Supplementary Material

Discovery of IDO1 and DNA Dual Targeting Antitumor Agents

Kun Fang[†ab], Shanchao Wu[†b], Guoqiang Dong[b], Ying Wu[b], Shuqiang Chen[b], Jianhe Liu[*d], Wei Wang[*ac] and Chunquan Sheng[*b]

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Chemical Synthesis and Structural Characterization of the Intermediates and Target Compounds

General. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker AVANCE300 and AVANCE600 spectrometer (Bruker Company, Germany), using TMS as an internal standard and CD_3OD or DMSO-d_6 as solvents. Chemical shift are given in ppm (δ). Elemental analyses were performed with a MOD-1106 instrument and were consistent with theoretical values within 0.4%. The mass spectra were recorded on an Esquire 3000 LC-MS mass spectrometer. TLC analysis was carried out on silica gel plates GF254 (Qingdao Haiyang Chemical, China). Silica gel column chromatography was performed with Silica gel 60 G (Qingdao Haiyang Chemical, China). Unless otherwise noted, all materials were obtained from commercial suppliers and used without further purification. Chemical names were created using ChemDraw Ultra 10.0 software.

***N*,*N*-dimethyl-1-(5-nitro-1*H*-indol-3-yl)methanamine (7a).** A solution of dimethylamine (12.5 g, 0.09 mol) and formaldehyde (7.2 mL, 0.08 mol) in acetic acid (50 mL) was stirred at 0 °C for 30 min. To this solution was added **6a** (10 g, 0.06 mol) slowly. After stirring for 3 days at room temperature, the mixture was poured into ice-water (200 mL) and the pH of the solution was adjusted to 13 by 50% aqueous NaOH. The solid formed was collected by filtration, washed with water and dried to give the desired product (9.6 g, 73 %) as a yellow solid. ^{1}H NMR (DMSO-d_6, 600 MHz) δ : 11.67 (s, 1H), 8.61 (d, J = 2.3 Hz, 1H), 8.00 (dd, J = 9.0, 2.4 Hz, 1H), 7.54 (d, J = 9.0 Hz, 1H), 7.52 (s, 1H), 3.61 (s, 2H), 2.18 (s, 3H).

Diethyl 2-acetamido-2-((5-nitro-1*H*-indol-3-yl)methyl)malonate (8a). NaOH powder (0.5 g, 11.4 mmol) was added to a stirred solution of **7a** (5 g, 22.8 mmol) and diethyl 2-acetamidomalonate (5.5 g, 25.1 mmol) in anhydrous tobuene (100 mL). The resulting solution was stirred to reflux for 8 h under nitrogen atmosphere. TLC showed that the reaction was complete. The reaction mixture was washed with hot-water (3 × 100 mL) and cooled to ambient temperature. The precipitate was collected by filtration, washed with water and dried to give the desired product (5.7 g, 64 %) as a yellow solid. ^{1}H NMR (DMSO-d_6, 600 MHz) δ : 11.71 (s, 1H), 8.26 (d, J = 2.2 Hz, 1H), 8.14 (s, 1H), 7.97 (dd, J = 9.0, 2.4 Hz, 1H), 7.51 (d, J = 9.0 Hz, 1H), 7.29 (s, 1H), 4.08-4.15 (m, 4H), 3.66 (s, 2H), 1.92 (s, 3H), 1.16 (t, J = 7.1 Hz, 6H); ESI-MS (m/s): 392.41 [M + H].

Diethyl 2-acetamido-2-((1-methyl-5-nitro-1*H*-indol-3-yl)methyl)malonate (9a). To a solution of **8a** (5 g, 12.8 mmol) in DMF (20 mL), NaH (60% dispersion in mineral oil, 4.4 g, 19.2 mmol) was added slowly at 0 °C, after stirring 0.5 h, MeI (5.5 g, 38.4 mmol) was added. The mixture was further stirred at room temperature overnight and then poured into ice-water (150 mL). Then, the aqueous layer was extracted with ethyl acetate (3 × 100 mL) and the combined organic layers were washed with brine, dried over sodium sulfate and concentrated under reduced pressure. The crude product was purified by column chromatography on SiO_2 (PE: EtOAc = 3: 1) to afford the desired product (4.0 g, yield 77%) as a yellow solid. ^{1}H NMR (DMSO-d_6, 600 MHz) δ : 8.26 (d, J = 2.3 Hz, 1H), 8.16 (s, 1H), 8.03 (dd, J = 9.1, 2.2 Hz, 1H), 7.61 (d, J = 9.0 Hz, 1H), 7.31 (s, 1H), 4.11-4.17 (m, 4H), 3.83 (s, 3H), 3.65 (s, 2H), 1.92 (s, 3H),

1.18 (t, J = 7.0 Hz, 6H); ESI-MS (m/s): 406.43 [M + H].

2-Amino-3-(1-methyl-5-nitro-1*H*-indol-3-yl)propanoic acid (11a).

The solution of **9a** (5 g, 12.3 mmol) in 4% aqueous NaOH (30 mL) and EtOH (30 mL) was heated at 100 °C for 8 h under nitrogen atmosphere. TLC showed that the reaction was complete. The reaction mixture was concentrated to remove EtOH under reduced pressure. The residue was cooled to 0 °C and the pH was slowly adjusted to 2–3 with 2N HCl. The solid formed was collected by filtration, washed with water and dried to give 2-acetamido-3-(1-methyl-5-nitro-1*H*-indol-3-yl)propanoic acid (3.1 g, crude), which was used directly for the next step without further purification.

The solution of 2-acetamido-3-(1-methyl-5-nitro-1*H*-indol-3-yl)propanoic acid (3.1 g, crude) in 6N HCl (50 mL) was refluxed for 8 h. After cooling at 0 °C, the pH of the solution was slowly adjusted to 4–5 with 4N NaOH. The solid formed was collected by filtration, washed with water and dried to afford the desired product (2.0 g, yield 76%) as a yellow solid. ^{1}H NMR (D_2O-d_6, 600 MHz) δ : 8.53 (s, 1H), 8.05 (d, J = 8.8 Hz, 1H), 7.44 (d, J = 9.0 Hz, 1H), 7.31 (s, 1H), 4.05 (t, J = 6.2 Hz, 1H), 3.82 (s, 3H), 3.42 (dd, J = 15.5, 4.8 Hz, 1H), 3.32 (dd, J = 15.4, 7.5 Hz, 1H).

Ethyl 2-amino-3-(1-methyl-5-nitro-1*H*-indol-3-yl)propanoate (12a). To a solution of **11a** (2.0 g, 7.6 mmol) in EtOH (30 mL), $SOCl_2$ (1.1 mL, 15.2 mmol) was added dropwise at 0 °C. The reaction mixture was further refluxed for 3 h. After cooling to ambient temperature, the solid formed was collected by filtration, washed with water and dried to afford the desired product (2.1 g, yield 93%) as a yellow solid. ^{1}H NMR (DMSO-d_6, 600 MHz) δ : 8.66 (s, 2H), 8.54 (d, J = 2.2 Hz, 1H), 8.05 (dd, J = 9.1, 2.3

Hz, 1H), 7.64 (d, *J* = 9.0 Hz, 1H), 7.52 (s, 1H), 4.26 (t, *J* = 5.9 Hz, 1H), 4.06-4.12 (m, 2H), 3.84 (s, 3H), 3.42 (dd, *J* = 15.0, 5.5 Hz, 1H), 3.34 (dd, *J* = 15.0, 7.0 Hz, 1H), 1.12 (t, *J* = 7.1 Hz, 3H); ESI-MS (m/s): 292.27 [M + H].

Ethyl 2-((*tert*-butoxycarbonyl)amino)-3-(1-methyl-5-nitro-1*H*-indol-3-yl)propanoate (13a). Boc anhydride (1.8 g, 8.2 mmol) was added a stirred solution of **12a** (2.0 g, 6.9 mmol) and triethylamine (1.1 mL, 8.2 mmol) in DCM (100 mL) at 0 ℃. The reaction was warmed to ambient temperature and allowed to stir for 12 h. The reaction mixture was washed with 1N HCl (2 × 100 mL) and water (3 × 100 mL). The organic layer was dried over anhydrous Na_2SO_4, filtered, and concentrated to afford the desired product (2.6 g, yield 95%) as a yellow solid. ^{1}H NMR (DMSO-d_6, 600 MHz) δ : 8.45 (d, *J* = 1.7 Hz, 1H), 7.93 (dd, *J* = 8.8, 1.9 Hz, 1H), 7.72 (d, *J* = 8.8 Hz, 1H), 7.60 (s, 1H), 7.90 (d, *J* = 7.9 Hz, 1H), 4.18-4.22 (m, 1H), 4.02-4.09 (m, 2H), 3.90 (s, 3H), 3.06-3.18 (m, 2H), 1.34 (s, 8H), 1.11 (t, *J* = 7.1 Hz, 3H); ESI-MS (m/s): 392.17 [M + H].

Ethyl 3-(5-(bis(2-hydroxyethyl)amino)-1-methyl-1*H*-indol-3-yl)-2-((tert-butoxycarbonyl)ami- no)propanoate (15a). A mixture of **13a** (2.5 g, 6.4 mmol) and 10% Pb/C (0.25 g, 10% weight of **13a**) in ethyl acetate (100 mL) was stirred at room temperature overnight under hydrogen atmosphere. TLC showed that the reaction was complete. Pb/C was removed through filtration and washed twice with ethyl acetate (30 mL). The combined filtrate was concentrated under reduced pressure to afford ethyl 3-(5-amino-1-methyl-1*H*-indol-3-yl)-2-((*tert*-butoxycarbonyl)amino)propanoate (2.2 g, crude) as a colorless oil that was used in the next step without further

purification.

This oily material (2.2 g, crude) and MeOH (30 mL) were added into a thick wall pressure bottle cooled in an ice water bath. Then ethylene oxide (2 mL) was slowly dropwise added with violent stirring. After that, the reaction mixture was stirred at room temperature for further 24 h. TLC showed that the reaction was complete. The solvent was evaporated and the residue was purified by column chromatography on SiO_2 (DCM: MeOH = 100: 5) to afford the desired product (2.6 g, yield 95%) as a brown solid. ^{1}H NMR (DMSO-d_6, 600 MHz) δ : 7.25 (d, J = 8.6 Hz, 1H), 7.05 (d, J = 7.7 Hz, 1H), 6.80 (s, 1H), 6.58 (d, J = 8.5 Hz, 1H), 6.51 (s, 1H), 4.68 (t, J = 5.0 Hz, 2H), 4.09-4.13 (m, 1H), 3.99-4.08 (m, 2H), 3.59 (s, 3H), 3.54-3.57 (m, 4H), 3.42-3.44 (m, 4H), 2.99 (dd, J = 14.6, 5.2 Hz, 1H), 2.89 (dd, J = 14.3, 8.8 Hz, 1H), 1.11 (t, J = 7.1 Hz, 3H); ESI-MS (m/s): 450.23 [M + H].

2-Amino-3-(5-(bis(2-chloroethyl)amino)-1-methyl-1*H*-indol-3-yl)propanoic acid (17). To a stirring solution of compound **15a** (2.5 g, 5.6 mmol) in $CHCl_3$ (100 mL), $POCl_3$ (1.6 mL, 16.7 mmol) was added and the mixture was refluxed for 3 h. TLC showed that the reaction was complete. The solvent was concentrated under reduced pressure to afford ethyl 3-(5-(bis(2-chloroethyl)amino)-1-methyl-1*H*-indol-3-yl)-2-((*tert*-butoxycarbonyl)amino)propanoate (2.7 g, crude) as a brown oil that was used in the next step without further purification.

This oily material (2.7 g, crude) was suspended in 2N HCl (25 mL) and the mixture was then refluxed for 3 h. After cooling at 0 °C, the pH of the solution was slowly adjusted to 4–5 with 2N NaOH. The solid formed was collected by filtration, washed

with water and dried to afford the desired product (1.6 g, yield 80%) as a gray solid. ^{1}H NMR (CD_3OD-d_6, 600 MHz) δ : 7.28 (d, J = 9.0 Hz, 1H), 7.16 (d, J = 1.9 Hz, 1H), 7.03 (s, 1H), 6.89 (dd, J = 9.0, 2.2 Hz, 1H), 3.84 (dd, J = 9.1, 4.0 Hz, 1H), 3.73 (s, 3H), 3.69-3.72 (m, 4H), 3.63-3.67 (m, 4H), 3.43 (dd, J = 15.3, 3.9 Hz, 1H), 3.10 (dd, J = 15.2, 9.2 Hz, 1H); ^{13}C NMR (DMSO-d_6, 150 MHz) δ : 169.74, 140.03, 131.60, 128.90, 128.53, 111.38, 110.23, 107.71, 103.60, 54.71, 53.99, 41.76, 40.05, 32.35, 26.63; HRMS (ESI, positive) m/z calcd for $C_{16}H_{20}Cl_2N_3O_2$ (M-H): 356.0924; found 356.0928.

2-Amino-3-(6-(bis(2-chloroethyl)amino)-1-methyl-1H-indol-3-yl)propanoic acid (18). Compound **18** was synthesized using the method described for the preparation of compound **17** using **6a**. Gray solid (1.1 g, yield 73%). ^{1}H NMR (DMSO-d_6, 600 MHz) δ : 7.41 (d, J = 8.6 Hz, 1H), 6.92 (s, 1H), 6.67 (d, J = 2.0 Hz, 1H), 6.63 (dd, J = 8.8, 2.2 Hz, 1H), 3.71-3.76 (m, 8H), 3.62 (s, 3H), 3.39-3.42 (m, 1H), 3.21 (dd, J = 14.9, 3.9 Hz, 1H), 2.88 (dd, J = 8.7, 14.8 Hz, 1H); ^{13}C NMR (DMSO-d_6, 150 MHz) δ : 170.37, 142.92, 138.79, 127.03, 120.91, 120.05, 109.05, 107.85, 93.86, 55.19, 53.72, 42.05, 32.67, 27.49; HRMS (ESI, positive) m/z calcd for $C_{16}H_{20}Cl_2N_3O_2$ (M-H): 356.0945; found 356.0949.

Protocols for biological assays

IDO1 Enzyme Activity Assay

IDO1 catalyzes the oxidative cleavage of the pyrrole ring of the indole nucleus of tryptophan to yield *N*'-formylkynurenine. The assays were performed at room temperature as described in the literature using 20 nM IDO1 (NOVUS BioscienceInc.

H00003620-P01) and 2 mM D-Trp in the presence of 20 mM ascorbate, 3.5 uM methylene blue and 0.2 mg/mL catalase in 50 mM potassium phosphate buffer (pH 6.5). The initial reaction rates were recorded by continuously following the absorbance increase at 321 nm due to the formation of *N*'-formlylkynurenine. The IC_{50} values were calculated using nonlinear regression with normalized dose-response fit using Prism GraphPad software.

***In Vitro* Cytotoxicity Assay**

This assay was determined by the Cell Counting Kit-8 (CCK-8) method. Cells were plated in 96-well microtiter plates at a density of 6~10 × 10^3/well and incubated in a humidified atmosphere with 5% CO_2 at 37 °C for 24 h. Test compounds were added onto triplicate wells with different concentrations and 0.1% DMSO for control. After they had been incubated for 48 h, 10 μL of the CCK-8 solution was added to each well and the plate was incubated for additional 1-4 h in the incubator. The absorbance (OD) was read on a WellscanMK-2 microplate reader (Labsystems) at 450 nm. The concentration causing 50% inhibition of cell growth (IC_{50}) was determined by the Logit method. All experiments were performed three times.

***In Vitro* Cell Cycle Assay**

LLC (3 × 10^5 /well) cells were incubated in six-well plates (Corning) for 24 h, and then treated with 0.1% DMSO (as control), various concentrations of compounds **17** or **18** for 24 h. The treated cells were collected, resuspended, and incubated for 30 min at 37 °C with 25 μg/mL PI and 10 μg/mL RNase buffer. For each sample, at least 1 × 10^4 cells were analyzed using flow cytometry (BD Accuri C6).

In Vitro Cell Apoptosis Assay

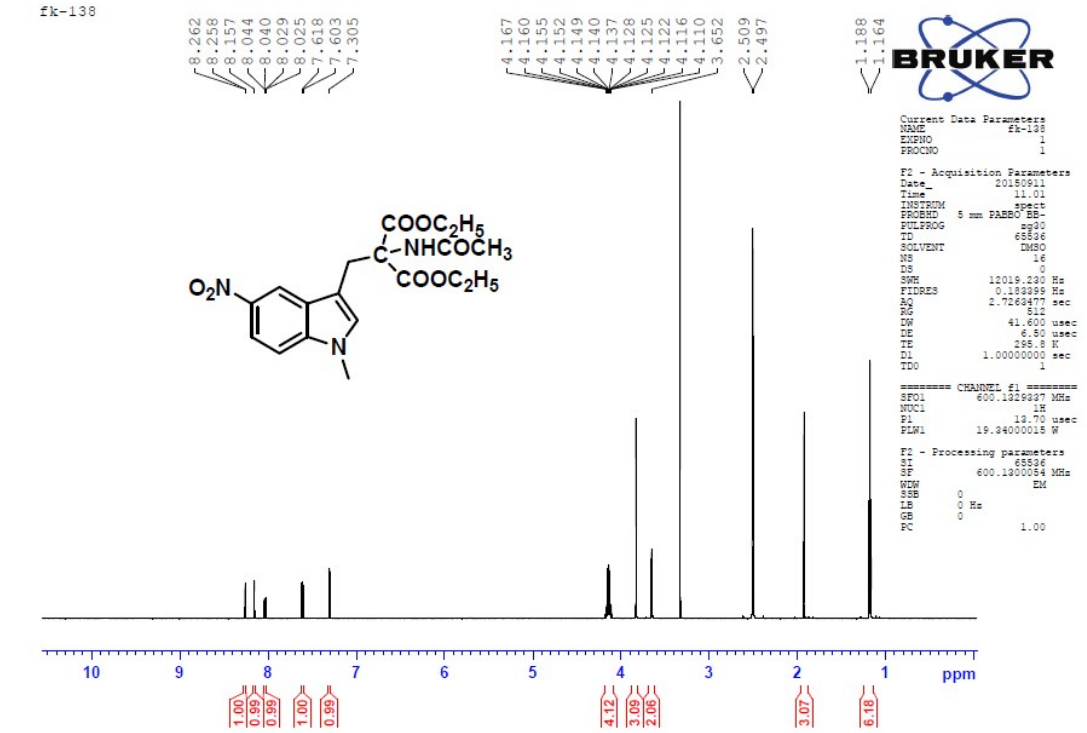
LLC (obtained from Shcellbank, 3×10^5 /well) cells were incubated in six-well plates for 24 h, and then treated with 0.1% DMSO (as control), various concentrations of compounds **17** or **18** in fresh growth medium. After 48 h, the cells were then harvested by trypsinization and washed twice with cold PBS. After centrifugation and removal of the supernatants, cells were resuspended in 400 μL of 1 × binding buffer, which was then added to 5 μL of annexin V-FITC and incubated at room temperature for 15 min. After adding 10 μL of PI, the cells were incubated at room temperature for another 15 min in the dark. The stained cells were analyzed by a flow cytometer (BD Accuri C6).

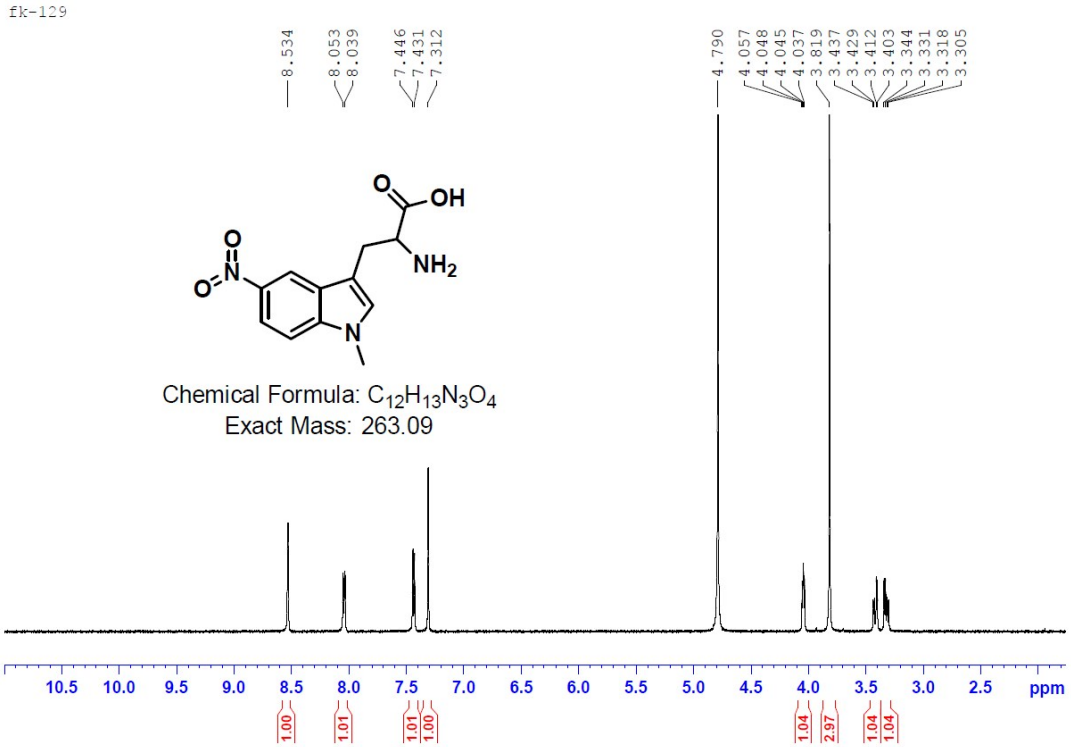
In Vivo Antitumor Assay

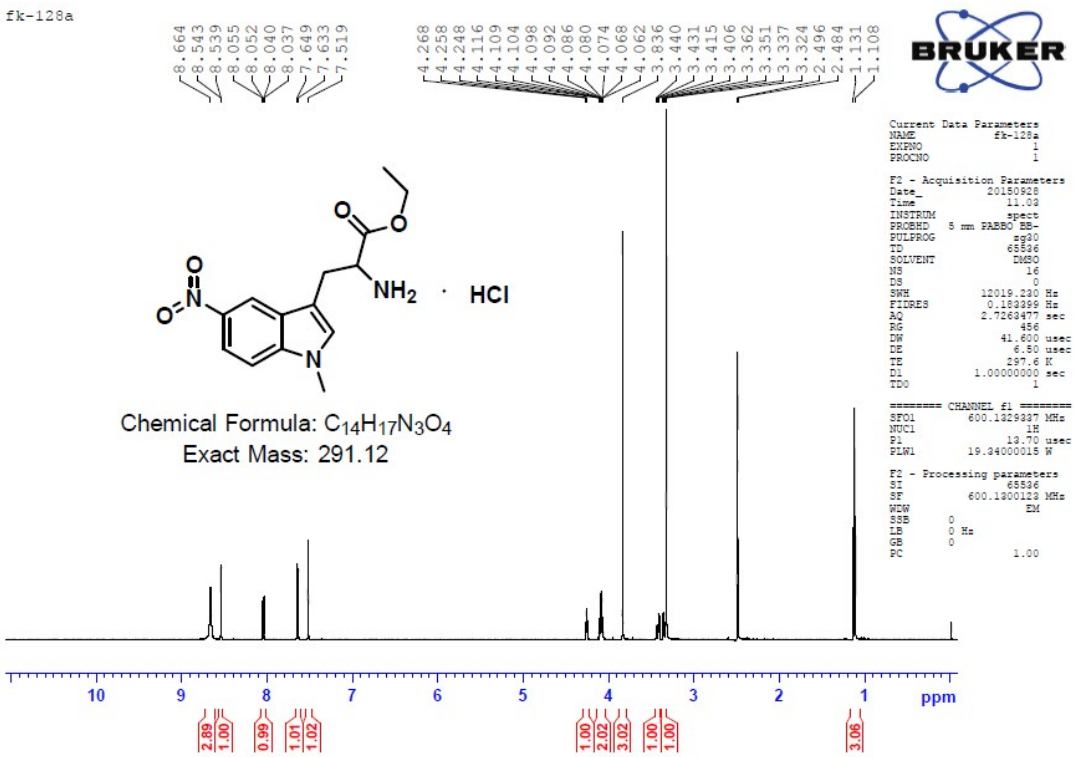
C57BL/6 male mice (certificate SCXK-2013-0018, n = 6 animals/group) were purchased from Shanghai Laboratory Animal Center, SLAC. LLC cells used for implantation were harvested during log phase growth and resuspended in phosphate-buffer saline. Each mice were inoculated s.c. into the right forelimb with 5×10^5 tumor cells. When tumor volumes approached ~50 mm^3, the mice were assigned into groups randomly and treated orally with compound **17** (20 mg/kg, qd), compound **18** (20 mg/kg, qd), 1-MT (400 mg/kg, bid) or melphalan (20 mg/kg, qd), and the blank control group received an equal volume of water containing 0.5% carboxyl methyl cellulose and 0.5% Tween 80 twice every day. Compounds were reconstituted in 0.5% carboxyl methyl cellulose and 0.5% Tween 80 in water. Tumor volumes were monitored by caliper measurement of the length and width and calculated using the

formula of TV = $^1/_2 \times a \times b^2$, where a is the tumor length and b is the width. Tumor volumes and body weights were monitored every 4 days over the course of treatment. After treatment, the mice were sacrificed.

Spectral data



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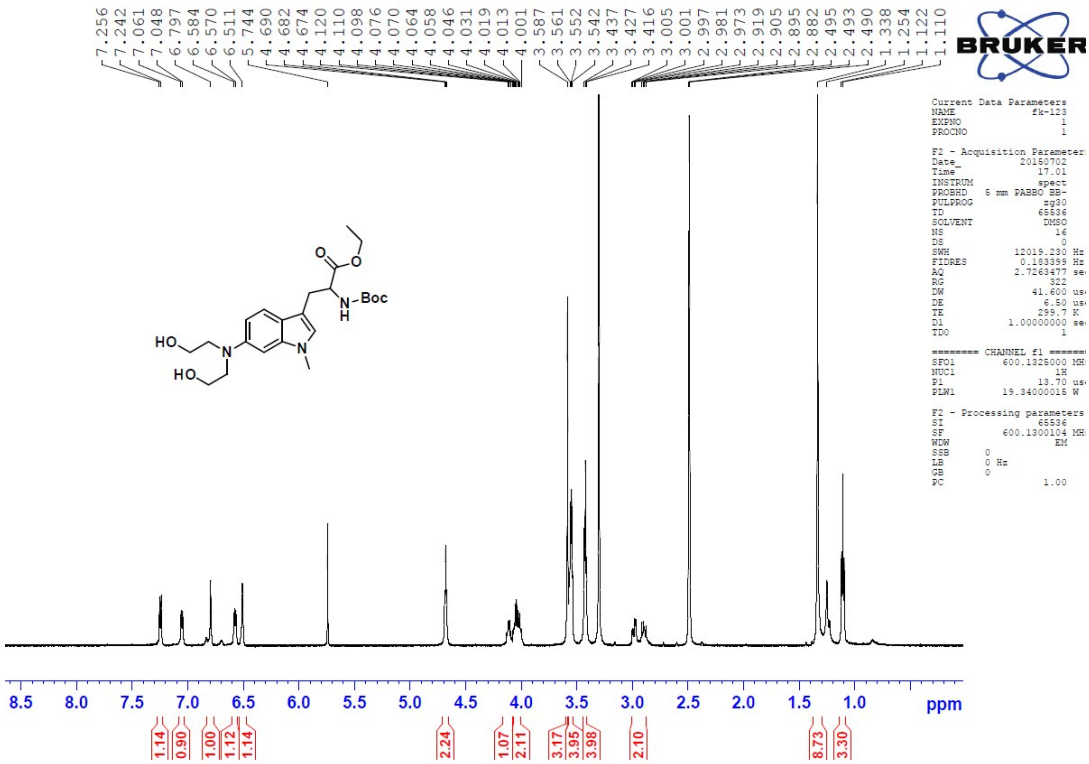
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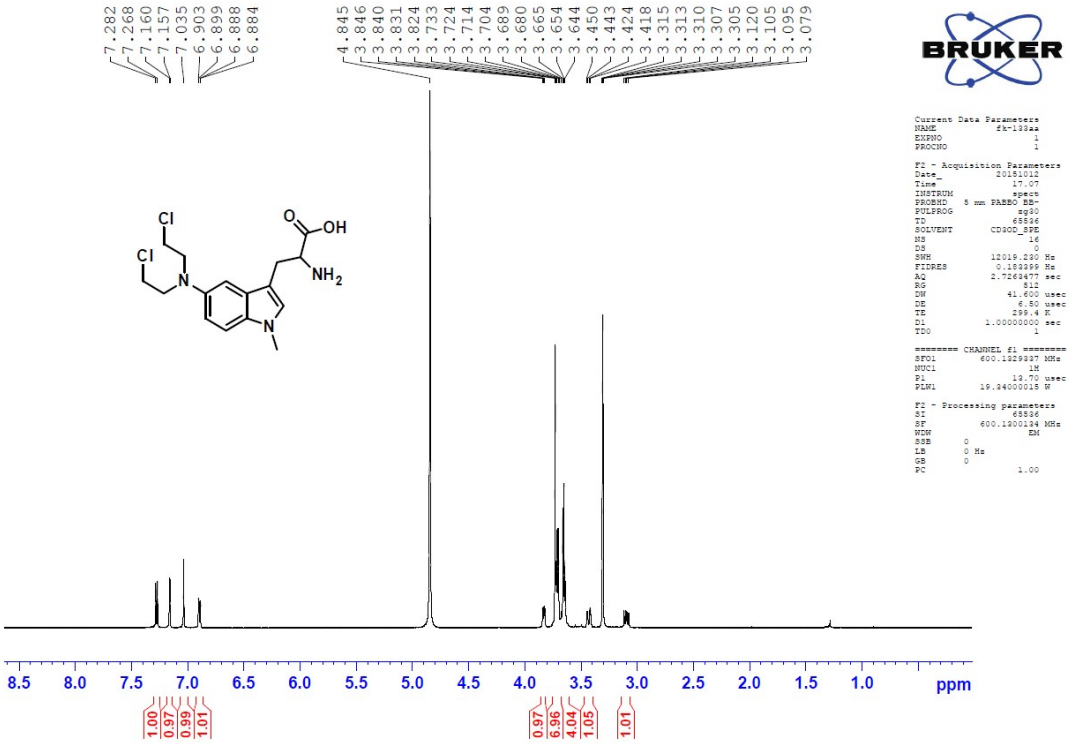
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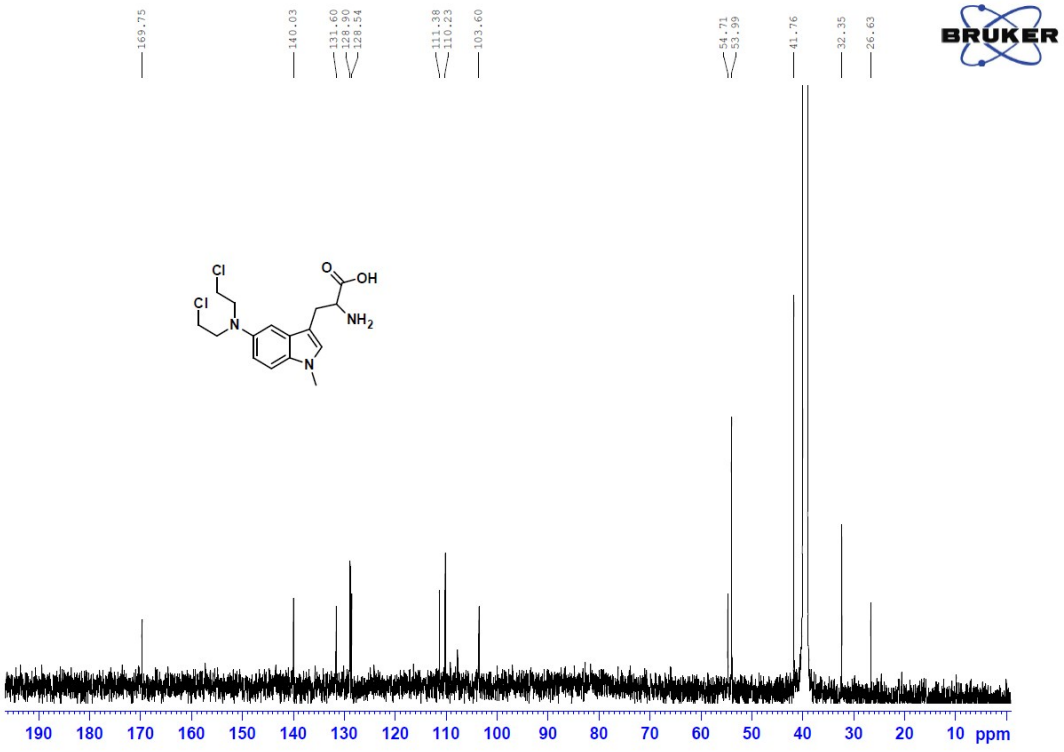
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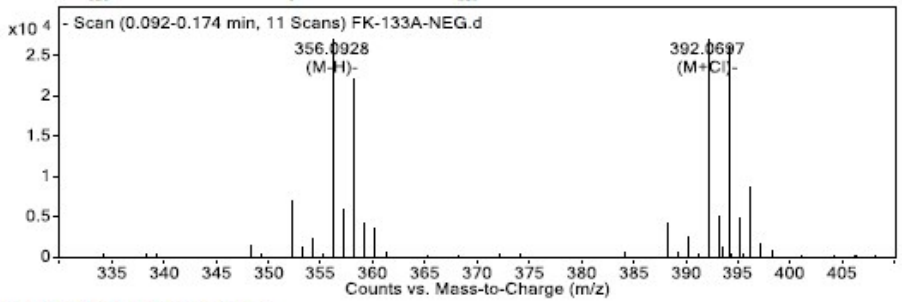

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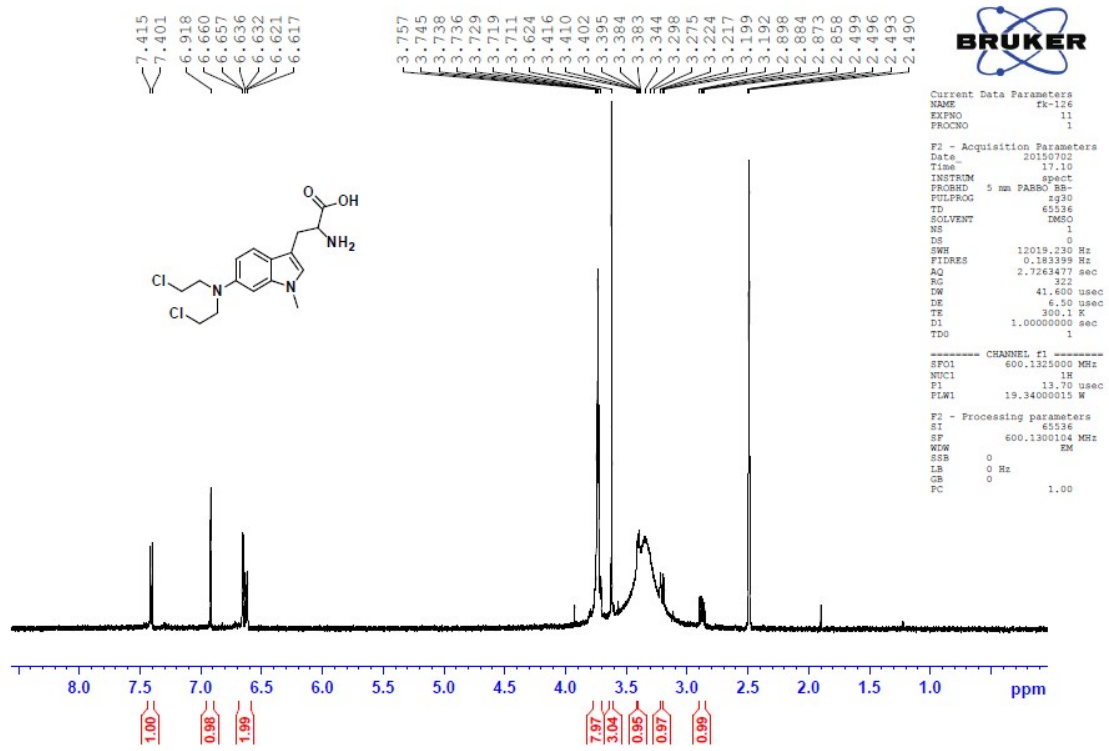
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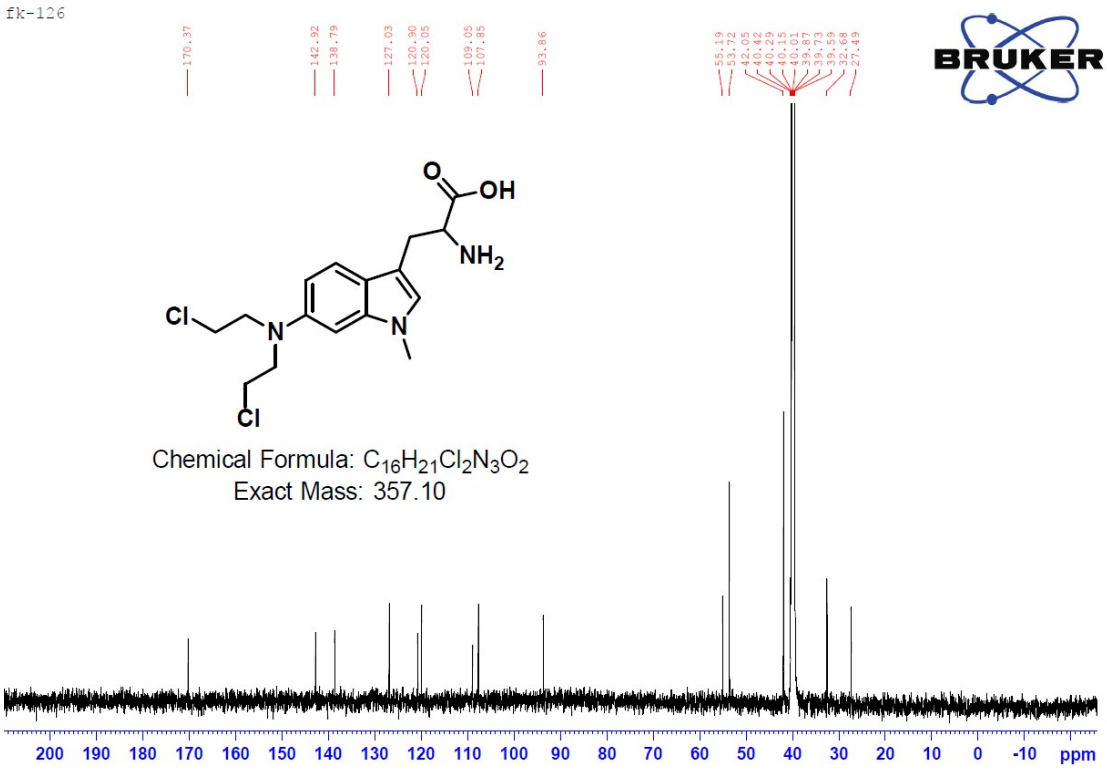
3.745
3.738
3.736
3.729
3.719
3.711
3.624
3.416
3.410
3.402
3.395
3.384
3.383
3.344
3.298
3.275
3.224
3.217
3.199
3.192
2.898
2.884
2.873
2.858
2.499
2.496
2.493
2.490
BRUKER
Current Data Parameters
NAME fk-126
EXPNO 11
PROCNO 1
F2 - Acquisition Parameters
Date_ 20150702
Time 17.10
INSTRUM spect
PROBHD 5 mm PABBO BB-
PULPROG zg30
TD 65536
SOLVENT DMSO
NS 1
DS 0
SWH 12019.230 Hz
FIDRES 0.183399 Hz
AQ 2.7263477 sec
RG 322
DW 41.600 usec
DE 6.50 usec
TE 300.1 K
D1 1.00000000 sec
TD0 1
======== CHANNEL f1 ========
SFO1 600.1325000 MHz
NUC1 1H
P1 13.70 usec
PLW1 19.34000015 W
F2 - Processing parameters
SI 65536
SF 600.1300104 MHz
WDW EM
SSB 0
LB 0 Hz
GB 0
PC 1.00
O
OH
NH2
Cl
N
Cl
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
ppm
1.00
0.98
1.99
7.97
3.04
0.95
0.97
0.99


fk-126
170.37
142.92
138.79
127.03
120.90
120.05
109.05
107.85
93.86
55.19
53.72
42.05
41.42
40.29
40.15
40.01
39.87
39.73
39.59
32.68
27.49
BRUKER
O
OH
NH2
Cl
N
N
Cl
Chemical Formula: C16H21Cl2N3O2
Exact Mass: 357.10
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
ppm

Qualitative Analysis Report

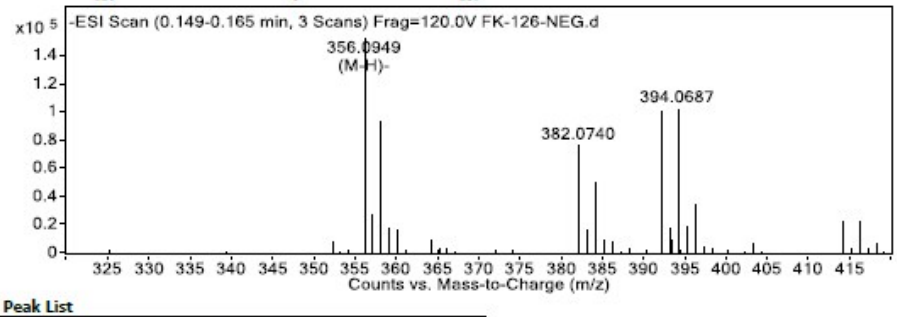
Data Filename	FK-126.d	**Sample Name**	FK-126
Sample Type	Sample	**Position**	P1-D3
Instrument Name	Instrument 1	**User Name**	
Acq Method	TEST-POS-WL.m	**Acquired Time**	7/3/2015 9:37:27 AM
IRM Calibration Status	Success	**DA Method**	Default.m
Comment			

Sample Group Info.

Data Filename	FK-126-NEG.d	**Sample Name**	Unavailable
Sample Type	Unavailable	**Position**	Unavailable
Instrument Name	Unavailable	**User Name**	Unavailable
Acq Method		**Acquired Time**	Unavailable
IRM Calibration Status	Success	**DA Method**	Default.m
Comment	Sample information is unavailable		

User Spectra

Fragmentor Voltage	Collision Energy	Ionization Mode
120	0	ESI



Peak List

m/z	Abund	Formula	Ion
112.9858	251048.9		
356.0949	152007.9	C16 H20 Cl2 N3 O2	(M-H)-
358.092	93005.9		
382.074	76836.9		
392.0716	101195.5		
394.0687	103034.4		
713.1949	84383.7		
715.1927	108700.9		
955.9722	84393.1		
966.0007	74738.2		

Formula Calculator Element Limits

Element	Min	Max
C	0	70
H	0	120
O	1	30
N	0	30
Cl	0	5

Formula Calculator Results

Formula	Best	Mass	Tgt Mass	Diff (ppm)	Ion Species	Score
C16 H21 Cl2 N3 O2	TRUE	357.1021	357.1011	-2.85	C16 H20 Cl2 N3 O2	95.8

Fragmentor Voltage	Collision Energy	Ionization Mode
120	0	ESI