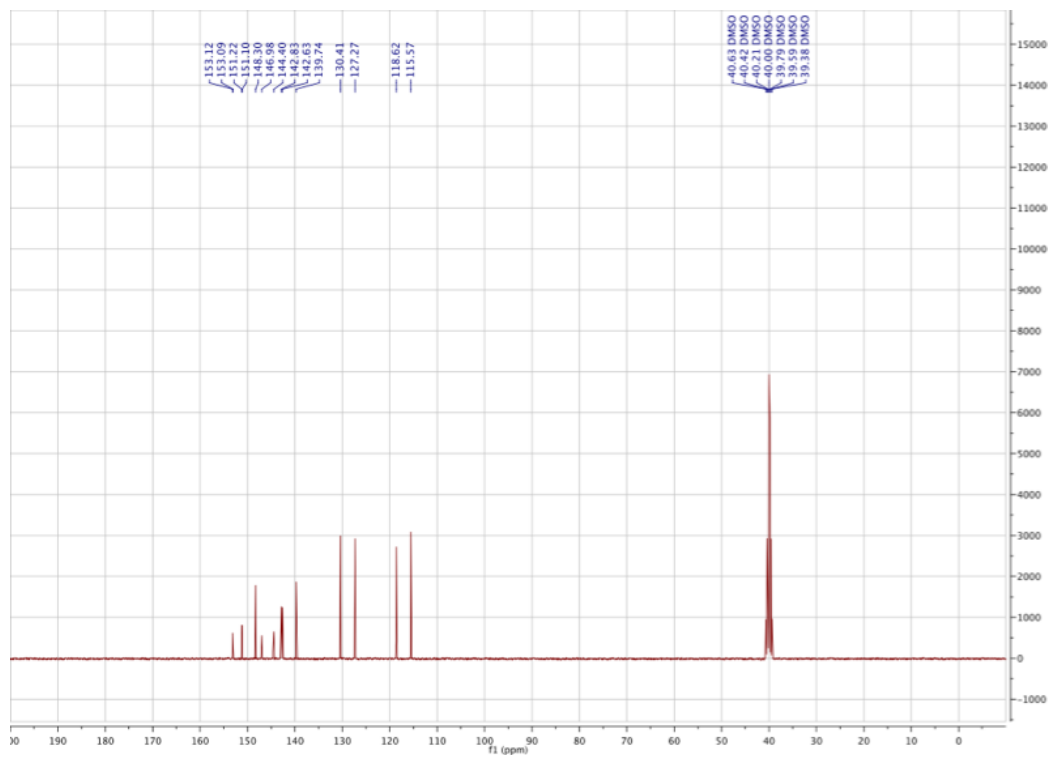
compound 16



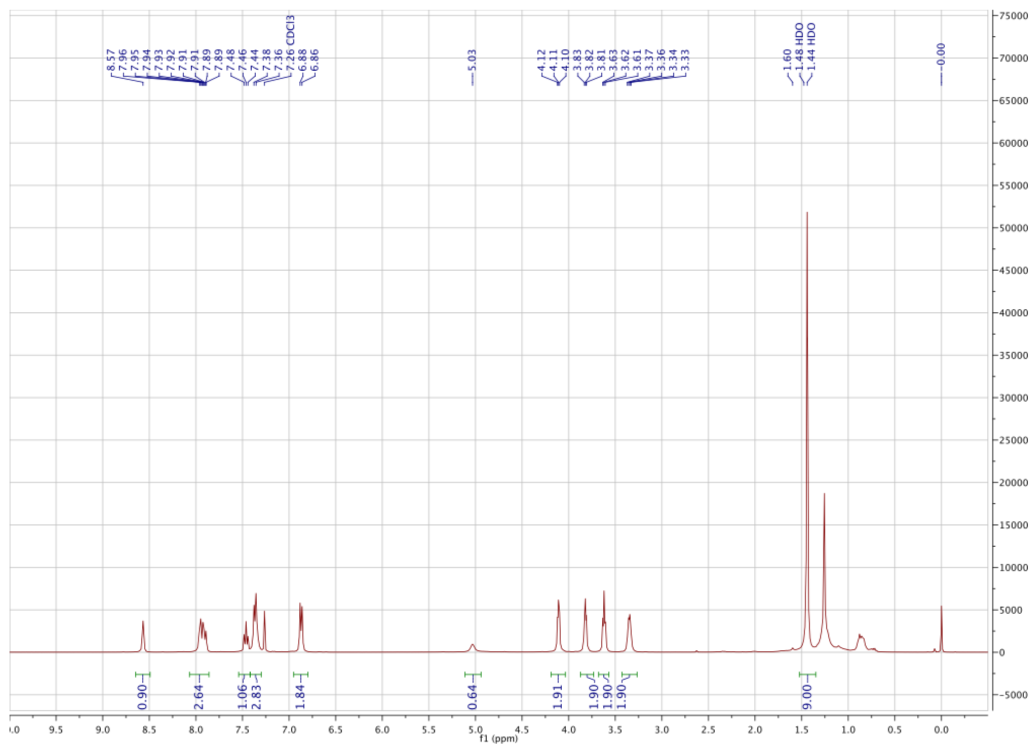


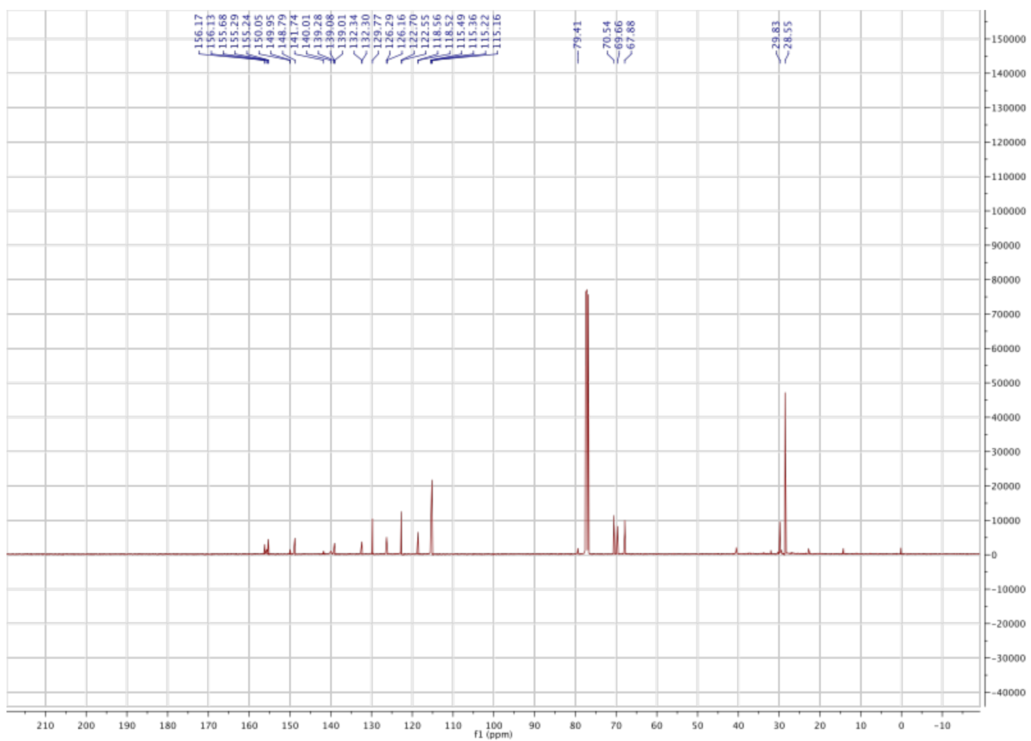
Compound 17



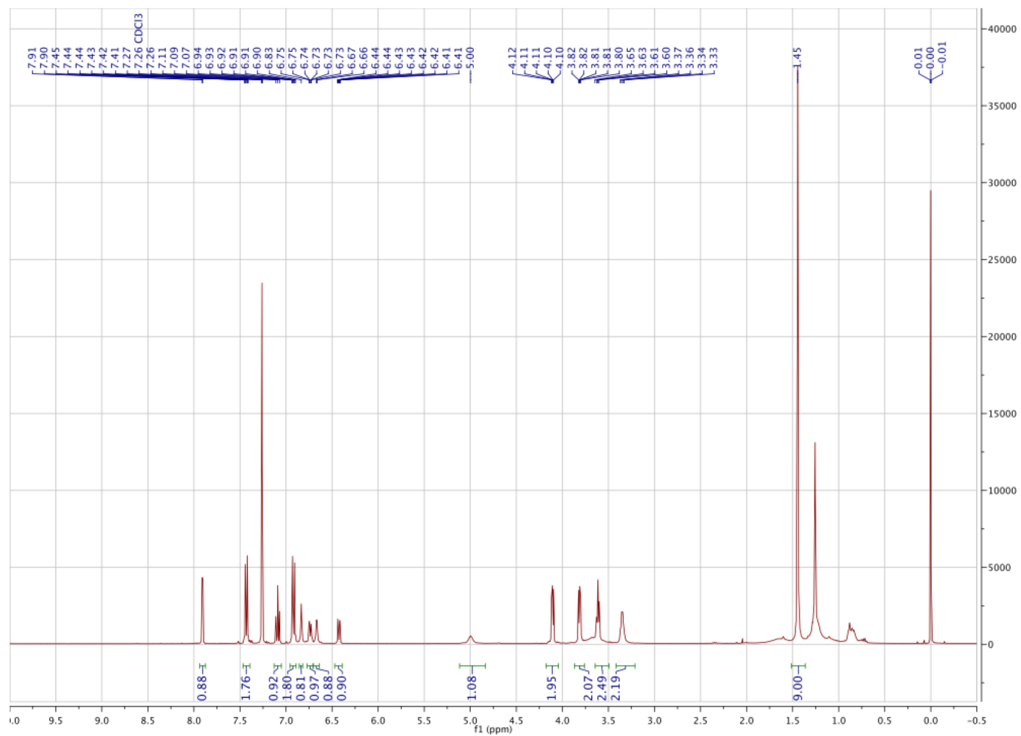


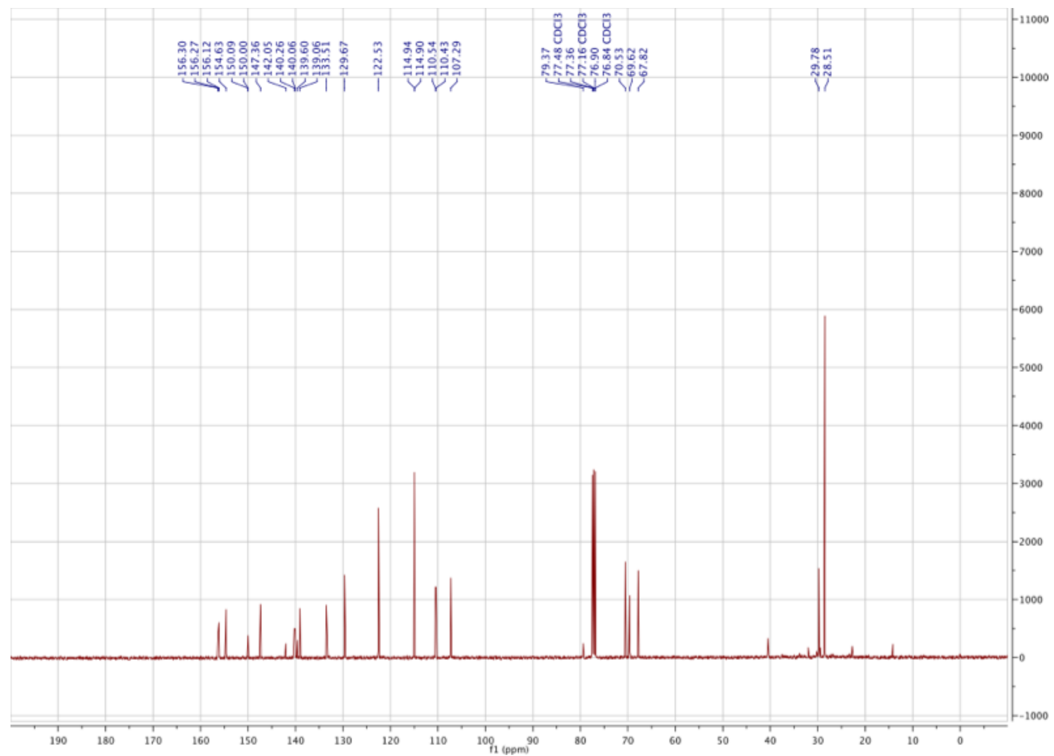
compound 18.





compound 19





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