iScience, Volume *23*

Supplemental Information

A New Compound with Increased Antitumor Activity by Cotargeting MEK and Pim-1

Yanan Li, Ying Cheng, Maoqi Zhang, Xiaoli He, Li Kong, Kexiang Zhou, Yunfu Zhou, Lin Li, Hongqi Tian, Xiaomin Song, and Yukun Cui

SUPPLEMENTAL FIGURES

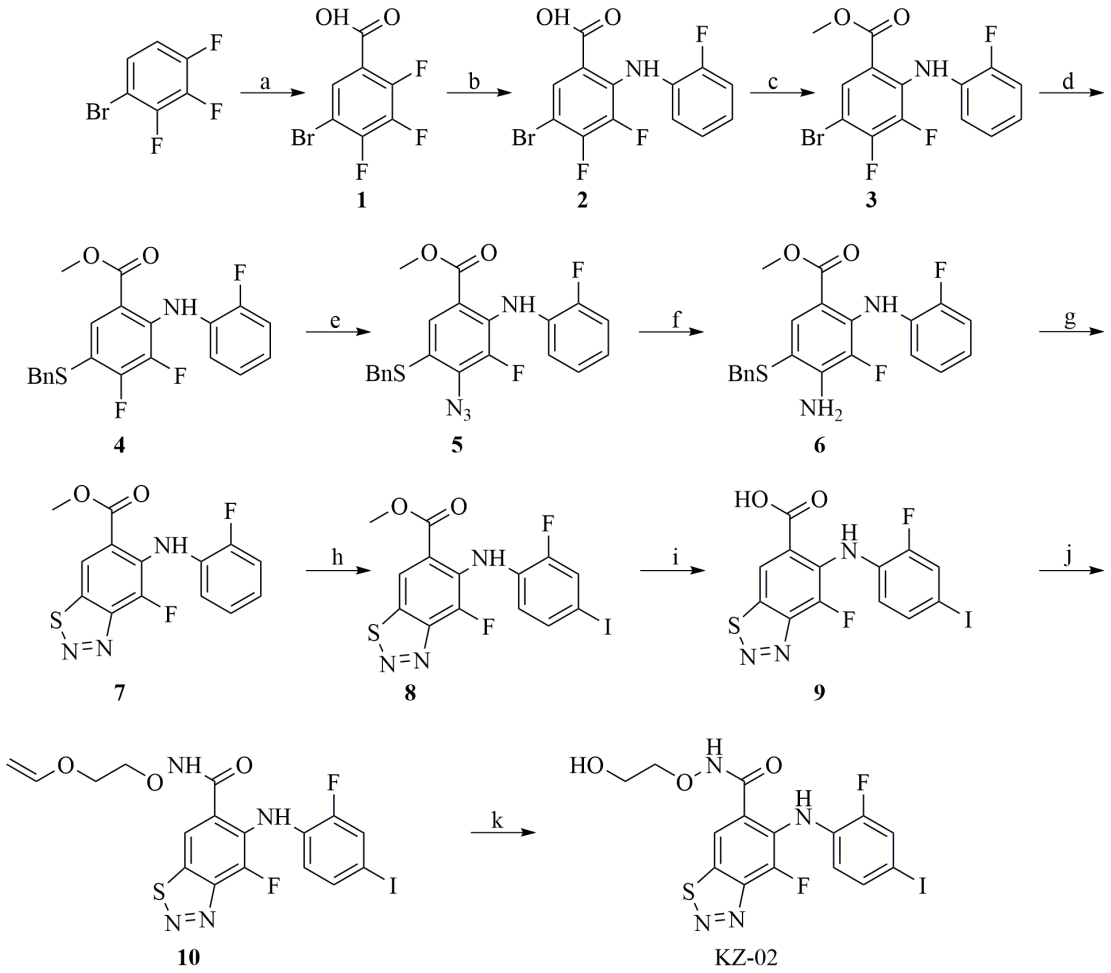


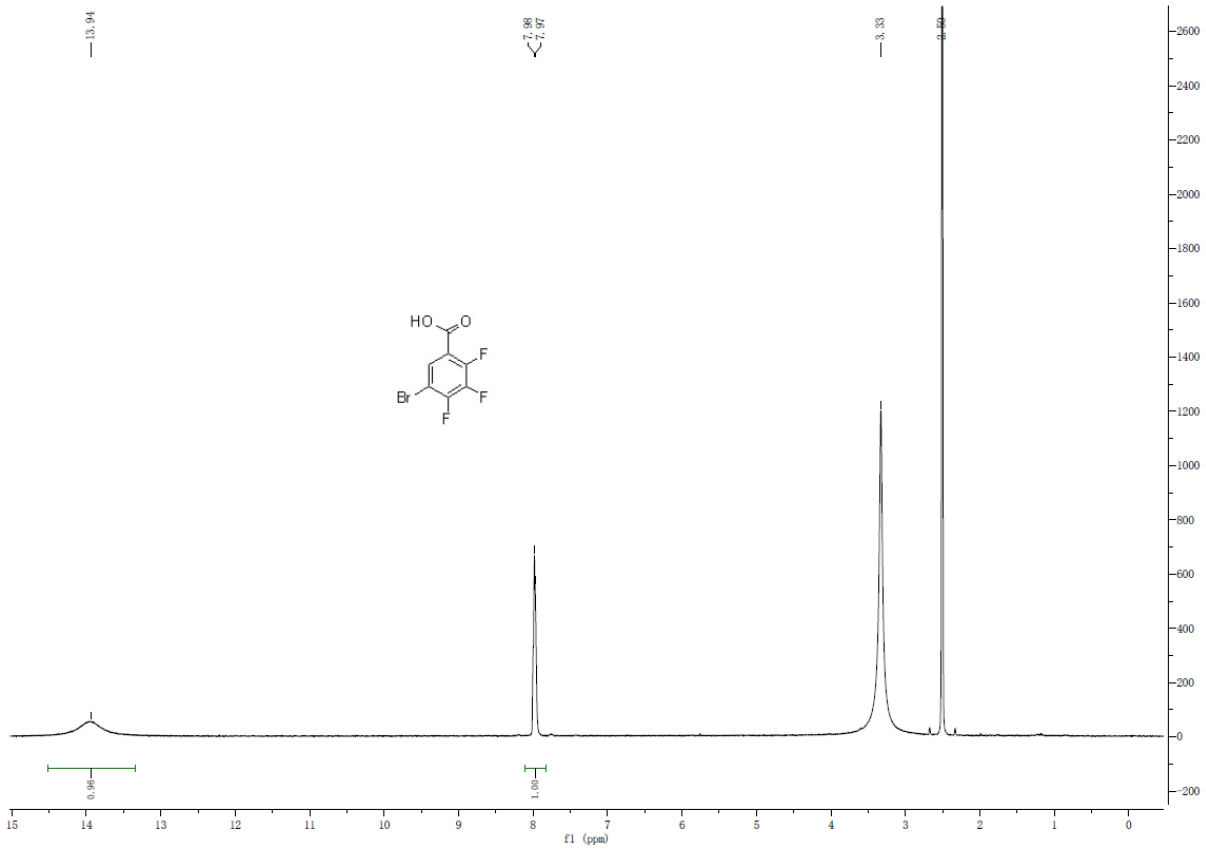
Figure S1. The Synthesis Route of KZ-02. The Analysis Spectra for Key Intermediates and Target Compound are Shown Below. Related to Figure 1.

(a) N_2, LDA, dry ice, dry THF, –78ºC to r.t. (b) N_2, 2-fluoroaniline, LiHMDS, –78ºC to r.t. (c) Thionyl chloride, 85ºC; DCM, Methanol, 0ºC. (d) N_2, phenylmethanethiol, DIPEA, Xantphos, Tris (dibenzylideneacetone) dipalladium, 1,4-dioxane, 90ºC. (e) NaN_3, DMA, 90ºC. (f) H_2, 10% Pd/C, Methanol, r.t. (g) con. HCl, H_2O, r.t; $NaNO_2/H_2O$, 10 ºC. (h) NIS, TFA, DMF, r.t. (i) 1M LiOH, THF/MeOH=4/1(v/v), r.t. (j) O-(2-(vinyloxy) ethyl) hydroxylamine, EDCI, HOBT, DCM, r.t. (k) 1M HCl, DCM, r.t. Abbreviations: LDA, Lithium diisopropylamide; THF, Tetrahydrofuran; LiHMDS, lithium hexamethyldisilamide; DCM, dichloromethane; DIPEA, N, N-Diisopropylethylamine; DMA, Dimethylacetamide; TFA, Trifluoroacetic acid; DMF, N, N-dimethyllformamide; EDCI, 1-Ethyl-3-(3-dimethylaminopropyl) carbetamide; NIS, N-Iodosuccinimide; HOBt, Hydroxybenzotriazole; r.t., room temperature. Analysis spectra for key intermediates and target compound are shown in

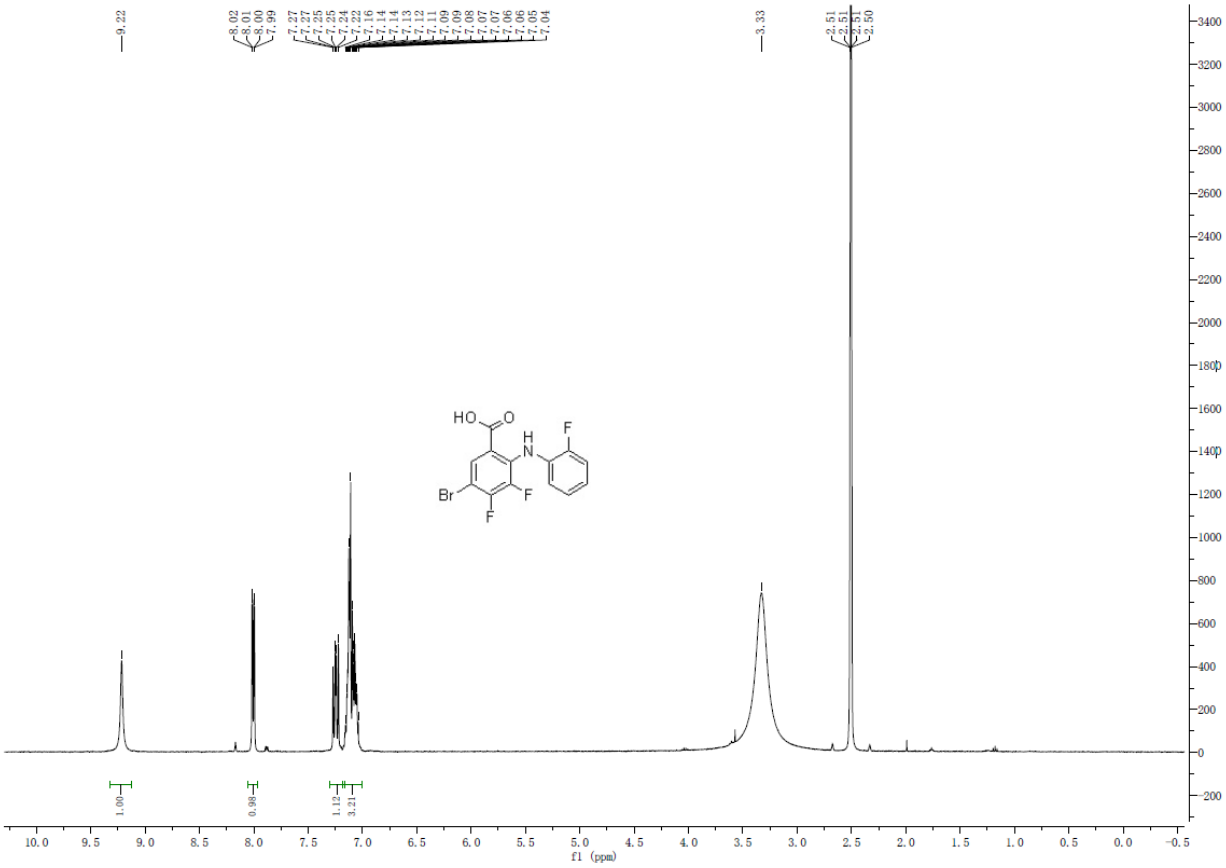
Data S1.

Data S1. Analysis Spectra for Key Intermediates and Target Compound. Related to Figure S1.

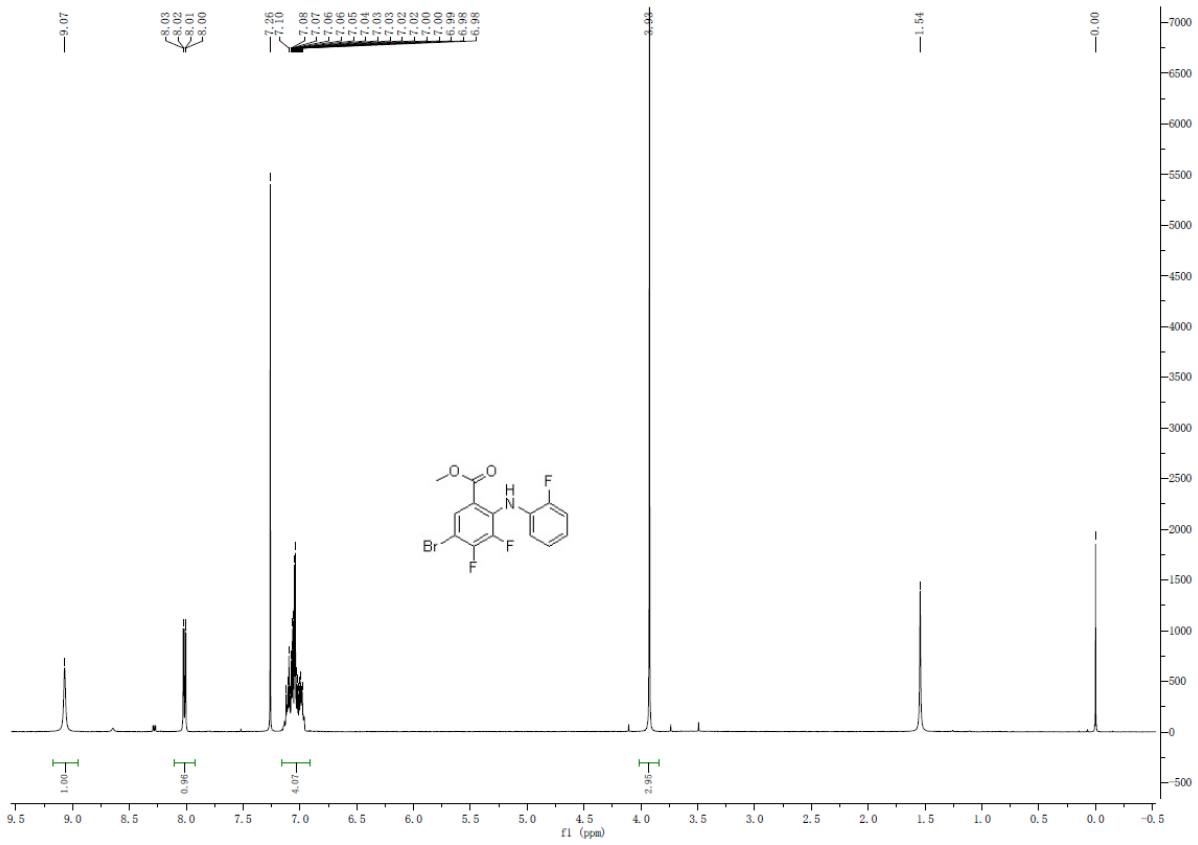
^{1}H NMR spectra of compound **1**



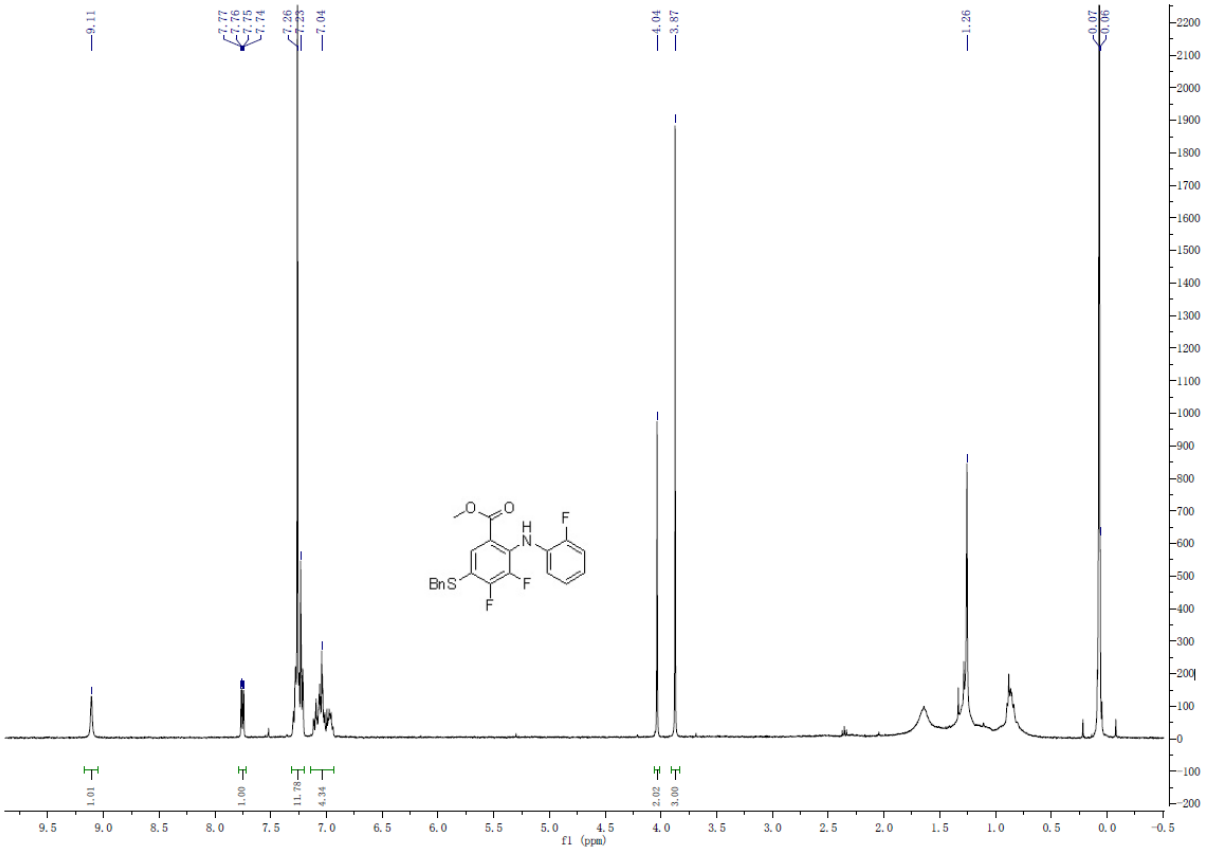
^{1}H NMR spectra of compound **2**



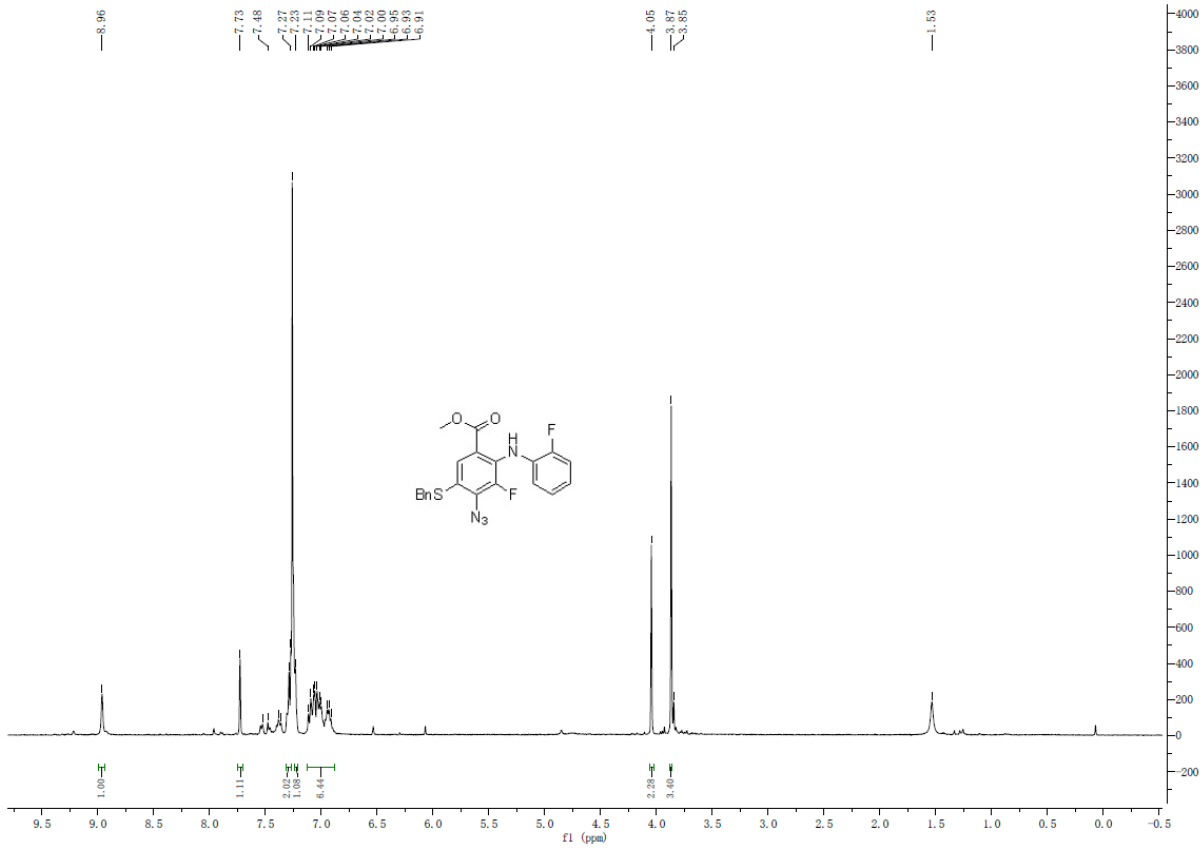
^{1}H NMR spectra of compound **3**



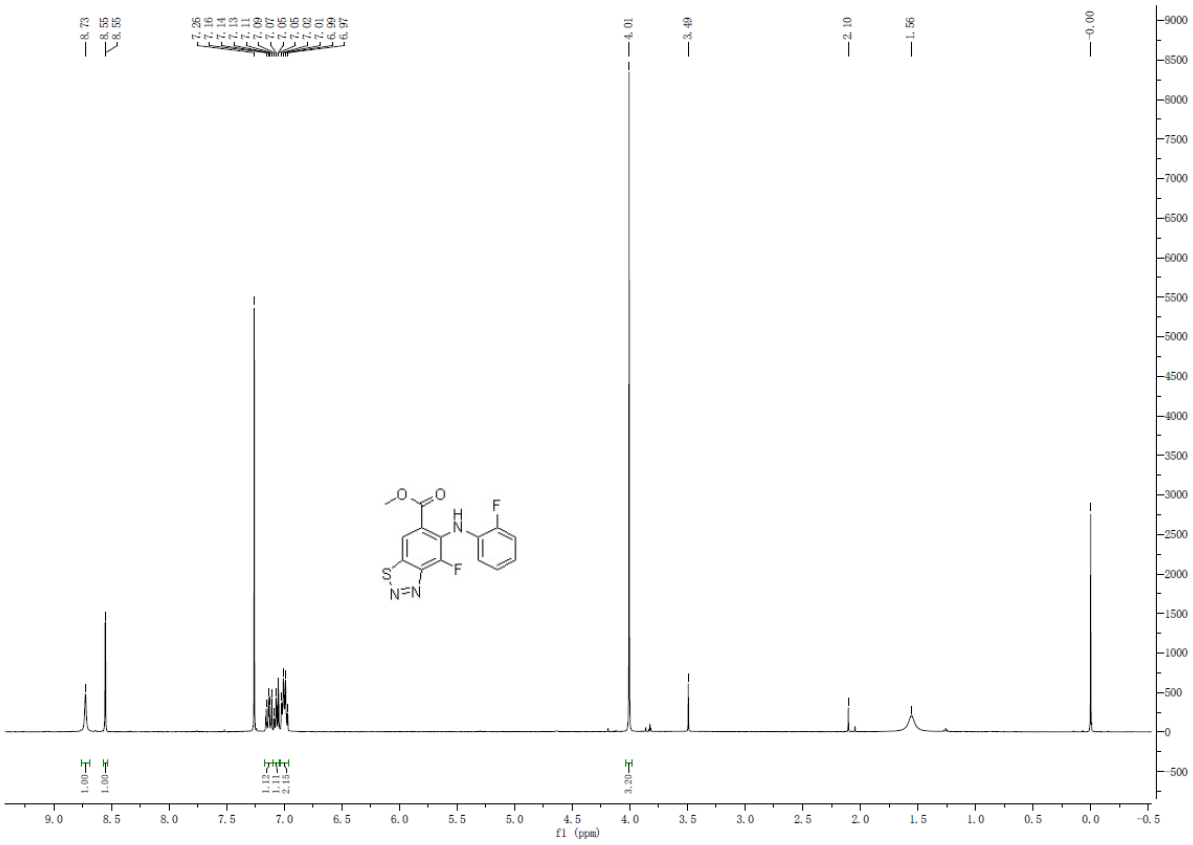
^{1}H NMR spectra of compound **4**



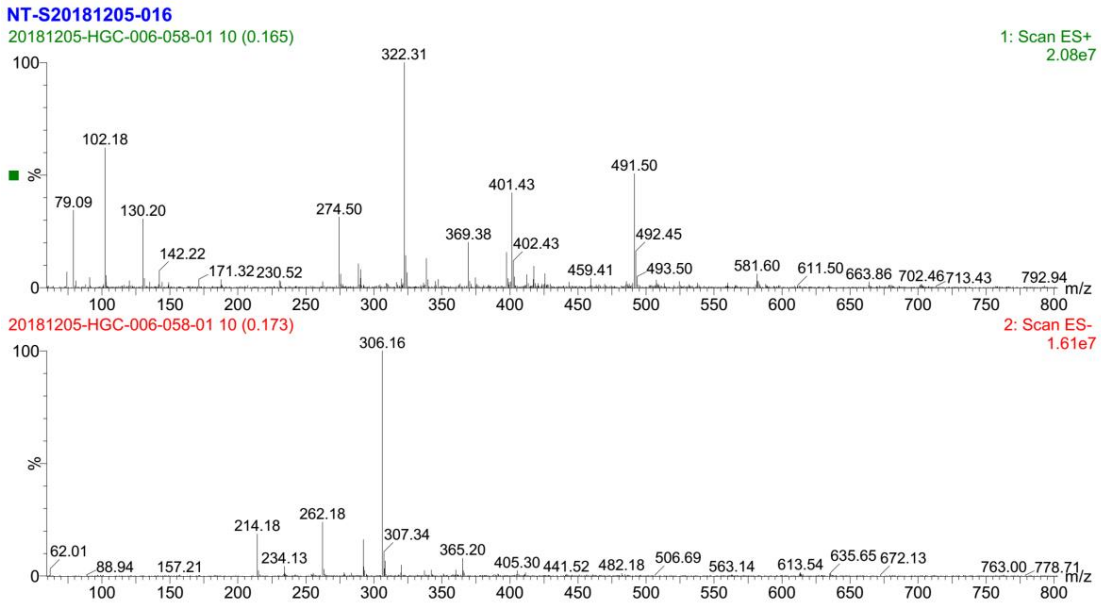
^{1}H NMR spectra of compound **5**



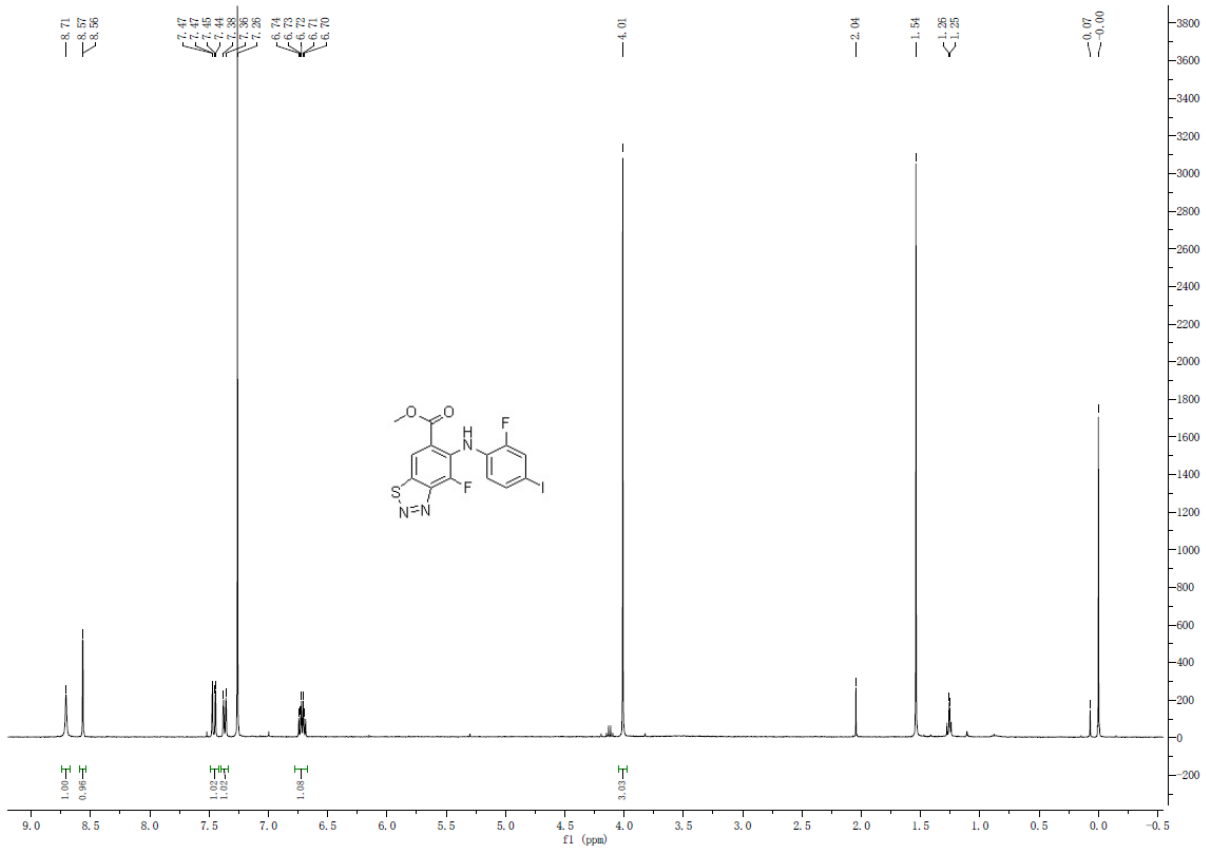
^{1}H NMR spectra of compound **7**



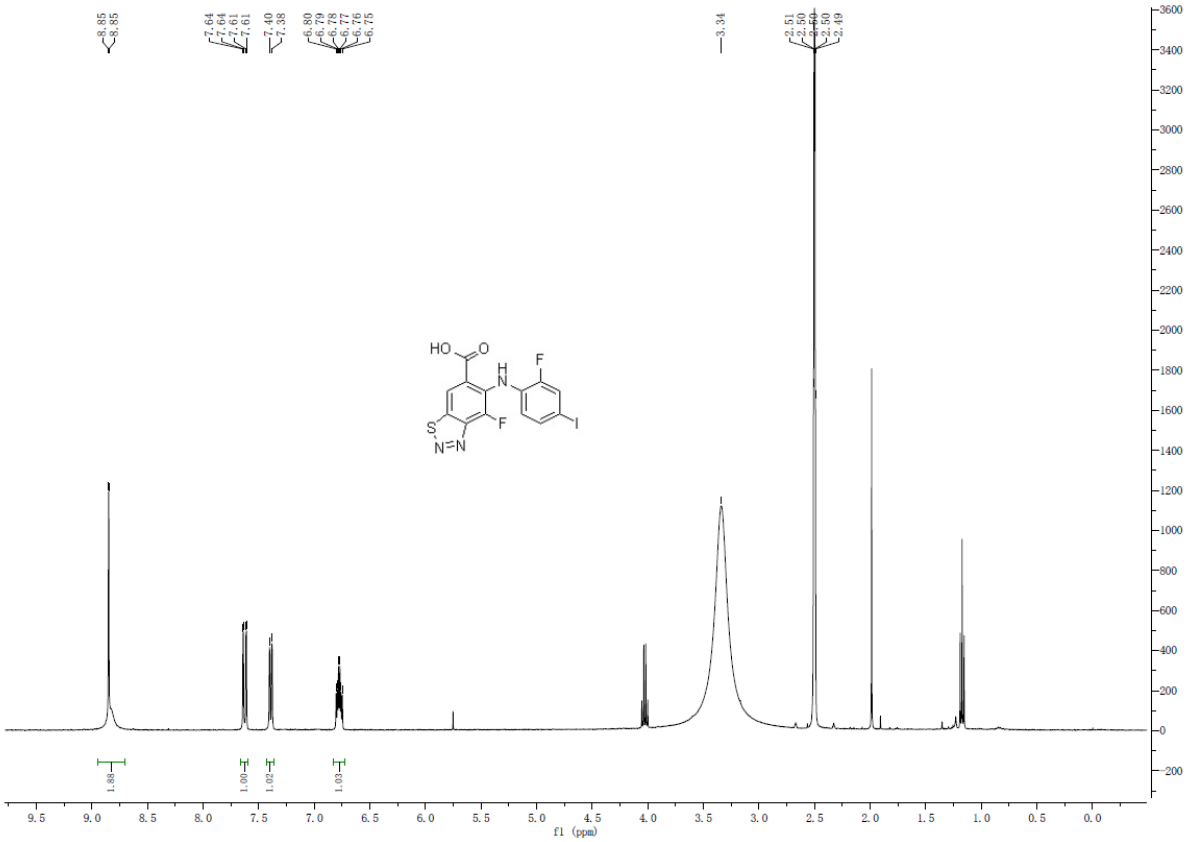
MS spectra of compound **7**



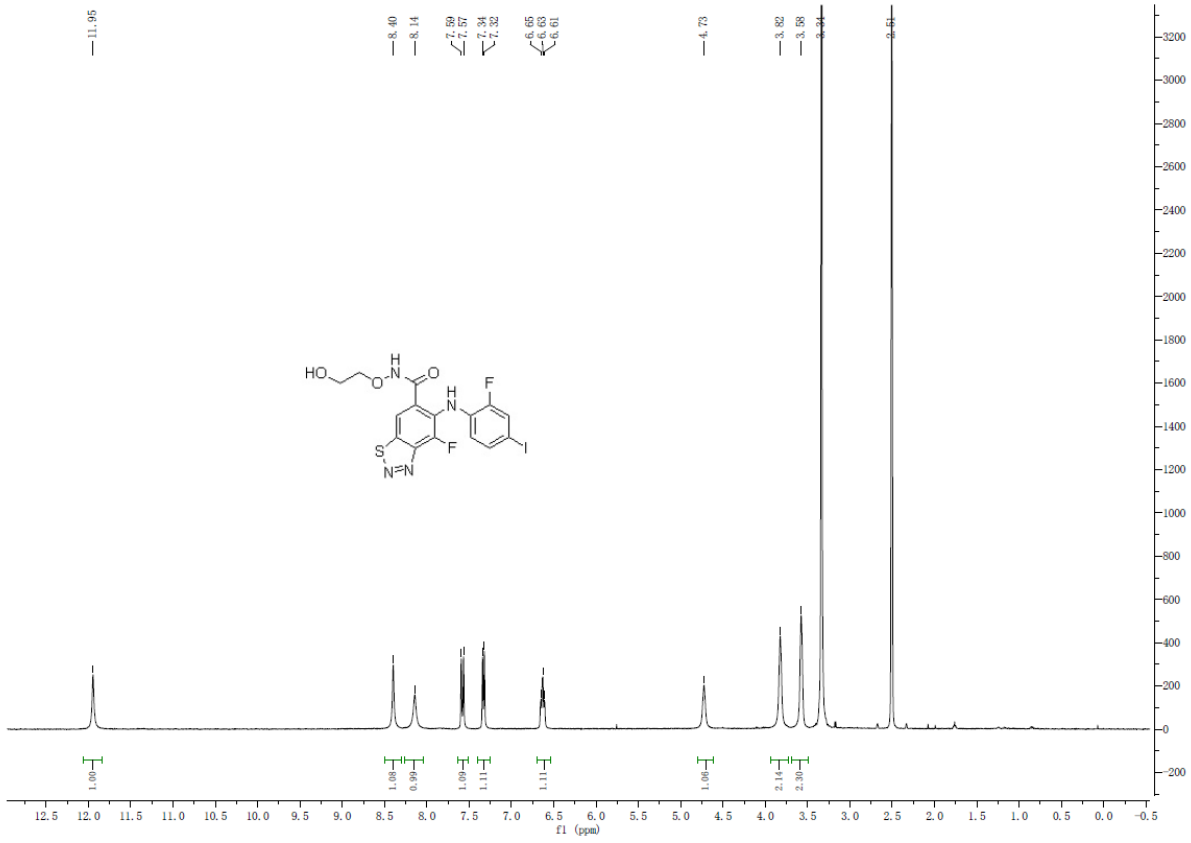
^{1}H NMR spectra of compound **8**



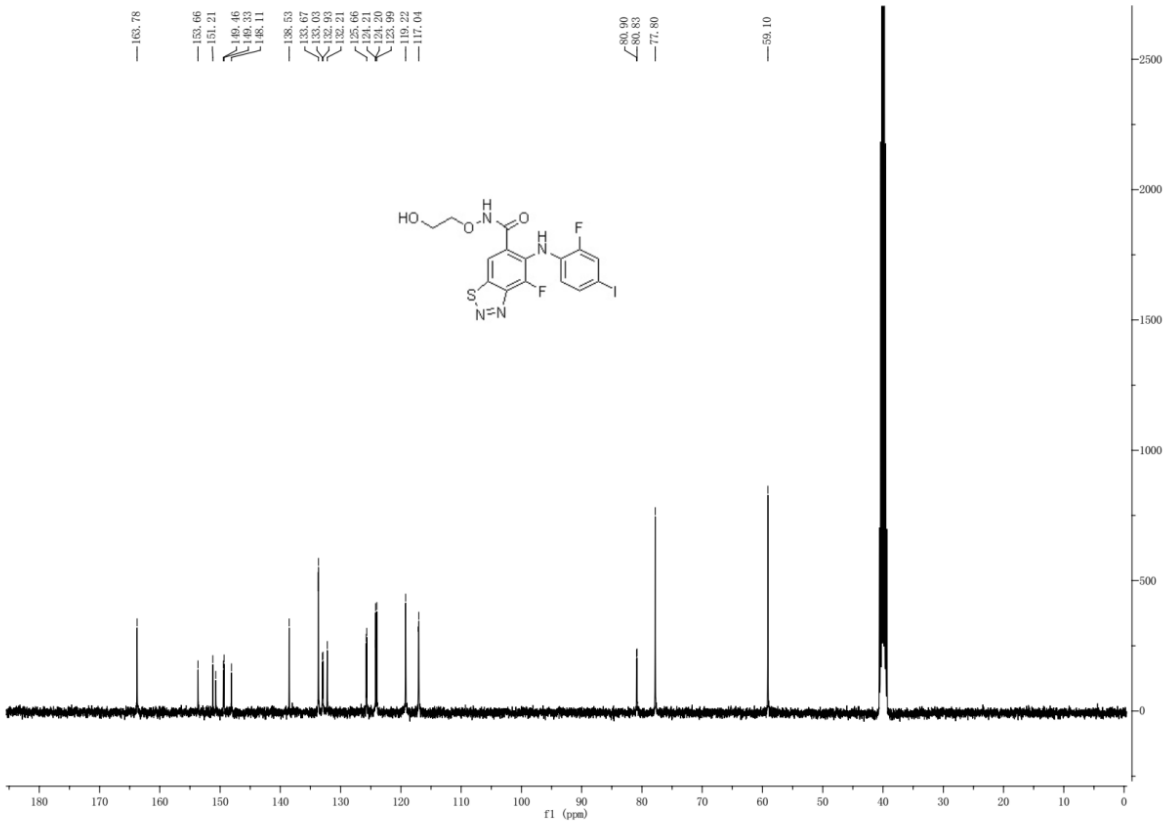
^{1}H NMR spectra of compound **9**



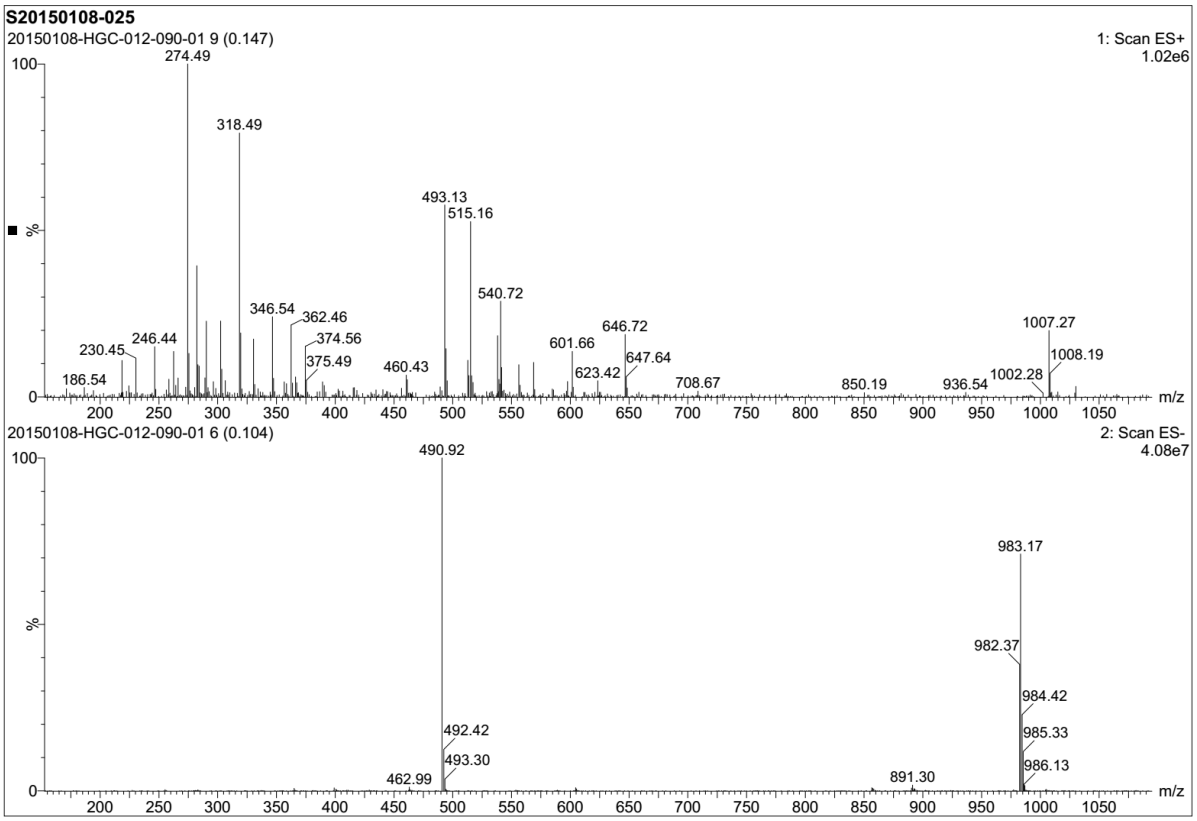
^{1}H NMR spectra of **KZ-02**



^{13}C NMR spectra of **KZ-02**



MS spectra of **KZ-02**



HRMS Spectra of **KZ-02**

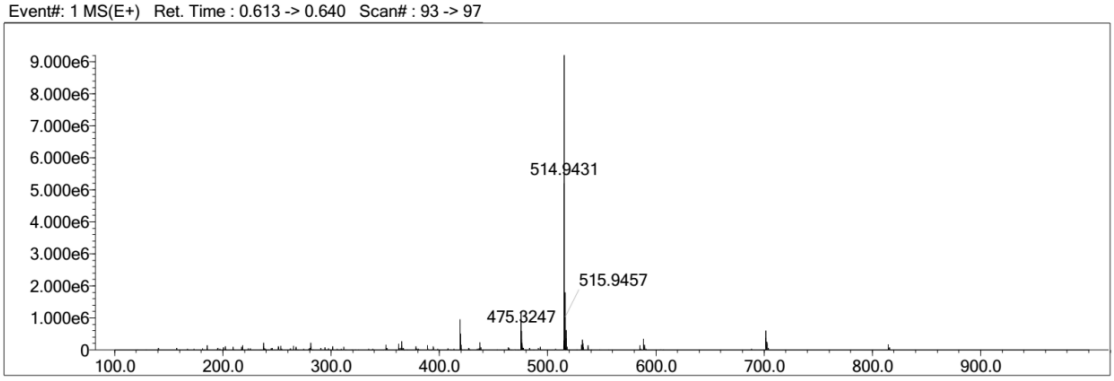
Elmt	Val.	Min	Max	Elmt	Val.	Min	Max	Elmt	Val.	Min	Max	Elmt	Val.	Min	Max	Use Adduct
H	1	0	11	18O	2	0	0	P	3	0	0	Se	2	0	0	H
B	3	0	0	F	1	2	2	S	2	1	1	Br	1	0	0	Na
C	4	0	15	Na	1	0	0	Cl	1	0	0	I	3	1	1	K
N	3	0	4	Mg	2	0	0	Ti	2	0	0					NH4
O	2	0	3	Si	4	0	0	Fe	2	0	0					Br

Error Margin (ppm): 500
HC Ratio: 0.0 - 3.0
Max Isotopes: all
MSn Iso RI (%): 75.00

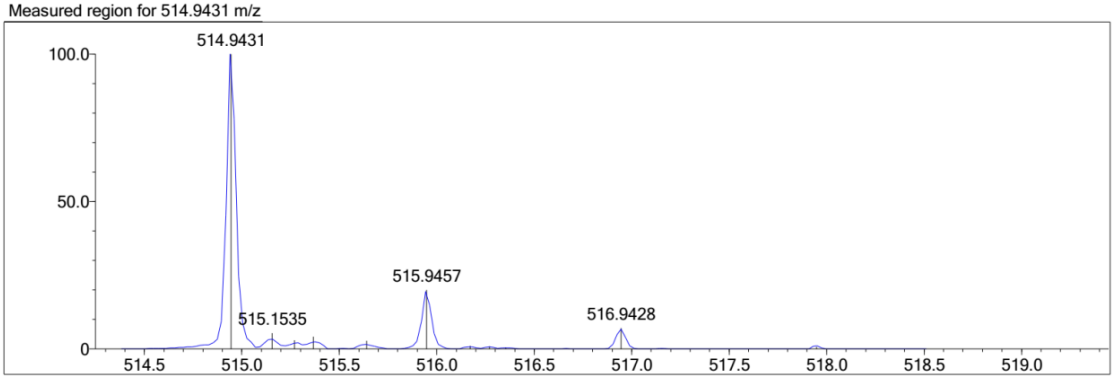
DBE Range: 0.0 - 50.0
Apply N Rule: yes
Isotope RI (%): 1.00
MSn Logic Mode: AND

Electron Ions: both
Use MSn Info: yes
Isotope Res: 10000
Max Results: 50

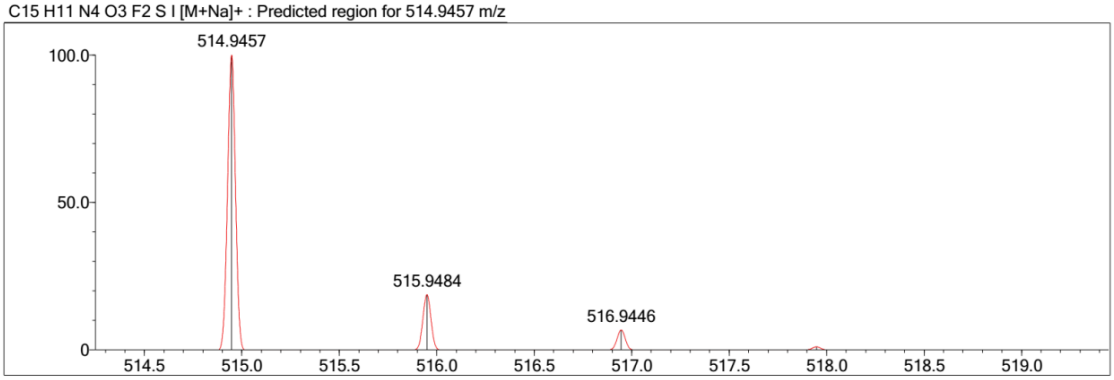
Event#: 1 MS(E+) Ret. Time : 0.613 -> 0.640 Scan# : 93 -> 97



Measured region for 514.9431 m/z



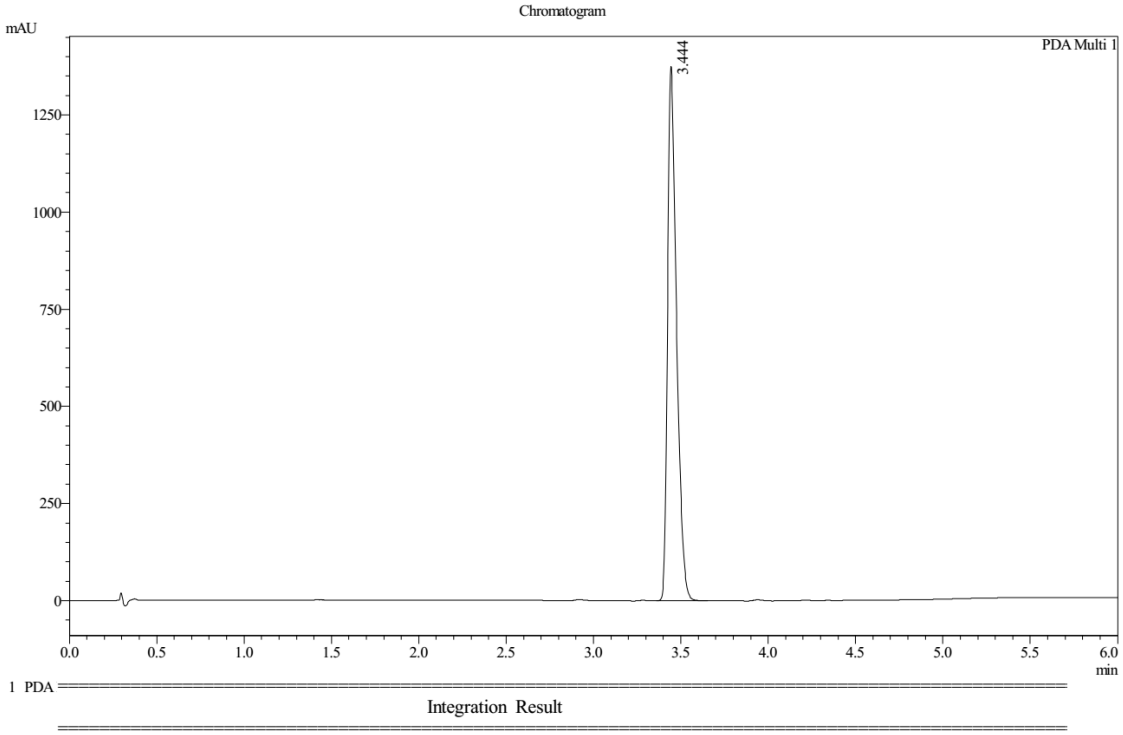
C15 H11 N4 O3 F2 S I [M+Na]+ : Predicted region for 514.9457 m/z



Rank	Score	Formula (M)	Ion	Meas. m/z	Pred. m/z	Df. (mDa)	Df. (ppm)	Iso	DBE
1	76.57	C15 H11 N4 O3 F2 S I	[M+Na]+	514.9431	514.9457	-2.6	-5.05	85.55	12.0

HPLC Spectra of **KZ-02**

Instrument & Column: HPLC-01(11#-603) Xbriage RP18, 5um



PDA Ch1 220nm 4nm

Peak#	Ret. Time	Height	Height %	USP Width	Area	Area %
1	3.444	1335027	100.000	0.097	4925937	100.000

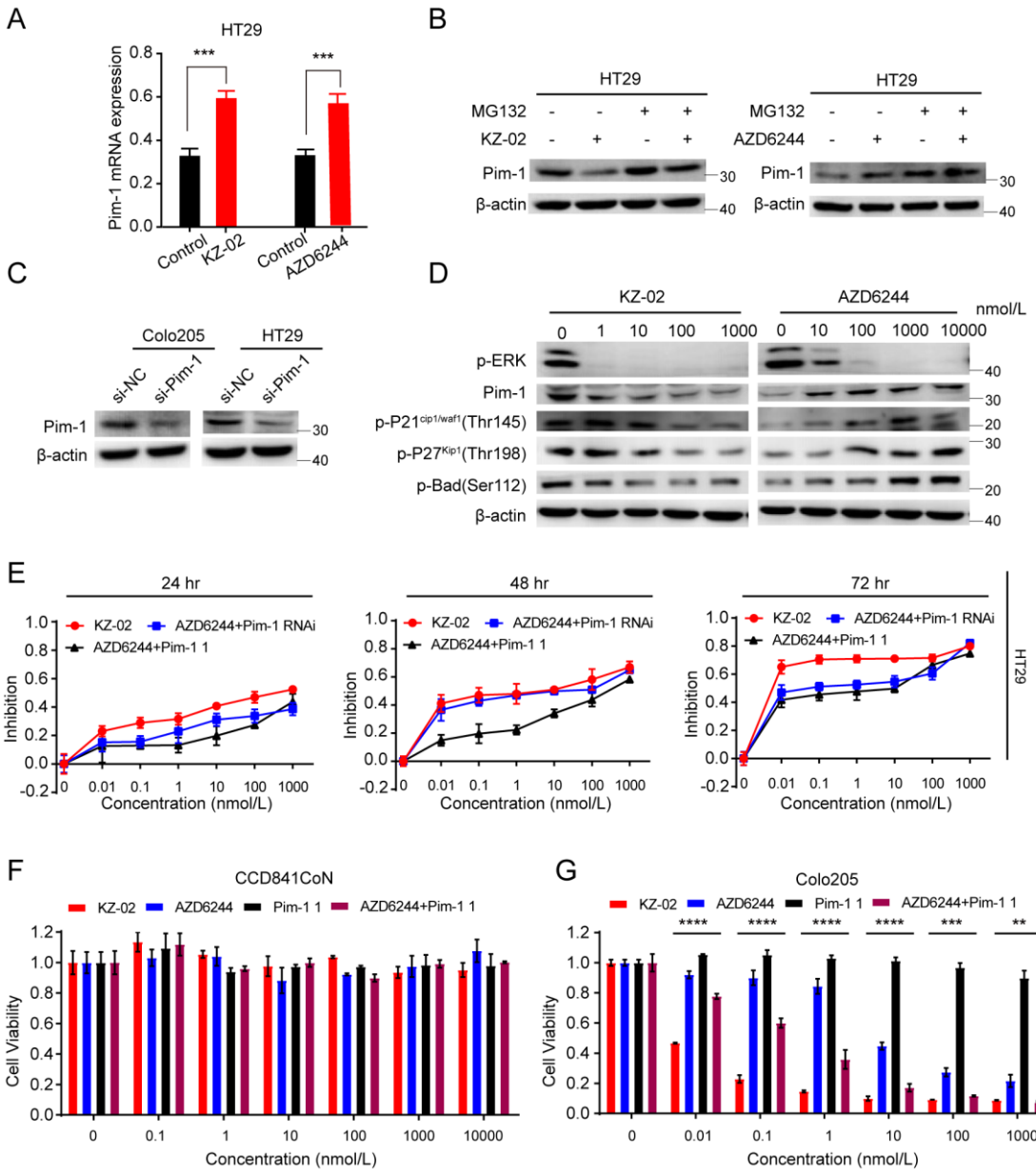


Figure S2. KZ-02 Inhibits Pim-1 Activation in HT29 Cells and Specifically Targets Tumor Cells. Related to Figure 7.

(a, b, d, e) Same experiments in HT29 cells as showed in Figures 7b-7e, which were in Colo205 cells. Error bars are based on the standard deviations of triplicate samples.
(c) Pim-1 protein levels after being knocked down were detected by western blot analysis.
(f, g) Benign colon cell CCD841CoN (f) or malignant colon cell Colo205 (g) were treated with various concentrations of KZ-02, AZD6244, Pim-1 1 or AZD6244+Pim-1 1 for 48 h, and cell proliferation inhibition was then determined by MTT assay. Error bars are based on the standard deviations of triplicate samples. Data are represented as mean ± SD. Comparisons of two groups are performed with Student's t-test. *P<0.05, **P<0.01, ***P<0.001,****P<0.0001.

SUPPLEMENTAL TABLE

Table S1. *In Vitro* Kinase Selectivity Screening for KZ-02. Related to Figure 3.

Kinase	% inhibition	Kinase	% inhibition	Kinase	% inhibition	Kinase	% inhibition
MEK1/MAP2K1	**100**	ERK2 (P42MAPK)	21	Ron kinase	6	IGF1R kinase	6
ALK	-1	ERK5 (MAPK7)	14	Src kinase	-8	IRK (InsR)	3
AKT1/PKBalpha	2	FAK	34	TIE2 kinase	3	IRR kinase	15
AKT2/PKBbeta	4	FGFR1 kinase	10	mTOR kinase (FRAP1)	1	JAK1	18
AKT3/PKBgamma	7	FGFR2 kinase	22	TRKA	-2	JAK2	4
ALK4 (ACVR1B)	33	MusK	26	TRKB	11	JAK3	9
AurA/Aur2 kinase	26	p38alpha kinase	7	TRKC	23	JNK1	16
AurC/Aur3 kinase	0	p38beta 2 kinase	-7	Tyk2 (JTK1)	11	JNK2	38
Axl kinase	1	p70S6K	15	MKK6 /p38alpha	26	JNK3	18
CHK1	-2	p70S6Kbeta	13	FGFR3 kinase	17	KDR kinase (VEGFR2)	3
CHK2	8	PDGFRalpha kinase	6	Tyro3 /Sky kinase	25	Lck kinase	-14
c-kit kinase	15	PDGFRbeta kinase	-1	FGFR4 kinase	4	LTK	-10
c-Met kinase	14	**Pim-1 kinase**	**89**	FLT-1 kinase (VEGFR1)	20	Lyn A kinase	3
DDR2 kinase	6	Pim-2 kinase	35	FLT-3 kinase	-4	Lyn B kinase	-2
EGFR kinase	20	PKA	-2	FLT-4 kinase (VEGFR3)	27	MEK5 (MAP2K5)	16
EphA1 kinase	7	PKCalpha	-15	Fms/CSFR kinase	14	Abl kinase	-7
EphA2 kinase	-15	RAF-1 kinase	33	GSK3alpha	64	Mer kinase	-2
EphB1 kinase	10	Ret kinase	10	GSK3beta	16		
EphB2 kinase	5	ROCK1	1	HER2/ErbB2 kinase	-29		
ERK1	1	ROCK2	2	HER4/ErbB4 kinase	3		

TRANSPARENT METHODS

Inhibitors, antibodies and reagents. KZ-02 was synthesized according to the scheme in the **Figure S1.** AZD6244 (MEK inhibitor, catalog No.S1008, purity >98%) was purchased from Selleck Chemicals. Pim-1 1 (Pim-1 inhibitor, sc-204330, purity >98%) was purchased from Santa Cruz Biotechnology. Compound C (AMPK inhibitor, S7840, purity >98%) was purchased from Selleck Chemicals. Recombinant human IL-23 (catalog#200-23, Lot#07125227, 10 μg) was purchased from PeproTech.

Immunoblotting was performed using the following antibodies: β-actin (sc-47778, Santa Cruz, dilution 1:5000), phospho-ERK1/2 (Thr202/Tyr204) (#4370, Cell Signaling, dilution 1:2000), ERK1/2 (#4695, Cell Signaling, dilution 1:1000), phospho-AMPK (Thr172) (#2535S, Cell Signaling, dilution 1:1000), AMPK (#5831S, Cell Signaling, dilution 1:1000), phospho-STAT3 (Tyr705) (#4113S, Cell Signaling, dilution 1:1000), STAT3 (#1264S, Cell Signaling, dilution 1:1000), Pim-1 (sc-28777, Santa Cruz, dilution 1:200), phospho-Cdc25C (Ser216) (#4901, Cell Signaling, dilution 1:1000), Cdc25C (#4688, Cell Signaling, dilution 1:1000), and GAPDH (#AM1020B, ABGENT, dilution 1:2000).

Lipofectamine®3000 transfection reagent (#L-3000-015) was purchased from Thermo Fisher Scientific. McCoy's 5A cell culture medium, 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide (MTT), SABC immunohistochemical kit, DAB color-developing reagent, methanol, isopropyl alcohol, dimethyl sulfoxide (DMSO) and other reagents were purchased from Sigma.

Cell lines and culture. All cells were originally purchased from the American Type Culture Collection (ATCC). Colo205 human colon cancer cells were cultured in Roswell Park Memorial Institute 1640 (RPMI-1640, Invitrogen) medium supplemented with 10% inactivated FBS. HT29 human colon cancer cells were grown in McCoy's 5A medium (Sigma) with the same supplementation. CCD841CoN human normal colon epithelial cells were maintained according to the recommendations of ATCC. All cells were routinely screened to avoid mycoplasma contamination and maintained in a humidified chamber with 5% CO_2 at 37^{o}C for up to 1 week after thawing before cell activity analysis or injection in mice.

Animals and tumor models. All mouse procedures were performed in accordance with institutional protocol guidelines at Shantou University. Male nude mice around 4 weeks obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. (China) were fed at laboratory animal center of Shantou University Medical College. Mice were treated with Colo205 or HT29 cells to

construct the tumor models. When the volume of each tumor was around 200 mm^3, sequential animal studies were carried out.

Clinical samples. Twenty paraffin-embedded specimens of CRC preserved at the Department of Pathology of Shantou University Medical College Cancer Hospital between January 2010 and August 2015 were included in this study. Specimens were examined for histological grade based on World Health Organization criteria. All specimens were fixed with paraformaldehyde, embedded in paraffin, and prepared as serial slices of 4 μm in thickness. All experimental subjects had complete clinical pathological data and were aged 45-75 years (mean: 58.65), and there were no significant differences between age groups. No patients received radiotherapy, chemotherapy, biotherapy, or any other operation before surgery for the cancer. The pathological diagnosis was performed by experts at the Department of Pathology of Shantou University Medical College Cancer Hospital. All procedures involved in this clinical trial were in compliance with the ethical regulations. Any study-related clinical procedures and the acquisition of clinical samples or data were performed after the patient signed an approved informed consent. Patient samples were collected according to the study protocol approved by the institutional review boards of Shantou University Medical College Cancer Hospital.

Preparation of KZ-02. The target compound was synthesized under 11 steps. The initial compounds of 1-bromo-2,3,4-trifluorobenzene and 2-fluoroaniline were purchased from Shanghai Bidepharmatech Chemical.

<Step 1: 5-bromo-2,3,4-trifluorobenzoic acid (Compound1)>Under N_2 atmosphere, 1-bromo-2,3,4-trifluorobenzene (13.64 g, 64.6 mmol) was dissolved in 120 mL anhydrous THF. The solution was cooled to –78 ^oC, and then LDA (2.0 M in THF, 33.9 mL, 67.97 mmol) was added dropwise. The mixture was stirred at this temperature for an additional 1 h, and then poured into dry ice carefully. The mixture was warmed to r.t and stirred for 1 h. The pH of the solution was adjusted to 2 – 3 with 10% HCl (300 mL), and extracted with ethyl acetate (200 mL×3). The organic phase was washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was washed with PE, filtrated, and dried to obtain compound **1** as a white solid (13.50 g, 82%). ^1H NMR (400 MHz, DMSO-d_6) δ 13.94 (s, 1H), 7.98 (t, J = 75.2 Hz, 1H).

<Step 2: 5-bromo-3,4-difluoro-2-((2-fluorophenyl)amino)benzoic acid (Compound 2)>Under N_2 atmosphere, to the solution of compound **1** (13.50 g, 52.9 mmoL) and 2-fluoroaniline (10.2 mL, 105.8 mmol) in 120 mL anhydrous THF at –78 ^oC, LiHMDS (1M in THF, 158.7 mL, 158.7 mmol) was added dropwise. The mixture was slowly warmed to r.t and stirred for 8 h. The mixture was quenched with 10% HCl

(aq., 100 mL) at 0 °C, extracted with ethyl acetate (200 mL×3). The organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was washed with PE, filtrated, and dried yielding to the desired compound **2** as a pale-yellow solid (13.73 g, 75%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.22 (s, 1H), 8.00 (dd, *J* = 7.4, 2.1 Hz, 1H), 7.29 – 7.20 (m, 1H), 7.18 – 7.01 (m, 3H).

<Step 3: Methyl 5-bromo-3,4-difluoro-2-((2-fluorophenyl)amino)benzoate (Compound 3)>A solution of compound **2** (13.73 g, 39.6 mmol) in 60 mL thionyl chloride and 300 mL MeOH was stirred at 85 °C for 12 h. The solution was cooled to room temperature and concentrated to remove most MeOH. The residue was filtrated, washed with MeOH, dried yielding to the desired compound **3** as an off-white solid (12.58g, 90%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.07 (s, 1H), 8.02 (dd, *J* = 7.1, 2.3 Hz, 1H), 7.17 – 6.95 (m, 4H), 3.93 (s, 3H).

<Step 4: Methyl 5-(benzylthio)-3,4-difluoro-2-((2-fluorophenyl)amino)benzoate (Compound 4)>Under N_2 atmosphere, to the solution of compound **3** (12.58 g, 35.80 mmol) in 30 mL 1,4-dioxane, phenylmethanethiol (4.44 g, 35.80 mmoL), DIPEA (9.21 g, 71.60 mmol), Xantphos (2.06 g, 3.56 mmol) and Tris (dibenzylideneacetone)dipalladium (1.63 g, 1.78 mmol) were added. After being stirred at 90°C for 8 h, the mixture was cooled to r.t, filtrated and washed with ethyl acetate. The organic phase was washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified by silica gel column chromatography (PE/EA 50/1, v/v) to obtain the compound **4** as an off-white solid (12.64 g, 88%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.11 (s, 1H), 7.75 (dd, *J* = 7.5, 2.1 Hz, 1H), 7.31 – 7.19 (m, 5H), 7.14 – 6.93 (m, 4H), 4.04 (s, 2H), 3.87 (s, 3H).

<Step 5: Methyl 4-azido-5-(benzylthio)-3-fluoro-2-((2-fluorophenyl) amino) benzoate (Compound 5)>To a solution of compound **4** (12.64 g, 31.36 mmol) in 30 mL DMA, NaN_3 (2.45 g, 37.63 mmol) was added. The mixture was heated to 90 °C and stirred for 3 h. After it was cooled to r.t, the mixture was poured into ice-water, extracted with ethyl acetate (100 mL×3). The organic phase was washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was used for next step without purification (10.38 g, 78%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.96 (s, 1H), 7.73 (s, 1H), 7.27 - 7.23 (m, 3H), 7.12 – 6.89 (m, 6H), 4.05 (s, 2H), 3.87 (s, 3H).

<Step 6: Methyl 4-amino-5-(benzylthio)-3-fluoro-2-((2-fluorophenyl)amino) benzoate (Compound 6)>Under N_2 atmosphere, to the solution of compound **5** (10.38 g, 24.36 mmol) in 100mL MeOH was added 10% Pd/C (1.55 g). The N_2 atmosphere was completely changed to H_2 atmosphere. The mixture was stirred at r.t for 6 hours. The mixture was filtrated and washed with MeOH. The organic phase was concentrated to give the compound **6** as an off-white solid, which was used for next without purification (9.79 g, 100%).

<Step 7: Methyl 4-fluoro-5-((2-fluorophenyl)amino)benzo[d][1,2,3]thiadiazole -6-carboxylate (Compound 7)>To a solution of compound **6** (2.07 g, 5.17 mmol) in acetic acid (60 mL), concentrated HCl (8 mL) and water (6 mL) were added. The mixture was stirred at r.t for 1 h, and then cooled to 5 – 10^{o}C with ice-water batch. The 10 mL solution of $NaNO_2$ (0.43 g, 6.21 mmol) in water was added dropwise to the mixture with the temperature less than 10 ^{o}C. At the end of the drip, the mixture was stirred at this temperature for an additional 3 h, and then adjusted the pH to 8 with sat. $NaHCO_3$, extracted with ethyl acetate (30 mL×3). The organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified by silica gel column chromatography (PE/EA 5/1) yielding to the compound **7** as a yellow solid (1.53 g, 92.1%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.73 (s, 1H), 8.55 (d, *J* = 1.5 Hz, 1H), 7.17 – 7.10 (m, 1H), 7.10 – 7.04 (m, 1H), 7.04 – 6.95 (m, 2H), 4.01 (s, 3H). MS (ESI): m/z: 322.31 $[M+H]^+$.

<Step 8: Methyl 4-fluoro-5-((2-fluoro-4-iodophenyl)amino)benzo[d][1,2,3] thiadiazole-6-carboxylate (Compound 8)>To a solution of compound **7** (1.53 g, 4.77 mmol) in 10 mL DMF, NIS (1.18 g, 5.24 mmol) and trifluoroacetic acid (0.5 mL) was added. The mixture was stirred at r.t for 4 h, and then quenched with sat. aq NH_4Cl, extracted with ethyl acetate (30 mL×3). The organic phase was washed with brine (30 mL×3), dried over anhydrous Na_2SO_4, filtered and concentrated to obtain the compound **8** as a yellow solid (1.90 g, 89%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.71 (s, 1H), 8.56 (d, *J* = 1.5 Hz, 1H), 7.46 (dd, *J* = 10.3, 1.9 Hz, 1H), 7.37 (d, *J* = 8.5 Hz, 1H), 6.71 (td, *J* = 8.6, 5.9 Hz, 1H), 4.01 (s, 3H).

<Step 9: 4-fluoro-5-((2-fluoro-4-iodophenyl)amino)benzo[d][1,2,3]thiadiazole-6 -carboxylic acid (Compound 9)>The compound 8 (1.90 g, 4.25 mmol) was dissolved in the THF/MeOH = 4/1(v/v) (20 mL), and then10 mL of 1M LiOH (10.00 mmol) was added to the solution. The mixture was stirred at r.t for 2 h. Adjusted the pH to 3–4 with 10% HCl, and extracted with ethyl acetate. The organic phase was washed with brine, dried over anhydrous Na2SO4, filtered and concentrated to obtain the compound **9** as a brown yellow solid (1.70 g, 94.0%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.85 (d, *J* = 0.9 Hz, 2H), 7.63 (dd, *J* = 10.9, 1.9 Hz, 1H), 7.39 (d, *J* = 8.5 Hz, 1H), 6.78 (td, *J* = 8.9, 5.0 Hz, 1H).

<Step 10: 4-fluoro-5-((2-fluoro-4-iodophenyl)amino)-N-(2-(vinyloxy)ethoxy)benzo [d][1,2,3]thiadiazole-6-carboxamide (Compound 10)>To a solution of compound **9** (1.7 g, 5.54 mmoL) in 20 mL DCM, EDCI (1.59 g, 8.31 mmol) and HOBT (1.21 g, 8.31 mmol) were added. The solution was stirred at r.t for 10 min and then O-(2-(vinyloxy) ethyl) hydroxylamine (0.69 g, 6.65 mmol) was added. The mixture was stirred at r.t for 4 h, and then quenched with sat. aq NH_4Cl (20 mL), extracted with DCM (15 mL×3). The organic phase was washed with brine, dried over

anhydrous Na_2SO_4, filtered and concentrated to obtain the compound **10** (2.44g, 85%), which was used for the next step without further purification.

<Step 11: 4-fluoro-5-((2-fluoro-4-iodophenyl)amino)-N-(2-hydroxyethoxy) benzo[d] [1,2,3] thiadiazole-6-carboxamide (KZ-02)> To a solution of compound **10** (410 mg, 0.79 mmoL) in 5 mL DCM, 5.0 mL of 1 M HCl (5.0 mmol) was added. The mixture was stirred at r.t for 1 h. The mixture was diluted with sat. aq $NaHCO_3$, extracted with DCM. The organic phase was washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified by silica gel column chromatography (DCM/MeOH 20/1) yielding to the final compound **KZ-02** as a yellow solid (343 mg, 88%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.95 (s, 1H), 8.40 (s, 1H), 8.14 (s, 1H), 7.58 (d, *J* = 11.1 Hz, 1H), 7.33 (d, *J* = 8.4 Hz, 1H), 6.63 (t, *J* = 7.5 Hz, 1H), 4.73 (s, 1H), 3.82 (s, 2H), 3.58 (s, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 163.78, 153.66 - 151.21 , 150.71-148.11, 149.39 , 148.11 , 138.53 , 133.67, 132.21, 125.71 , 124.10 , 119.22, 117.04, 80.86, 77.80 , 59.10 .. MS (ESI): m/z: 493.13 $[M+H]^+$; HRMS, $[M+Na]^+$ for $C_{15}H_{11}F_2IN_4O_3S$, calcd, 514.9457; found, 514.9431. HPLC: 100%.

The synthetic route was shown schematically in **Figure S1.** The target compound and key intermediates had been precisely characterized by reasonable testing methods including ^{1}H NMR (nuclear magnetic resonance), ^{13}C NMR, MS (mass spectrum), HRMS (high resolution mass spectrum), or HPLC (high performance liquid chromatography). The results were shown in Data S1.

***In vitro* cell proliferation assay.** MTT assays were performed using a Cell Proliferation Kit (Sigma) following the supplier's instructions. Briefly, Colo205 cells (2×10^3) were inoculated into each well of a 96-well plate. After being cultured for 24 h, the cells were treated with 0.01 nmol/L to 1000 nmol/L of KZ-02, AZD6244, Pim-1 1, AZD6244+Pim-1 1 (1:1) or DMSO control for 24, 48 and 72 h. Medium was then changed and replaced with 100 μL of fresh growth medium with 10% FBS and 20 μL of 5 mg/mL MTT solution. Cells were incubated for another 4 h, and the medium was replaced with 150 μL of DMSO. After 15 min of incubation at 37ºC, the optical absorbance at 570 nm was measured using a micro-plate reader. Results were presented as the percentage of cell inhibition. Data collected at 0 nmol/L was set to 0 in each panel.

The coefficient of drug interaction (CDI) was used to analyze the synergistic inhibitory effect of drug combinations and calculated according to the reported literature (Wang et al., 2008). CDI was calculated as follows: CDI = AB/(A ×B). According to the absorbance of each group, AB was the ratio of the combination group to the control group, A or B was the ratio of the single agent groups to the control group. Thus, a CDI value less than, equal to or greater than 1 indicates that the

drugs are synergistic, additive or antagonistic, respectively. A CDI of less than 0.7 indicates that the drugs are significantly synergistic.

***In vivo* mouse tumor injection and growth assay.** All animal experiments strictly adhered to local and federal regulations, and were approved by the local authorities before initiation. Balb/c mice weighing 20 g were inoculated subcutaneously with 1×10^7 Colo205 or HT29 cells in 100 μL of a serum-free media and Matrigel mixture (BD Biosciences, 1:1). When the tumors reached about 200 mm^3, tumor-bearing mice were separated into treatment groups of eight mice each, and the indicated inhibitor treatments were performed by oral gavage once daily (QD) for 20 days. The treatment groups consisted of vehicle (20% sulfobutyl ether-β-cyclodextrin (SBE-β-CD) in water), KZ-02 (1 mg/kg for Colo205), AZD6244 (10 mg/kg for Colo205, 20 mg/kg for HT29), Pim-1 1 (20 mg/kg for HT29), and AZD6244+Pim-1 1 (20 mg/kg for HT29). Tumor sizes were recorded every 3 days for 21 days by caliper, and volumes were calculated using the formula of (length × $width^2$)/2. Body weights were also determined at the same time points.

Kinase selectivity screening and kinase 50% inhibitory concentration (IC_{50}) determination *in vitro*. Screening for inhibitory activity against various kinases and the examination of IC_{50} of KZ-02 and AZD6244 against MEK1 and Pim-1 were completed by Cerep Drug Discovery Services Co. LTD (France).

Western blot analysis. Immunoblot analysis was performed as previously reported (Landriscina et al., 2010) and improved appropriately. Cells were washed with ice-cold PBS (phosphate-buffered saline), and resuspended in lysis buffer (20 mmol/L Tris(hydroxymethyl)aminomethane-HCl, pH=7.5, containing 1% sodium dodecyl sulfate (SDS), 50 mmol/L NaCl, 1 mmol/L ethylenediaminetetraacetic acid, 1 mmol/L phenylmethylsulfonyl fluoride, 10 mmol/L sodium fluoride, and 1 mmol/L sodium orthovanadate) by agitation at 4 ℃ for 1 h followed by centrifugation at 4 ℃, 11000 g for 15 min. Equal amounts (20 μg protein) of supernatant were then subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and the separated proteins transferred to nitrocellulose membranes. Membranes were routinely blocked in 5% nonfat milk in Tris-buffered saline (TBS) with 0.1% Tween-20 (TBST) for 1 h at room temperature with agitation and washed. Primary antibodies, including phospho-ERK1/2 (Thr202/Tyr204), ERK1/2, phospho-AMPK (Tyr172), AMPK, phospho-STAT3 (Tyr705), STAT3, Pim-1, phospho-Cdc25C (Ser216), Cdc25C, β-actin, and GAPDH, were added (diluted in 5% bovine serum albumin (BSA) in TBST). Membranes were incubated overnight at 4 ℃ with agitation, washed, and then incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000 dilution in 5% BSA in TBST) for 1 h at room temperature. Proteins were detected

using Enhanced Chemiluminescence Western Blotting Detection Reagent (GE Healthcare). Results were visualized and quantified by an ImageQuant LAS 4000 (GE Healthcare). Protein analysis of tumor tissue lysate was conducted following the same protocol, using protein extraction from tumors pretreated by AZD6244, Pim-1 1, or their combination for 15 days with blank vehicles as control.

Immunohistochemistry (IHC). Formalin-fixed paraffin-embedded tissue slices were treated according to manual of the SABC immunohistochemical kit, and results were analyzed using a double-blind method. Five high-power fields (×400) were selected at random, and two pathologists evaluated scores independently. PBS, instead of the primary antibody, was used as negative control, and specimens were scored according to the intensity of the dye color and the number of positive cells. The intensity of the dye color was graded as 0 (no color), 1 (light yellow), 2 (light brown), or 3 (brown), and the number of positive cells was graded as 0 (<1%), 1 (1-24%), 2 (25-49%), 3 (50-74%), or 4 (75-100%). The two grades were added together and specimens were assigned to one of 4 levels: 0-2 scores (-), 3-5 scores (+), 6-8 scores (++), 9-12 scores (+++). The positive expression rate was expressed as the percent of the addition of (++) and (+++) to the total number.

Quantitative real-time PCR (RT-PCR). RT-PCR was performed as described previously (Maddalena et al., 2011). Total RNA was extracted from cultured cells with TRIzol, and reverse transcription of purified RNA was performed using oligo (dT) priming and Superscript III reverse transcription according to the manufacturer's instructions (Invitrogen). Quantification of all gene transcripts was performed by quantitative PCR using the SYBR Premix kit (TaKaRa) and a Rotor-Gene RG-3000A apparatus (Corbett Research). The gene expressions were all normalized by GAPDH. Data were analyzed by applying the $2^{-\Delta C_T}$ calculation method. The primer pairs used for target gene amplification were as follows: human Pim-1: forward 5'-CCGAGTGTATAGCCCTCCAG-3', and reverse 5'-GGGCCAAGCACCATCTAATG-3', human IL-8: forward 5'-TTCAGAGACAGCAGAGCACA-3', and reverse 5'-AGCACTCCTTGGCAAAACTG-3', human IL-10: forward 5'-AAGGCGCATGTGAACTCCC-3', and reverse 5'-ACGGCCTTGCTCTTGTTTTC-3', human IL-16: forward 5'-TAGAATCTACAGCAGAGGCCA-3', and reverse 5'-TTTGTTCTGAGGCTGCTCCTT-3', human IL-23: forward 5'-CTCTGCTCCCTGATAGCCCT-3', and reverse 5'-TGCGAAGGATTTTGAAGCGG-3', GAPDH: forward 5'-AGGTCGGAGTCAACGGATTT-3', and reverse

5’-ATCTCGCTCCTGGAAGATGG-3’.

Transfection. siRNAs specific for ERK1, ERK2, Pim-1, and IL-23 used in this study were previously described (Ma et al., 2017; Steinmetz et al., 2004; Zhang et al., 2010) and synthesized by Shanghai GenePharma Co. Ltd. with the following target sequences: 5’-GACCGGAUGUUAACCUUUA-3’, 5’-GGUGUGCUCUGCUUAUGAU-3’, 5’-AGAACAUCUUGCAUCCAUGGAUGGU-3’, and 5’-AAUCUGCUGAGUCUCCCAGUGGUGA-3’ respectively. SiRNAs were transfected at 100 pmol per well (6-well plate). Cells were inoculated in plates and allowed to attach for 18-24 h, followed by transfection with siRNAs by Lipofectamine RNAiMAX according to the manufacturer’s protocols. At 48 h after transfection, cells were lysed for protein expression analysis by western blot.

Statistical analyses. Statistical analyses were performed on the GraphPad Prism 7 software, using two-sided Student’s t-test and two-way analysis of variance to detect statistically significant differences between groups. Detailed statistical procedure and data distribution used in each experiment was reported in respective figure legend. P values were calculated to visually indicate the statistical results, and the significance was defined as follows: *P<0.05, **P<0.01, ***P<0.001,****P<0.0001.

SUPPLEMENTAL REFERENCES

Landriscina, M., Laudiero, G., Maddalena, F., Amoroso, M.R., Piscazzi, A., Cozzolino, F., Monti, M., Garbi, C., Fersini, A., Pucci, P., *et al.* (2010). Mitochondrial chaperone Trap1 and the calcium binding protein Sorcin interact and protect cells against apoptosis induced by antiblastic agents. Cancer Res. *70*, 6577-6586.

Maddalena, F., Laudiero, G., Piscazzi, A., Secondo, A., Scorziello, A., Lombardi, V., Matassa, D.S., Fersini, A., Neri, V., Esposito, F., *et al.* (2011). Sorcin induces a drug-resistant phenotype in human colorectal cancer by modulating Ca(2+) homeostasis. Cancer Res. *71*, 7659-7669.

Ma, N., Yang, D., Okamura, H., Teramachi, J., Hasegawa, T., Qiu, L., and Haneji, T. (2017). Involvement of interleukin23 induced by Porphyromonas endodontalis lipopolysaccharide in osteoclastogenesis. Mol. Med. Rep. *15*, 559-566.

Steinmetz, R., Wagoner, H.A., Zeng, P., Hammond, J.R., Hannon, T.S., Meyers, J.L., and Pescovitz, O.H. (2004). Mechanisms regulating the constitutive activation of the extracellular signal-regulated kinase (ERK) signaling pathway in ovarian cancer and the effect of ribonucleic acid interference for ERK1/2 on cancer cell proliferation. Mol. Endocrinol. *18*, 2570-2582.

Wang, D., Wang, Z., Tian, B., Li, X., Li, S., and Tian, Y. (2008). Two hour exposure to sodium butyrate sensitizes bladder cancer to anticancer drugs. Int. J. Urol. *15*, 435-441.

Zhang, T., Zhang, X., Ding, K., Yang, K., Zhang, Z., and Xu, Y. (2010). PIM-1 gene RNA interference induces growth inhibition and apoptosis of prostate cancer cells and suppresses tumor progression in vivo. J. Surg. Oncol. *101*, 513-519.