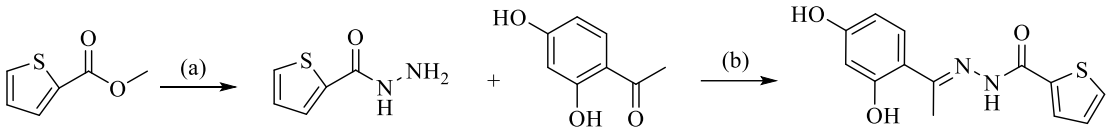
The Allosteric Inhibitor Targeting STAT3 Coiled-Coil Domain, Selectively Suppresses Proliferation of Breast Cancer

Supplementary Information

K116



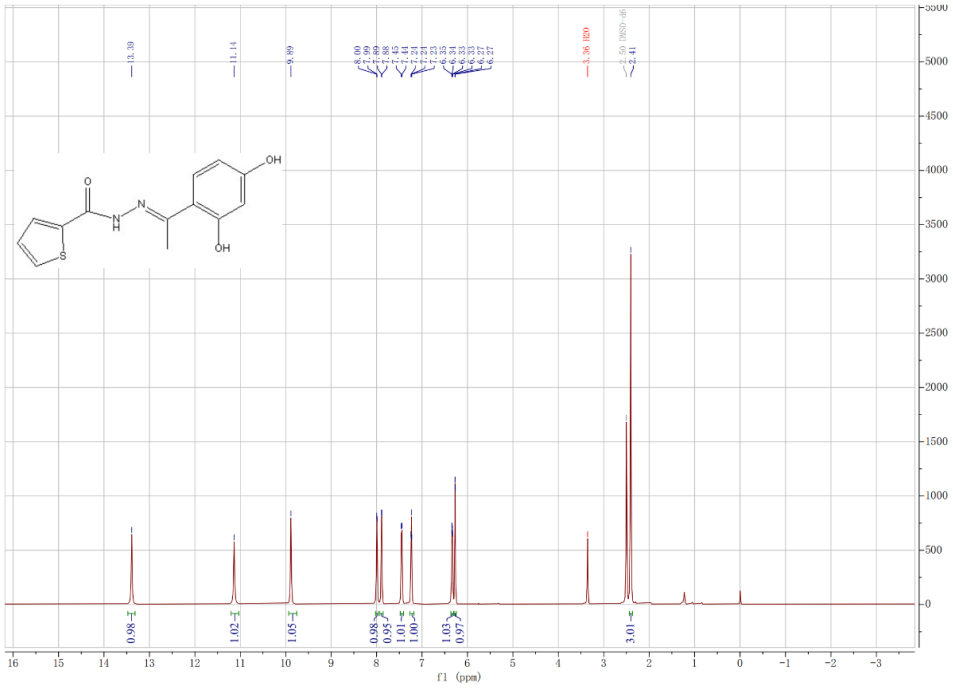
Reagents and conditions：(a) Hydrazinium hydroxide, MeOH, reflux; (b) AcOH, EtOH, reflux.

Step 1

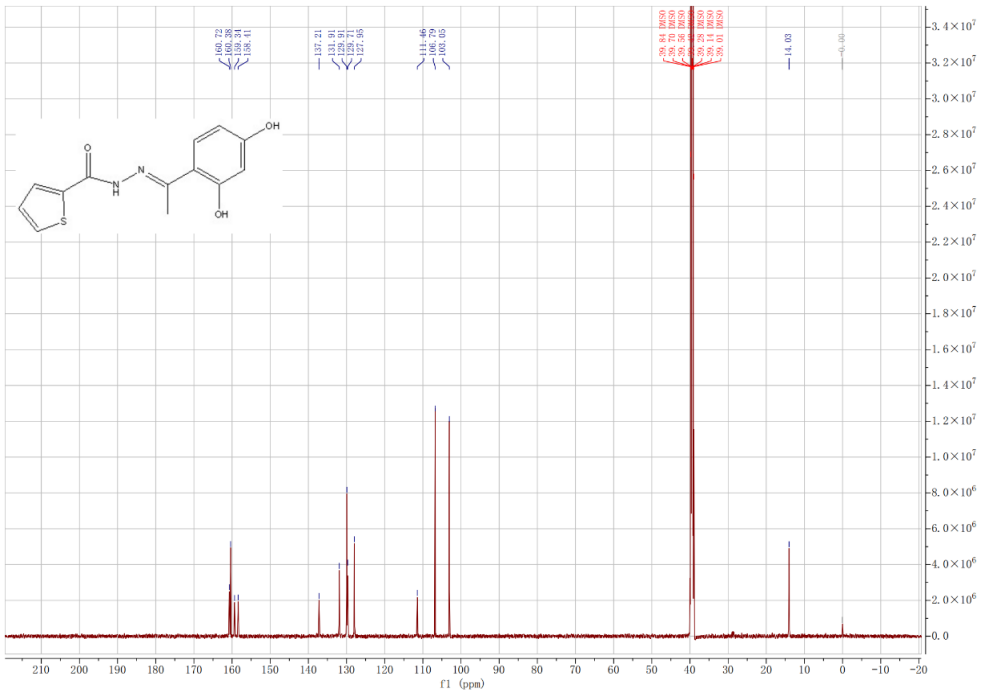
A mixture of methyl thiophene-2-carboxylate(500 mg, 3.52 mmol) and Hydrazine hydrate(1 ml) in 10 ml MeOH was refluxed for 4 hours. The mixture was concentrated under vacuum and filtered to afford thiophene-2-carbohydrazide (300.0 mg, 60.0% yield) as a clear crystal.

Step 2

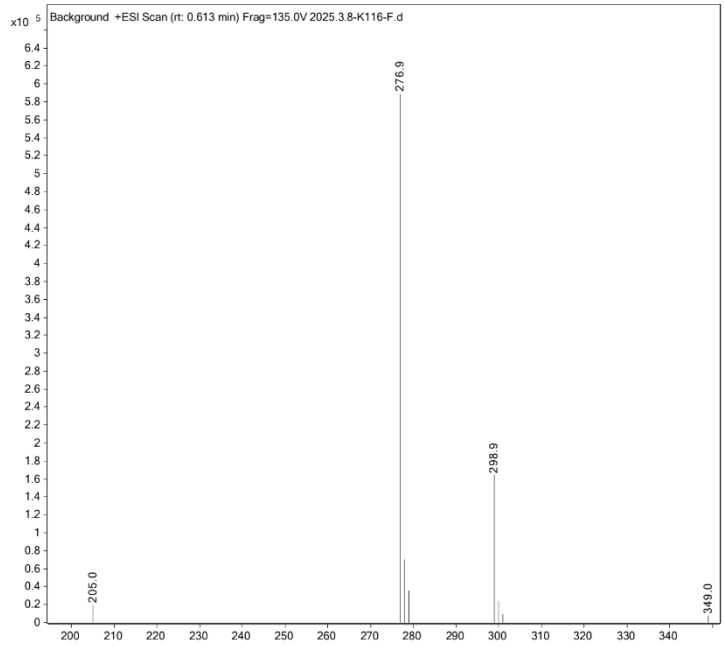
A mixture of hiophene-2-carbohydrazide (300 mg, 2.11 mmol), 1-(2,4-dihydroxyphenyl) ethan-1-one (385.2 mg, 2.53mmol) and AcOH (0.2 ml) in 10 ml EtOH was refluxed for 4 hours. The mixture was concentrated under vacuum and filtered to afford (*E*)-N'-(1-(2,4-dihydroxyphenyl)ethylidene)thiophene-2-carbohydrazide (K116) (430.0 mg) as a brown solid, 73.8% yield. $[M+H]^+$=276.99

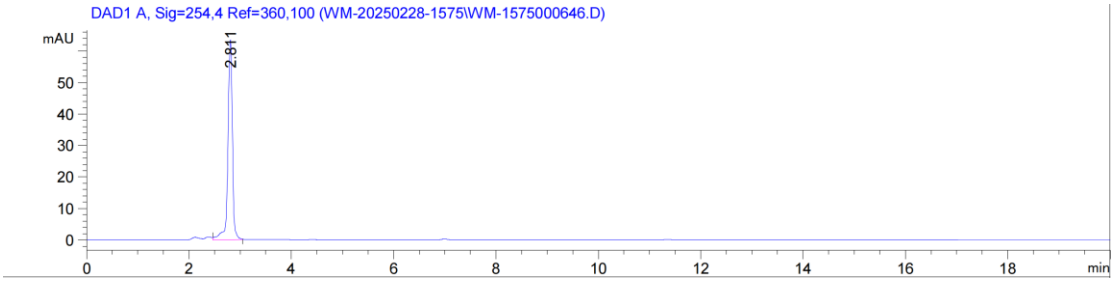


1H NMR (600 MHz, DMSO-d6) δ 13.39 (s, 1H), 11.14 (s, 1H), 9.89 (s, 1H), 8.00 (s, 1H), 7.89 (d, J = 5.1 Hz, 1H), 7.45 (d, J = 8.6 Hz, 1H), 7.24 (t, 1H), 6.37 - 6.31 (m, 1H), 6.30 - 6.25 (m, 1H), 2.41 (s, 3H).



13C NMR (151 MHz, DMSO-d6) δ 160.72, 160.38, 159.34, 158.41, 137.21, 131.91, 129.91, 129.71, 127.95, 111.46, 106.79, 103.05, 14.03.

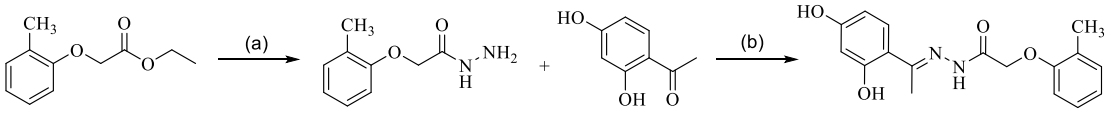




HPLC Conditions: XBridge C18 column (5 μm particle size, 4.6 mm × 250 mm; Waters, USA) with a mobile phase consisting of acetonitrile and water (70:30, v/v), with the water containing 0.1% TFA, at a flow rate of 1.0 mL/min.

Figure S1. The synthetic procedures and NMR, MS spectra copies and HPLC of K116

K134



Reagents and conditions: (a) Hydrazinium hydroxide 85%, EtOH, 80℃（b）Pyridine, EtOH, 80℃.

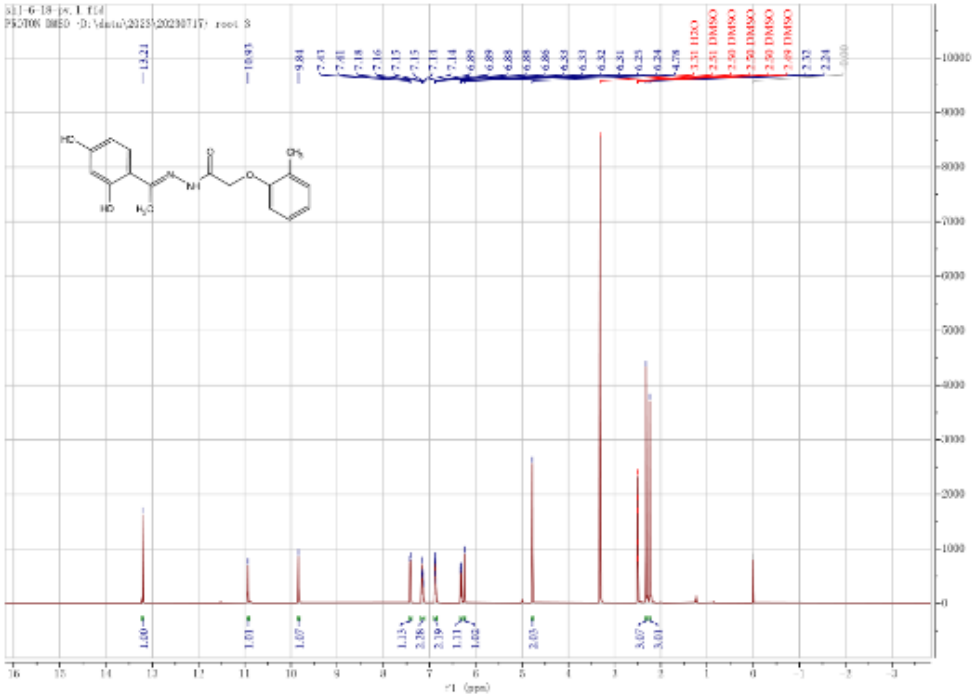
Step 1

To a stirred solution of ethyl 2-(o-tolyloxy)acetate (1eq) in anhydrous ethyl alcohol was added hydrazinium hydroxide 85% (2eq). The mixture was refluxed at 80℃ overnight. Cooled the reaction solution to room temperature and poured into ice water. Filtered the white precipitate and washed the white precipitate with ethyl alcohol and water.

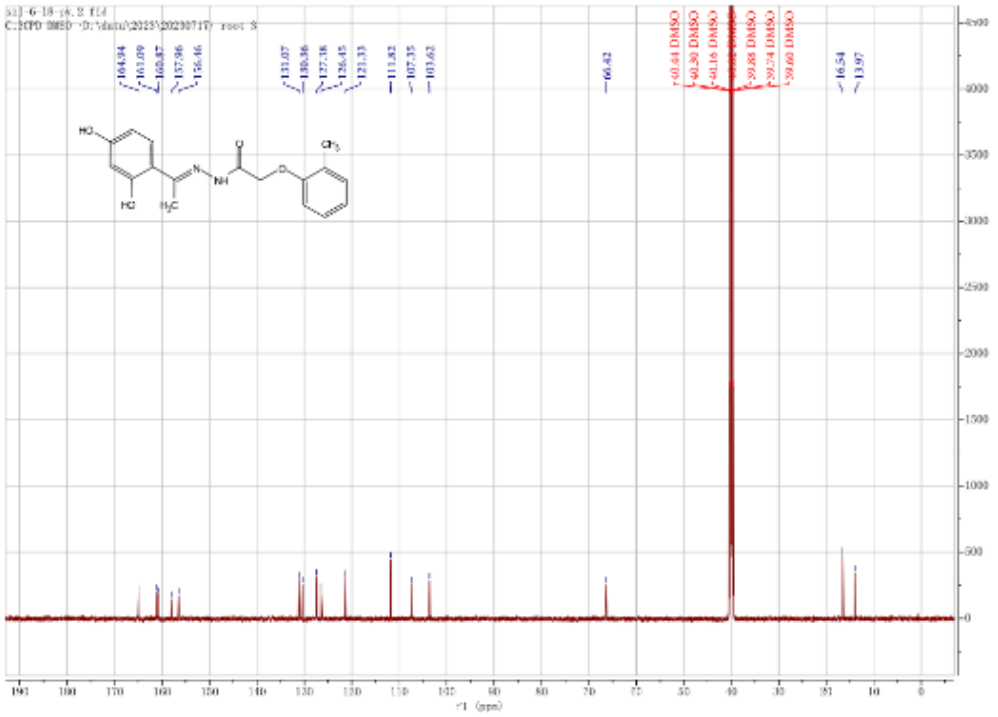
Dried in a vacuum oven to obtain 2-(o-tolyloxy)acetohydrazide as white solid, 53% yield.

Step 2

To a stirred solution of 2-(o-tolyloxy)acetohydrazide (1eq) in anhydrous ethyl alcohol was added 1-(2,4-dihydroxyphenyl)ethan-1-one (1.1eq) and anhydrous pyridine (0.1eq). The mixture was refluxed at 80℃ overnight. Cooled the reaction solution to room temperature. Filtered the white precipitate and washed with anhydrous ethyl alcohol. Dried in a vacuum oven to obtain (*E*)-N'-(1-(2, 4-dihydroxyphenyl)ethylidene)-2-(o-tolyloxy)acetohydrazide **(K134)** as white solid, 10% yield.



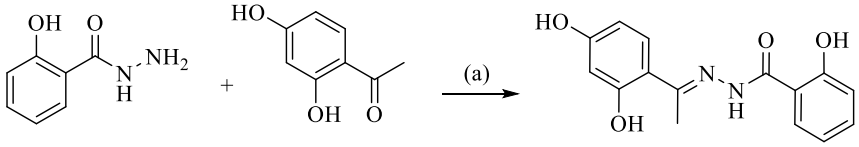
1H NMR (600 MHz, DMSO-d6) δ 13.21 (s, 1H), 10.93 (s, 1H), 9.84 (s, 1H), 7.42 (d, J = 8.8 Hz, 1H), 7.19 – 7.11 (m, 2H), 6.91 – 6.84 (m, 2H), 6.32 (dd, J = 8.7, 2.5 Hz, 1H), 6.25 (d, J = 2.5 Hz, 1H), 4.78 (s, 2H), 2.32 (s, 3H), 2.24 (s, 3H);



13C NMR (150 MHz, DMSO-d6) δ 164.94, 161.09, 160.87, 157.96, 156.46, 131.07, 130.36, 127.38, 126.45, 121.33, 111.82, 107.35, 103.62, 66.42, 16.54, 13.97; MS-ESI (m/z): 315.2 (M+H)+.

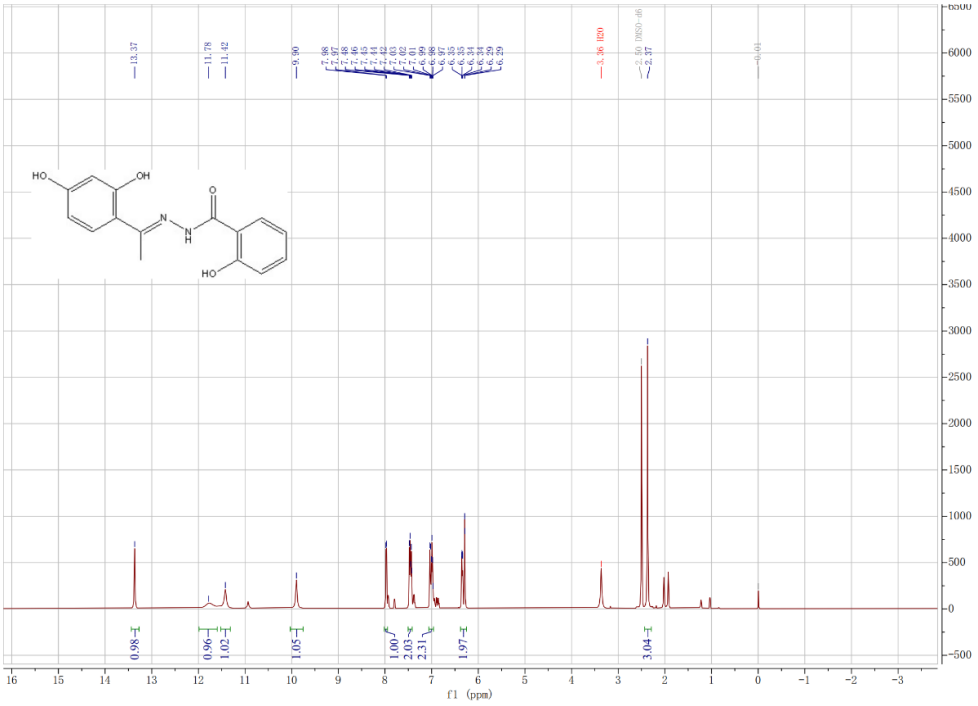
Figure S2. The synthetic procedures and the NMR spectrum data of K134.

K114

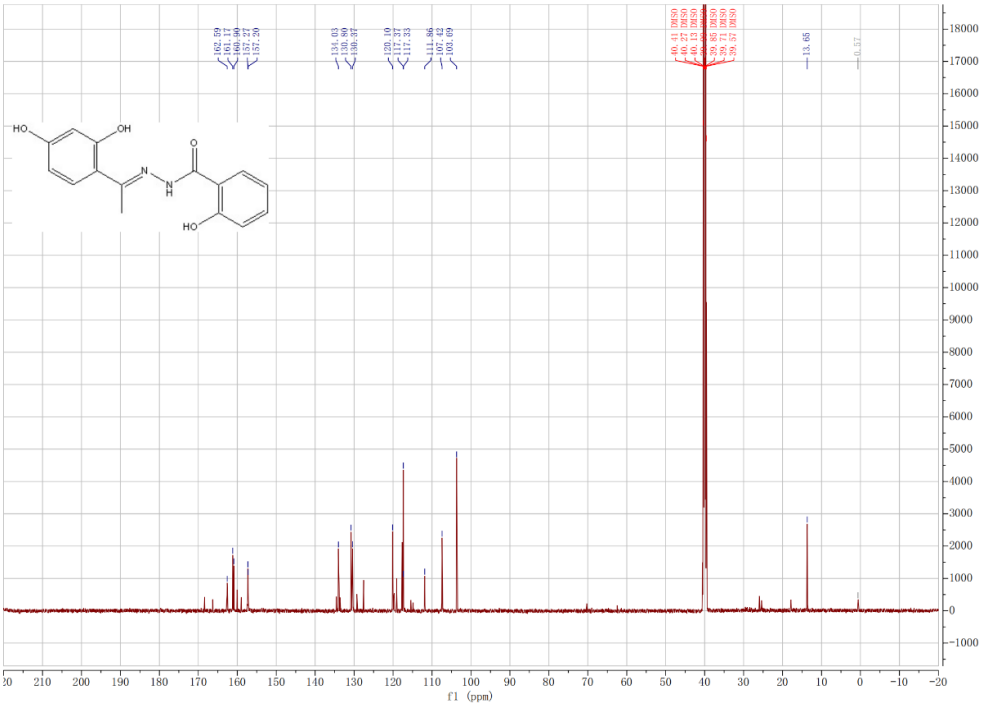


Reagents and conditions：(a) AcOH, EtOH, 80℃.

To a stirred solution of 2-hydroxybenzohydrazide (1 eq) in anhydrous ethyl alcohol was added 1-(2,4-dihydroxyphenyl)ethan-1-one (1 eq) and acetic acid (0.4eq). The mixture was refluxed at 80℃ for 3 hours. Cooled the reaction solution to room temperature. Filtered the white precipitate and washed with anhydrous ethyl alcohol. Dried in a vacuum oven to obtain (*E*)-N'-(1-(2,4-dihydroxyphenyl)ethylidene)-2-hydroxybenzohydrazide **(K114)** as white solid, 30% yield.



1H NMR (600 MHz, DMSO-d6) δ 13.37 (s, 1H), 11.78 (s, 1H), 11.42 (s, 1H), 9.90 (s, 1H), 7.97 (d, J = 7.8 Hz, 1H), 7.50 - 7.42 (m, 2H), 7.06 - 6.96 (m, 2H), 6.38 - 6.25 (m, 2H), 2.37 (s, 3H).



13C NMR (151 MHz, DMSO-d6) δ 162.59, 161.17, 160.90, 157.27, 157.20, 134.03, 130.80, 130.37, 120.10, 117.37, 117.33, 111.86, 107.42, 103.69, 13.65.

Figure S3. The synthetic procedures and the NMR spectrum data of K114.

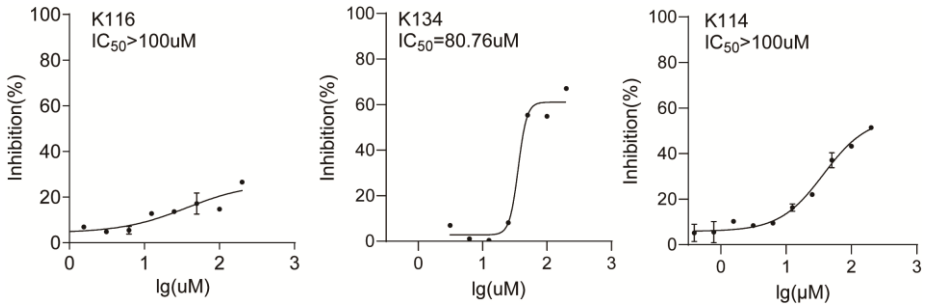


Figure S4. Inhibitory effect of K116, K134 and K114 on AC16 cells. The cell proliferation was analyzed using the CCK8 assay. Half-maximal inhibitory concentrations were determined using GraphPad Prism10. The data are representative of three independent studies. The data represent as the means ± SEMs, $^{*}P < 0.05$, $^{**}P < 0.01$.

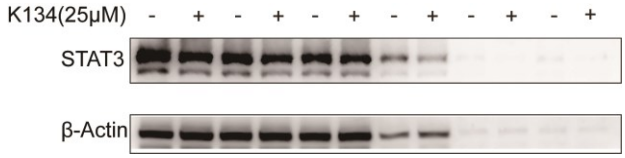


Figure S5. CETSA-WB experiment to further confirm the interaction between K134 (25μM) and STAT3.

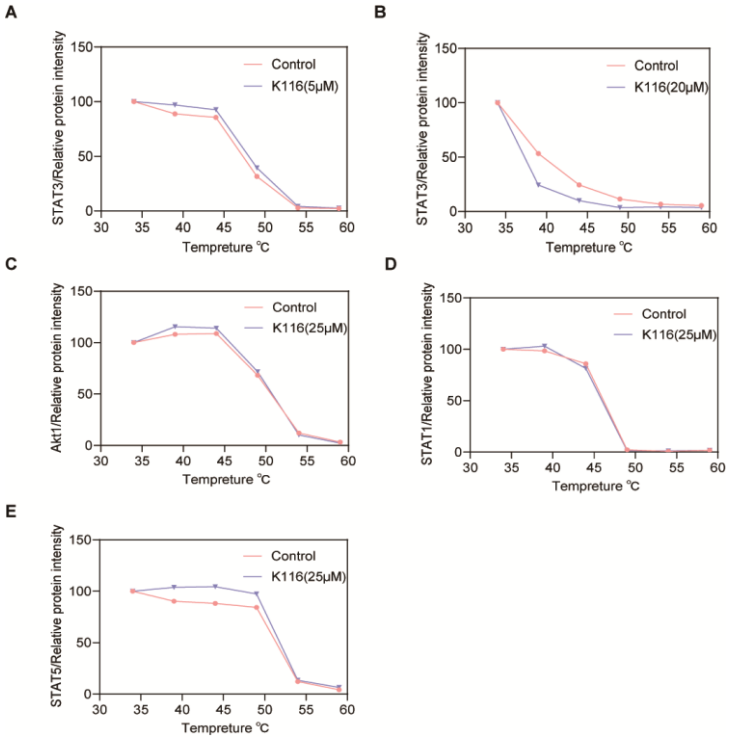


Figure S6. (A) The thermal stabilities of the STAT3 with or without K116 (5μM). (B) The thermal stabilities of the STAT3 with or without K116 (20μM). (C) The thermal stabilities of the Akt with or without K116 (25μM). (D) The thermal stabilities of the STAT1 with or without K116 (25μM). (E) The thermal stabilities of the STAT5 with or without K116 (25μM).

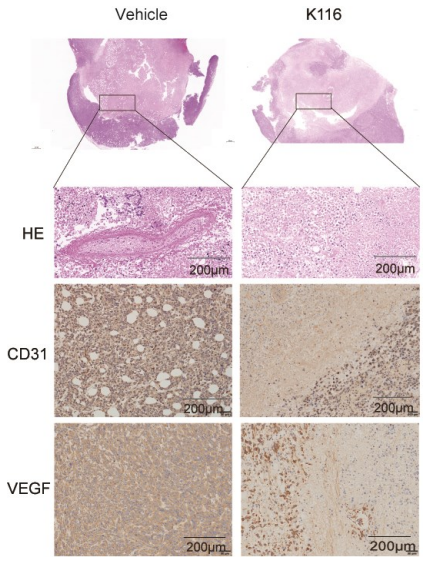


Figure S7. Representative tissue sections from representative agiogenesis of xenograft tumors stained with H&E. Immunohistochemical analysis was performed for CD31and VEGF staining after treatment of xenograft tumors with vehicle or K116. Representative images (×20 magnification) are shown. Scale bars = 200 μm.

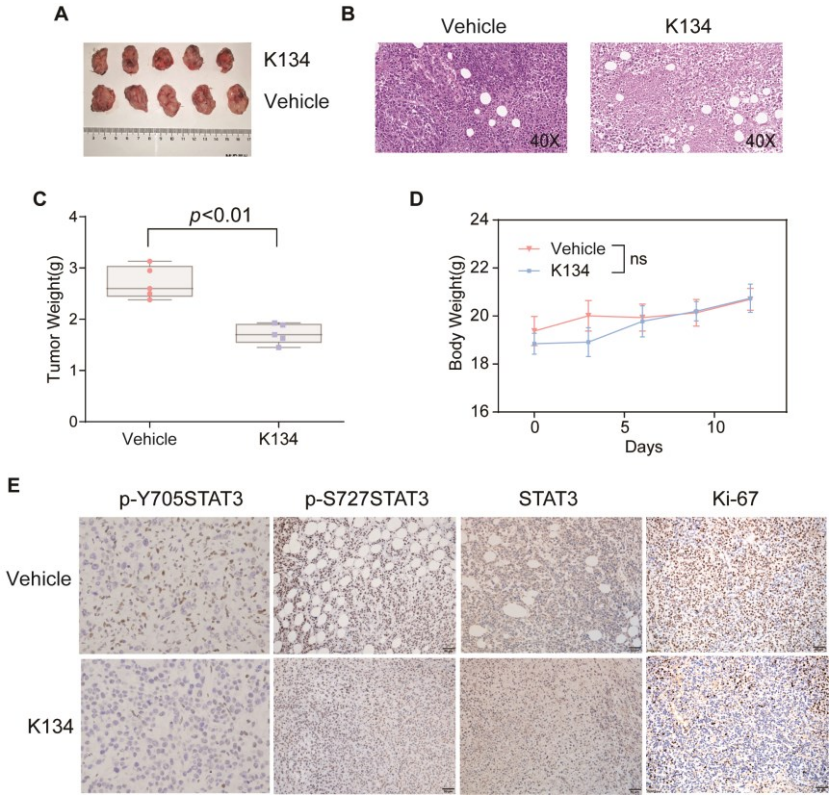


Figure S8. K134 displayed antitumor efficacy in 4T1 cell–derived xenografts. (A) Representative image of 4T1 xenograft tumors dissected from C57 mice treated intraperitoneally with vehicle or 30 mg/kg K134 every other day for 18 days (n = 5). (B) Representative tissue sections from representative xenograft tumors stained

with H&E. (C) Tumor weights in different groups of mice (n = 5). (D) Body weights in different groups of mice (n = 5). (E) Immunohistochemical analysis was performed for pY705STAT3, pS727STAT3, STAT3, and Ki-67 staining after treatment of xenograft tumors with vehicle or K134. Representative images (×20 magnification) are shown. Scale bars = 200 μm. The data are presented as means ± SEMs, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, ns, not significant.

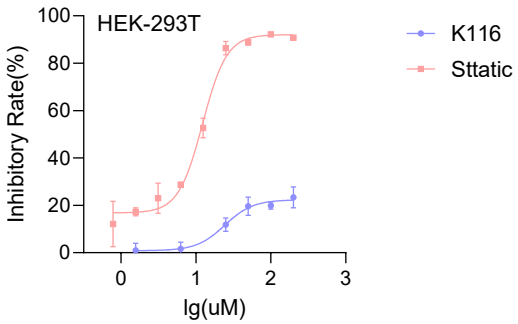


Figure S9. The cell viability of AC16 and HEK-293T cells treated with K116.

Table S1. IC_{50} values of K116 in cancer cell lines.

Cell line	IC_{50} ± SEMs (μM)
MDA-MB-468	4.8 ± 0.7
MCF-7	15.2 ± 2.2
4T1	6.7 ± 2.7
Huh7	>100
SMMC-7721	>100
BEL-7404	>100
A549	66.2 ± 2.9
MKN45	>100
HGC-27	>100

*The IC_{50} ± SEMs values of K116 to the indicated cancer cell lines were determined by CCK8 assays. The value of each K116 treatment group was calculated as a percentage change of the DMSO controls, which represents the viability of the cancer cell lines. The data represent the mean ±SEMs of three independent experiments.

Table S2. IC_{50} values of K134 in cancer cell lines.

Cell line	IC_{50} ± SEMs (μM)
MDA-MB-468	14.21 ± 1.9
4T1	9.37± 0.97
MCF-7	>100
MKN28	>100
MKN45	>100

*The IC_{50} ± SEMs values of K134 to the indicated cancer cell lines were determined by CCK8 assays. The value of each K134 treatment group was calculated as a percentage change of the DMSO controls, which represents the viability of the cancer cell lines. The data represent the mean ±SEMs of three independent experiments.

Table S3. IC_{50} values of K114 in cancer cell lines.

Cell line	IC_{50} ± SEMs (μM)
MDA-MB-468	9.98 ± 2.14
4T1	11.07 ± 0.96
MKN28	>100
MKN45	>100
HGC-27	>100

*The IC_{50} ± SEMs values of K114 to the indicated cancer cell lines were determined by CCK8 assays. The value of each K114 treatment group was calculated as a percentage change of the DMSO controls, which represents the viability of the cancer cell lines. The data represent the mean ±SEMs of three independent experiments.

Table S4. Primary antibodies used for western blots.

Antibody name	Company name	Catalog number	Dilution
Phospho-Stat3 (Tyr705)	Cell Signaling Technology	#9145	1:1000
Stat3 (124H6)	Cell Signaling Technology	#9139	1:1000
Phospho-Stat3 (Ser727)	Cell Signaling Technology	#9134	1:1000
Phospho-Stat1 (Tyr701)	Cell Signaling Technology	#9167	1:500
Phospho-Stat5 (Tyr694)	Cell Signaling Technology	#9359	1:500
Phospho-Akt (Ser473)	Cell Signaling Technology	#4060	1:1000
Phospho-Stat3 (Ser727)	Abcam	Ab32143	1:1000
β-actin	Proteintech	HRP-60008	1:5000