Supplementary Information

Discovery of the First-in-class PROTACs targeting Maternal Embryonic Leucine Zipper Kinase (MELK) for the Treatment of Burkitt lymphoma

[a] *State Key Laboratory of Bioactive Substance and Function of Natural Medicines, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100050, China*

*E-mail addresses: sunyonghui@imm.ac.cn

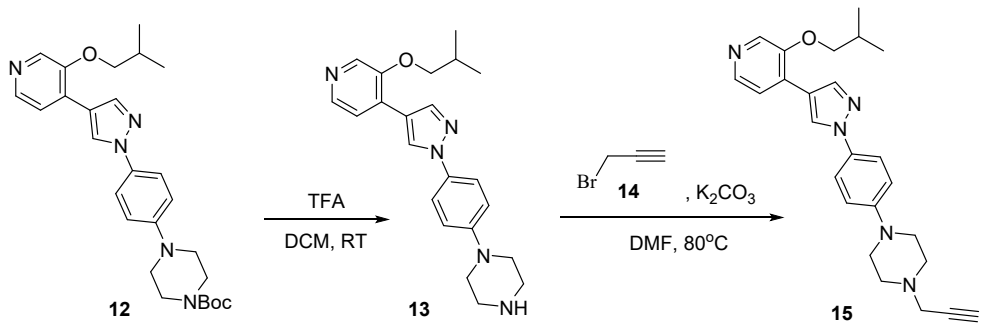
Table of contents

Supplementary information, Data S1

Materials and methods

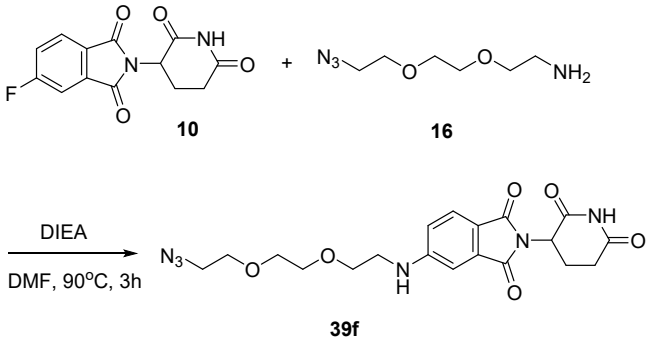
All commercial materials (Selleck, Bidepharm, J&K Chemical LTD, Energy Chemical, Alfa Aesar, and Aladdin.) were utilized without further purification. All amines were purchased. All solvents were analytical grade. The Pomalidomide derivatives were prepared by Crews' procedure. The NMR spectra was generated on a Bruker AVANCE NEO 700 MHz, JEOL-JNM-ECZ500R/S1 500 MHz spectrometer in $CDCl_3$ using solvent peak as standard. Mass spectral analyses were performed with Waters AQUITY UPLC SQD2. Flash column chromatography was performed on Qingdao Haiyang Chemical Co. Ltd silica gel 60 (200-300 mesh). Analytical TLC was performed on Yantai Chemical Industry Research Institute silica gel 60 F254 plates and the rotavapor was EYELA Rotavapor N-1300D-WB.

Synthesis of MELK degrader derivatives



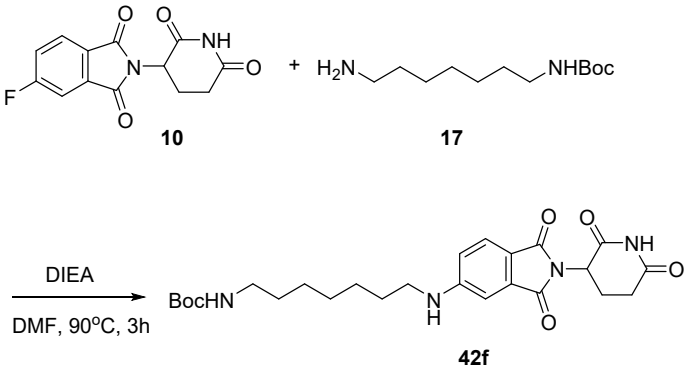
A mixture of compound **12** (100 mg) and TFA (4 mL) in dry DCM (12 mL) was stirred in a round bottom flask at room temperature and monitored by TLC. After the complete reaction was achieved, toluene (16 mL) was added and the mixture was evaporated in vacuum to afford compound **13**. Then, **13** was dissolved in dry DMF (10 mL) and potassium carbonate (118.6 mg, 0.63 mmol) was added. 80wt% 3-bromopropyne (37 μL, 0.34 mmol) was added slowly. The reaction mixture was then stirred for 6 h at 80 ℃. The reaction was quenched by water and the mixture was washed once with saturated aqueous NaCl, extracted by EtOAc. The organic layer was dried over anhydrous Na_2SO_4, and evaporated in vacuum to afford the crude

product, which was further purified by silica gel column chromatography (DCM: MeOH = 50: 1) to give the Intermediate **15**, IY= 86.2%.



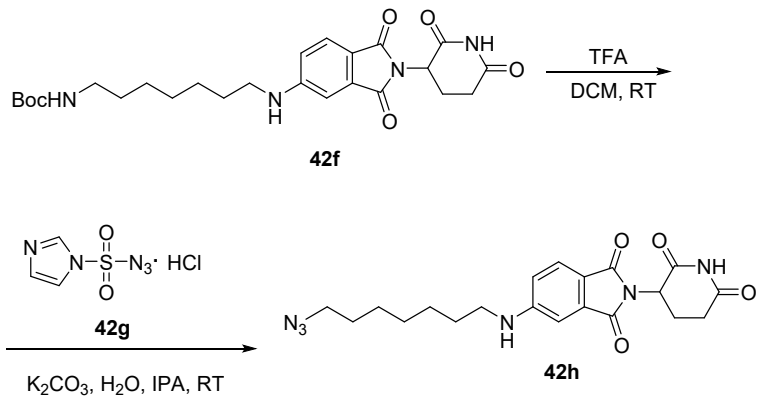
10 **16** **39f**

A mixture of **10** (55.2 mg), **16** (29.7 mg) and DIEA (119 μL) in dry DMF (5 mL) was stirred in a round bottom flask for 3 h at 90℃. The reaction was quenched by water and the mixture was washed once with saturated aqueous NaCl, extracted by EtOAc. The organic layer was dried over anhydrous Na_2SO_4, and evaporated in vacuum to afford the crude product, which was further purified by silica gel column chromatography (PE: EA = 1: 1) to give the intermediate **39f**, IY= 30.6%.

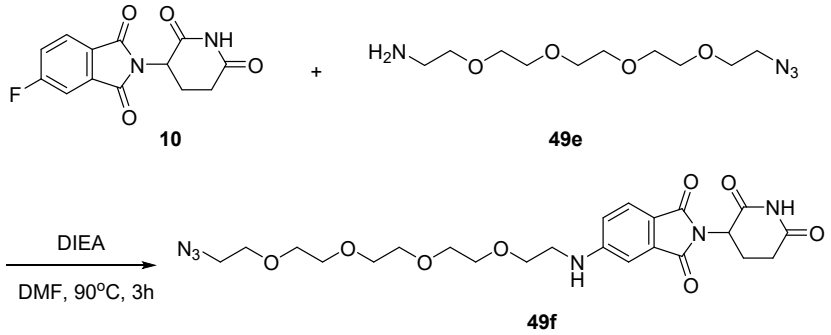


10 **17** **42f**

A mixture of **10** (55 mg), **17** (52 mg) and DIEA (139 μL) in dry DMF (5 mL) was stirred in a round bottom flask for 3 h at 95℃. The reaction was quenched by water and the mixture was washed once with saturated aqueous NaCl, extracted by EtOAc. The organic layer was dried over anhydrous Na_2SO_4, and evaporated in vacuum to afford the crude product, which was further purified by silica gel column chromatography (PE: EA = 2: 1) to give the intermediate **42f**, IY= 35.1%.

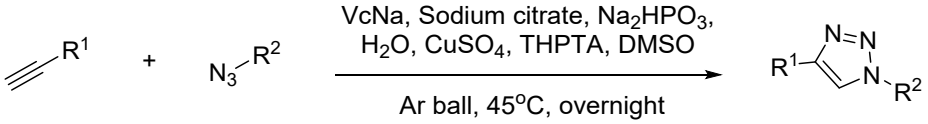


A mixture of **42f** (35 mg) and TFA (2 mL) in dry DCM (2 mL) was stirred in a round bottom flask at room temperature and monitored by TLC. After the complete reaction was achieved, toluene (4 mL) was added and the mixture was evaporated in vacuum. Then, the residue was dissolved in 4 mL IPA/H_2O (1:1). **42g** (22.5 mg) and K_2CO_3 (20 mg) was added. The mixture was stirred for 1 h at room temperature. The reaction was quenched by water and the mixture was washed once with saturated aqueous NaCl, extracted by EtOAc. The organic layer was dried over anhydrous Na_2SO_4, and evaporated in vacuum to afford the crude product .The crude material was purified by column chromatography (PE:EA=5:1) to afford **42h**, IY= 88.6%.

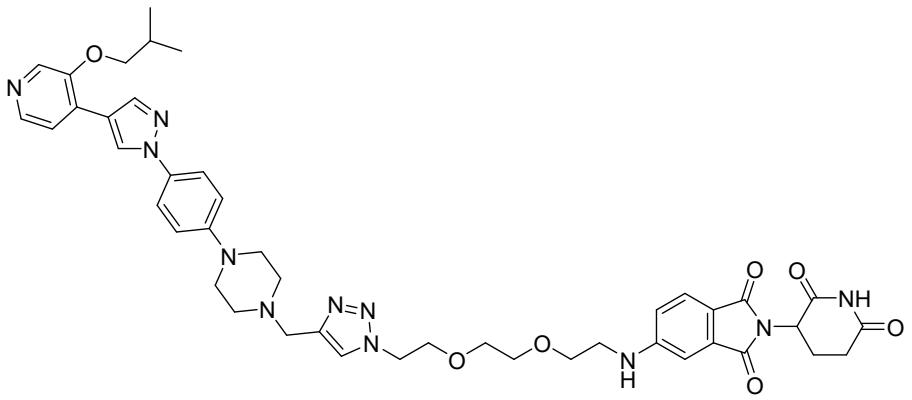


A mixture of **10** (55.2 mg), **49e** (53 mg) and DIEA (139 μL) in dry DMF (5 mL), stirring in a round bottom flask for 3 h at 90°C. The reaction was quenched by water and the mixture was washed once with saturated aqueous NaCl, extracted by EtOAc. The organic layer was dried over anhydrous Na_2SO_4, and evaporated in vacuum to afford the crude product, which was further purified by silica gel column chromatography (PE: EA = 1: 1) to afford **49f**, IY=35.1%.

I General procedure for 1,4-substituted triazole derivatives preparation



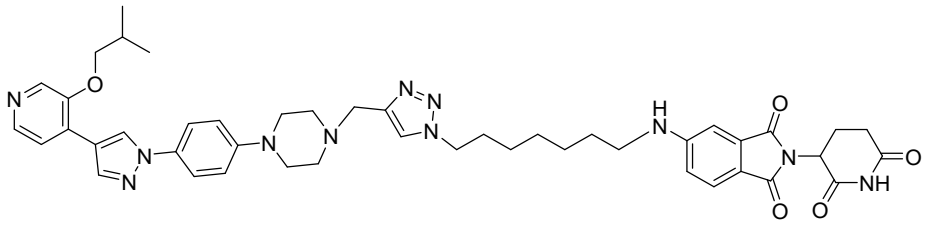
Dissolve VcNa (124 mg), citric acid (243.5 mg), and dibasic phosphate (350 mg) in water (5mL) for Buffer A, anhydrous copper sulfate (3.2 mg), and THPTA (8.7 mg) in water (1 mL) for Buffer B. A mixture of alkyne derivative in DMSO (50 mM, 400 μL) and Buffer A (167 μL) was stirred in a round bottom flask for 15 min at 45℃ under Ar. Then azide derivative in DMSO (50 mM, 400 μL) and Buffer B (84 μL) were added. The reaction mixture was stirred overnight at 45℃.The reaction was quenched by water and the mixture was washed once with saturated aqueous NaCl, extracted by EtOAc. The organic layer was dried over anhydrous Na_2SO_4, and evaporated in vacuum to afford the crude product, which was further purified by silica gel column chromatography (DCM: MeOH = 10: 1) to give one of the 1,4-substituted triazole derivative.



2-(2,6-dioxopiperidin-3-yl)-5-((2-(2-(2-(4-((4-(4-(4-(3-isobutoxypyridin-4-yl)-1H-pyrazol-1-yl)phenyl)piperazin-1-yl)methyl)-1H-1,2,3-triazol-1-yl)ethoxy)ethoxy) ethyl)amino)isoindoline-1,3-dione (MGP-39).

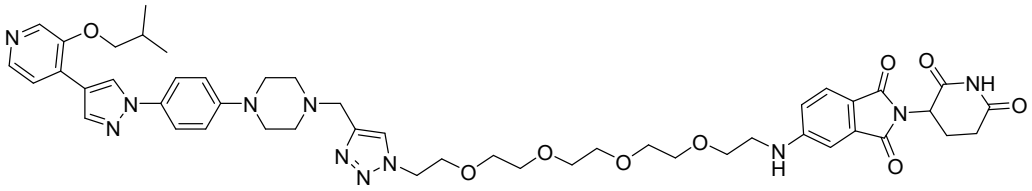
^{1}H NMR (700 MHz, Chloroform-*d*, ppm) δ 8.83 (s, 1H), 8.45 (s, 1H), 8.31 (s, 1H), 8.18 (s, 1H), 7.76 (s, 1H), 7.60 (d, J = 8.3 Hz, 1H), 7.58 (d, J = 8.8 Hz, 2H), 7.52 (s, 1H), 7.07 (s, 1H), 6.98 (d, J = 8.9 Hz, 2H), 6.79 (d, J = 8.2 Hz, 1H), 5.44 – 5.32 (m, 1H), 5.22 (s, 1H), 4.93 – 4.90 (m, 1H), 4.58 (t, J = 5.1 Hz, 2H), 3.98 (d, J = 6.3 Hz, 2H), 3.92 (t, J = 5.2 Hz, 2H), 3.82 (s, 2H), 3.68 (s, 2H), 3.64 – 3.61 (m, 4H), 3.50 –

3.42 (m, 2H), 3.28 (s, 3H), 2.89 – 2.84 (m, 1H), 2.84 – 2.75 (m, 4H), 2.74 – 2.68 (m, 1H), 2.31 – 2.25 (m, 2H), 2.14 – 2.09 (m, 1H), 2.03 – 1.98 (m, 1H), 1.12 (d, J = 6.7 Hz, 6H); ^{13}C NMR (176 MHz, Chloroform-*d*, ppm) δ 171.39, 168.83, 168.01, 167.39, 153.77, 151.30, 150.11, 143.82, 142.47, 139.73, 134.70, 134.65, 132.55, 128.44, 126.93, 125.46, 123.94, 120.68, 120.38, 118.59, 117.61, 116.87, 116.44, 106.24, 75.49, 70.50, 69.52, 53.16, 52.71, 50.15, 49.08, 48.76, 43.23, 31.50, 29.71, 28.60, 22.81, 19.52, 14.15; LC-MS: calculated for $C_{44}H_{51}N_{11}O_7$ $[M+H]^+$, 846.3973; found, 846.4037.



2-(2,6-dioxopiperidin-3-yl)-5-((7-(4-((4-(4-(4-(3-isobutoxypyridin-4-yl)-1H-pyrazol-1-yl)phenyl)piperazin-1-yl)methyl)-1H-1,2,3-triazol-1-yl)heptyl)amino) isoindoline-1,3-dione (MGP-42)

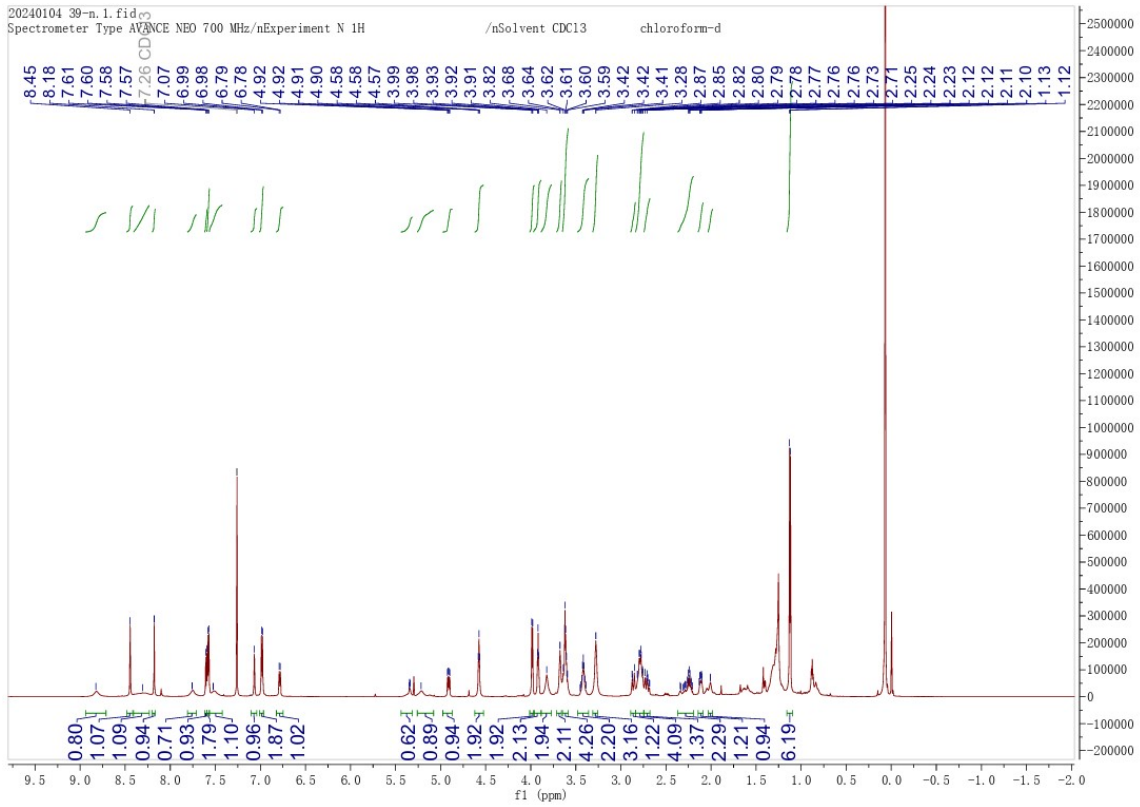
^{1}H NMR (500 MHz, chloroform-*d*, ppm) δ 8.44 (d, J = 1.4 Hz, 1H), 8.36 – 8.33 (m, 1H), 8.26 – 8.23 (m, 1H), 8.18 (d, J = 1.5 Hz, 1H), 7.62 – 7.52 (m, 4H), 7.48 (s, 1H), 7.03 – 6.97 (m, 2H), 6.94 (d, J = 2.1 Hz, 1H), 6.72 (dd, J = 8.3, 2.2 Hz, 1H), 5.30 (d, J = 1.6 Hz, 1H), 4.95 – 4.88 (m, 1H), 4.37 (t, J = 7.1 Hz, 2H), 3.98 (dd, J = 6.4, 1.5 Hz, 2H), 3.79 (d, J = 8.1 Hz, 2H), 3.27 (d, J = 5.3 Hz, 4H), 2.80 – 2.68 (m, 6H), 2.29 – 2.20 (m, 1H), 2.17 – 2.06 (m, 1H), 1.45 – 1.29 (m, 7H), 1.29 – 1.18 (m, 7H), 1.18 – 1.10 (m, 6H); ^{13}C NMR (176 MHz, Chloroform-*d*, ppm) δ175.61, 171.32, 168.69, 167.92, 167.34, 153.64, 151.20, 149.89, 142.33, 139.65, 134.58, 132.57, 129.89, 129.82, 128.26, 126.82, 125.40, 122.92, 120.58, 120.28, 117.99, 117.55, 116.48, 105.82, 75.39, 53.37, 52.99, 52.54, 50.15, 48.98, 43.25, 31.40, 30.04, 29.61, 28.50, 28.45, 27.12, 26.21, 22.71, 19.42; LC-MS: calculated for $C_{45}H_{53}N_{11}O_5$ $[M+H]^+$, 828.4231; found, 828.4296.

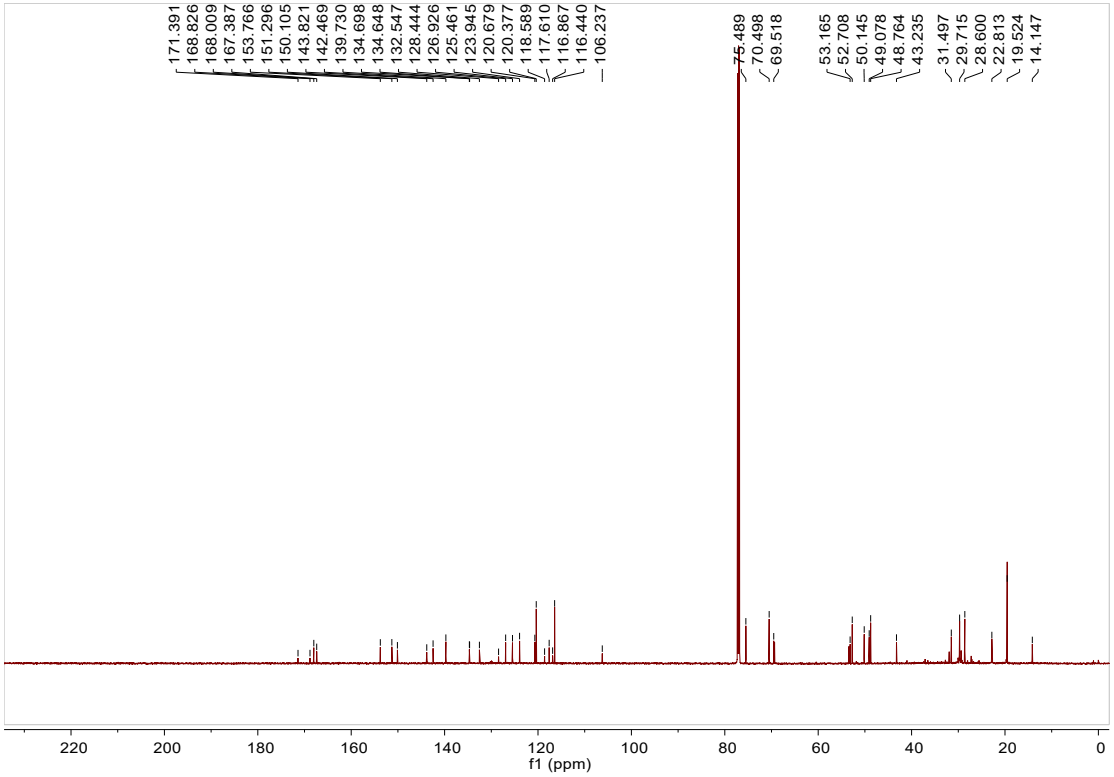


2-(2,6-dioxopiperidin-3-yl)-5-((14-(4-((4-(4-(4-(3-isobutoxypyridin-4-yl)-1H-pyrazol-1-yl)phenyl)piperazin-1-yl)methyl)-1H-1,2,3-triazol-1-yl)-3,6,9,12-tetraoxatetradecyl)amino)isoindoline-1,3-dione (MGP-49)

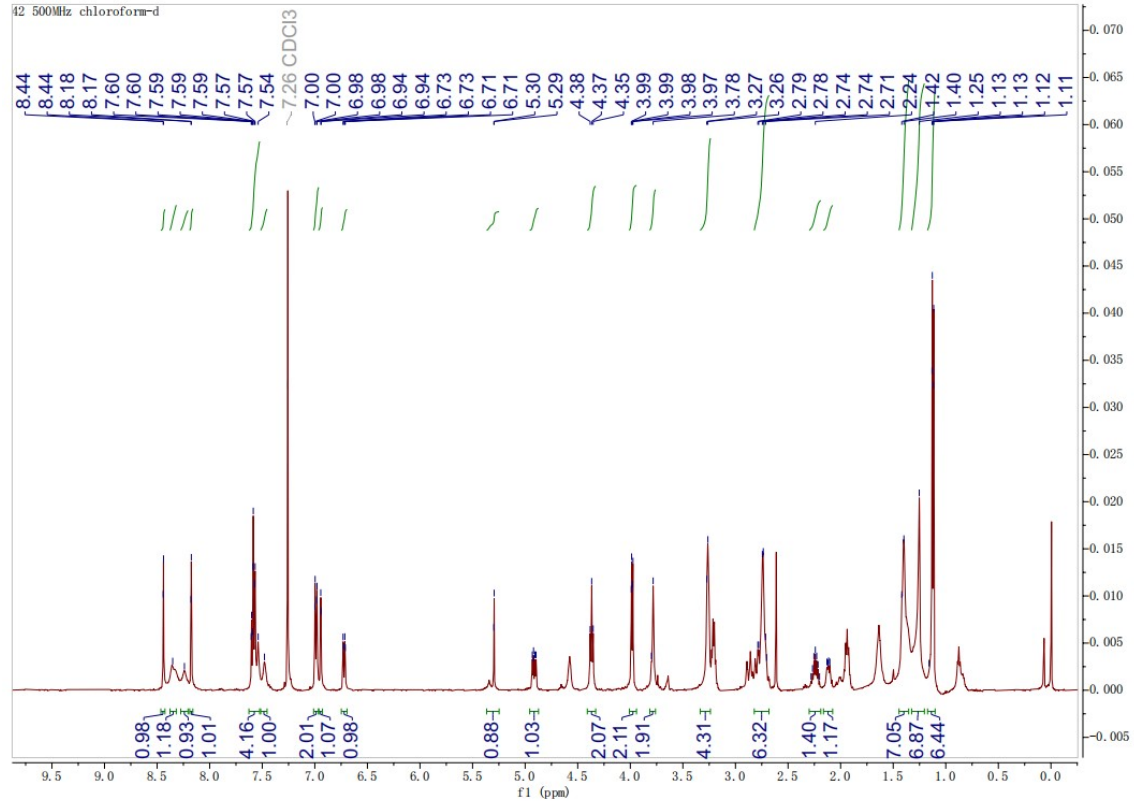
^{1}H NMR (700 MHz, Chloroform-*d*, ppm) δ 9.25 (s, 1H), 8.44 (s, 1H), 8.32 (s, 1H), 8.24 (d, J = 4.9 Hz, 1H), 8.18 (s, 1H), 7.70 (s, 1H), 7.61 (d, J = 8.3 Hz, 1H), 7.58 (d, J = 8.9 Hz, 2H), 7.47 (d, J = 4.9 Hz, 1H), 7.02 (s, 1H), 6.99 (d, J = 8.9 Hz, 2H), 6.76 (d, J = 8.3 Hz, 1H), 5.47 (t, J = 5.4 Hz, 1H), 4.91 (dd, J = 12.5, 5.4 Hz, 1H), 4.57 (t, J = 5.5 Hz, 2H), 3.99 (d, J = 6.3 Hz, 2H), 3.93 – 3.87 (m, 2H), 3.78 (s, 2H), 3.73 (t, J = 4.9 Hz, 2H), 3.69 – 3.60 (m, 12H), 3.40 – 3.36 (m, 2H), 3.26 (s, 4H), 2.86 (d, J = 16.6 Hz, 1H), 2.82 – 2.68 (m, 6H), 2.28 – 2.22 (m, 1H), 2.17 – 2.11 (m, 1H), 1.13 (d, J = 6.7 Hz, 6H); ^{13}C NMR (176 MHz, Chloroform-*d*, ppm) δ171.43, 168.93, 168.12, 167.42, 153.94, 149.76, 142.28, 142.28, 139.76, 134.46, 130.00, 129.92, 129.89, 129.77, 128.44, 126.94, 125.66, 120.38, 118.04, 117.64, 116.76, 115.62, 115.61, 107.11, 75.50, 70.54, 70.44, 69.46, 68.85, 53.45, 52.33, 50.05, 49.04, 48.32, 43.12, 31.92, 31.49, 29.72, 28.60, 27.23, 22.86, 19.52, 14.14; LC-MS: calculated for $C_{48}H_{59}N_{11}O_9$ $[M+H]^+$, 934.4497; found, 934.4563.

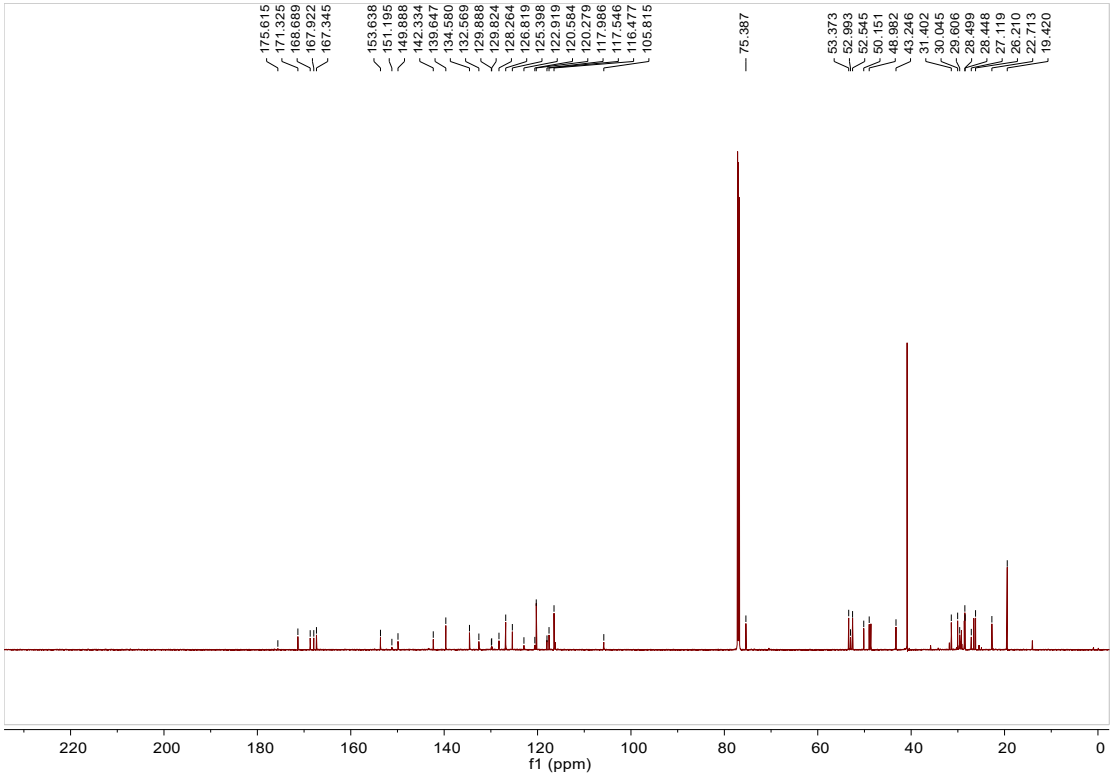
NMR data of compound MGP-39.



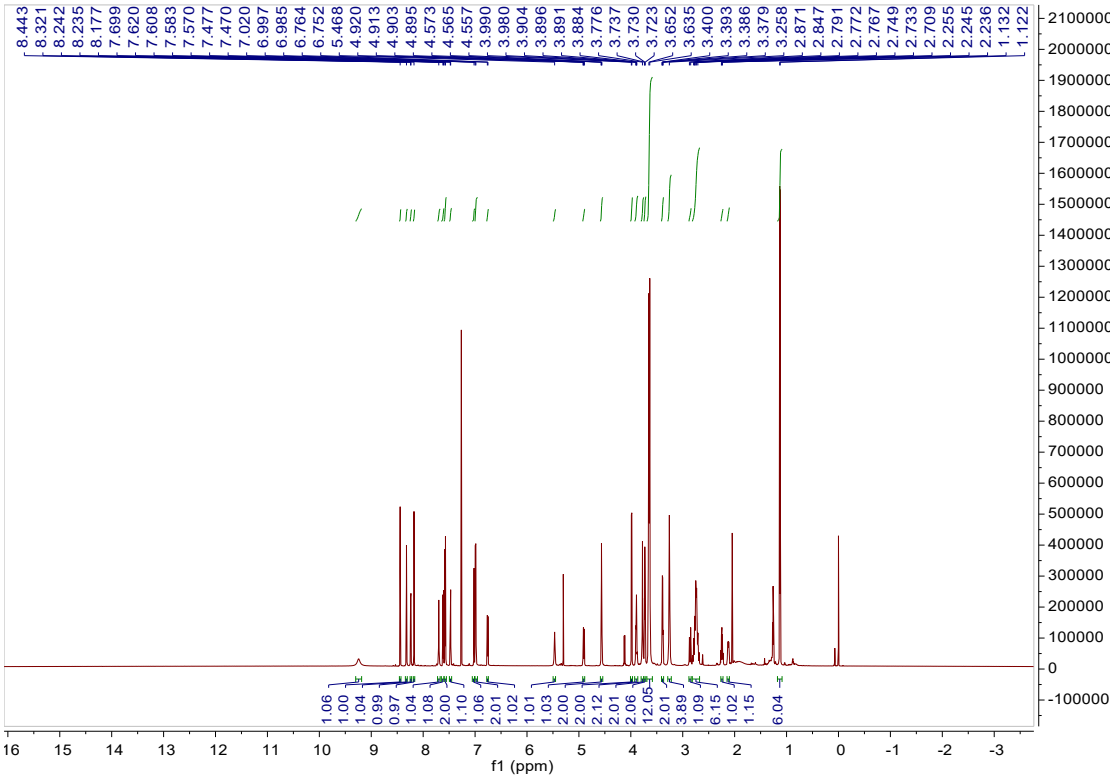


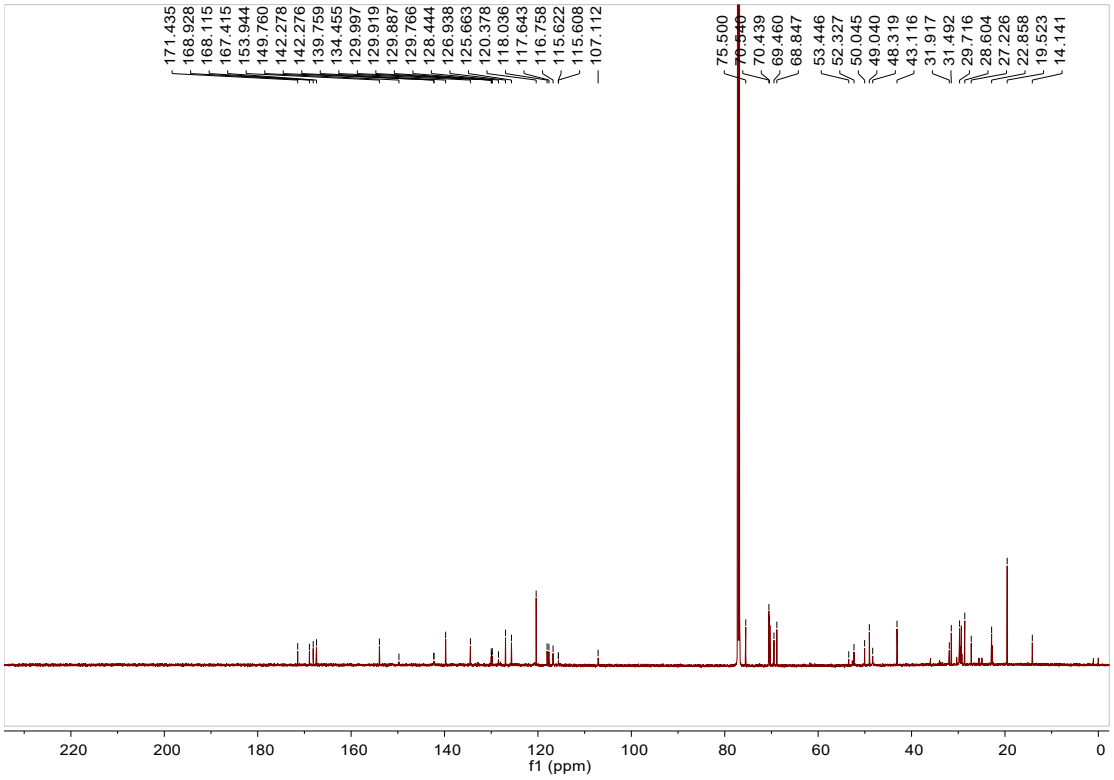
NMR data of compound MGP-42



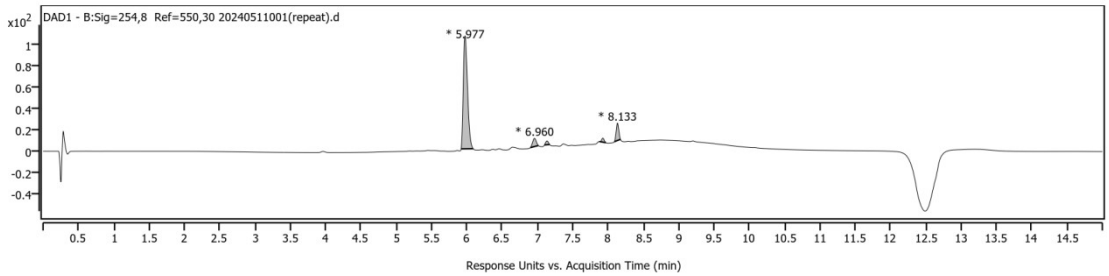


NMR data of compound MGP-49





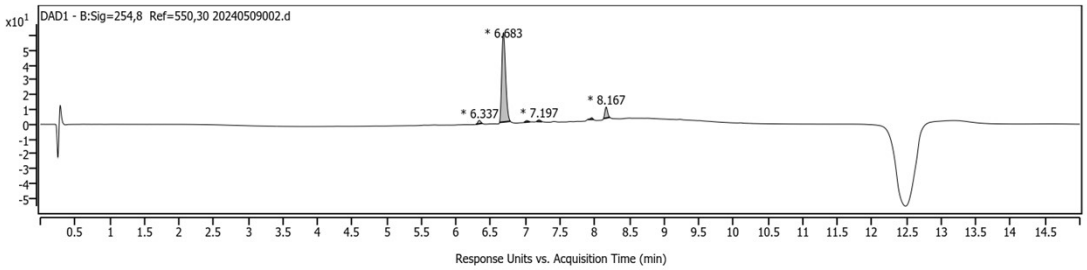
HPLC data of compound MGP-39



Chromatogram Peaks

Peak	Start	RT	End	Height	Area	Area %	SNR
1	5.920	5.977	6.090	105	435	100.00	
2	6.900	6.960	7.013	8	27	6.15	
3	7.100	7.137	7.173	4	9	2.10	
4	7.877	7.927	7.970	4	9	2.11	
5	8.090	8.133	8.177	16	41	9.44	

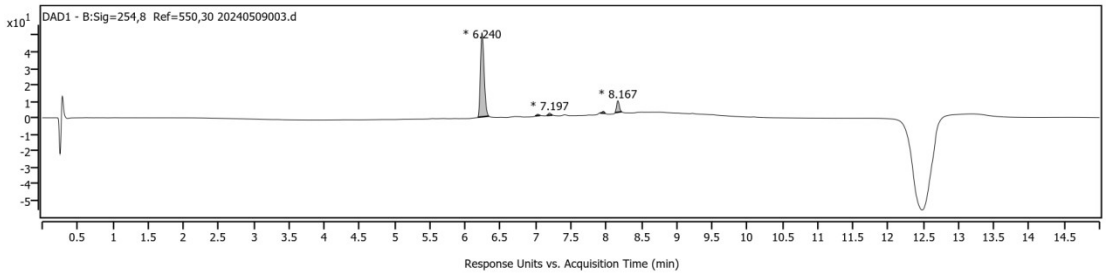
HPLC data of compound MGP-42



Chromatogram Peaks

Peak	Start	RT	End	Height	Area	Area %	SNR
1	6.287	6.337	6.377	2	6	2.71	
2	6.627	6.683	6.783	60	233	100.00	
3	6.980	7.023	7.067	1	3	1.33	
4	7.160	7.197	7.240	1	3	1.33	
5	7.900	7.957	7.983	1	2	1.06	
6	8.127	8.167	8.217	7	20	8.44	

HPLC data of compound MGP-49



Chromatogram Peaks

Peak	Start	RT	End	Height	Area	Area %	SNR
1	6.180	6.240	6.337	51	187	100.00	
2	6.993	7.030	7.067	1	2	1.14	
3	7.157	7.197	7.240	1	4	1.90	
4	7.913	7.960	7.990	1	2	1.32	
5	8.127	8.167	8.220	7	19	9.92	

Supplementary information, Data S2

Cell culture

The human RAMOS and MDA-MB-231 cell lines were provided by Prof. Susan K. Pierce (NIH). DOHH2 cell line was kindly provided by Prof. Wanli Liu (Tsinghua University). RPMI 1640, DMEM, Pen Strep and 2-Mercaptoethanol were purchased from Gibco®, MEM nonessential amino acid solution (10 mM, 100×) was purchased from Solarbio®, FBS was purchased from Biological Industries. RAMOS and DOHH2 cell lines were cultured in RPMI 1640 media with 10% FBS, 1% penicillin-streptomycin, 1% nonessential amino acid and 50 μM 2-Mercaptoethanol at 37°C incubator containing 5% CO_2. MDA-MB-231 cell line was cultured in DMEM media with 10% FBS, 1% penicillin-streptomycin, 1% nonessential amino acid and 50 μM 2-Mercaptoethanol at 37°C incubator containing 5% CO_2.

Cell viability assay

In cell viability experiments, cells were suspended in 45 μL of culture medium in 96-well cell culture plate (3000 cells/well). The stock compound in DMSO was diluted with 45 μL of culture medium, and then added to cells cultured in 96-well cell culture plate. Cells were incubated at 37°C incubator with 5% CO_2. After 72~96 hours, 10 μL of CCK-8 reagent (Tianjing meilunbio) was added to each well of the 96-well cell culture plate. After incubated for 4 hours, data was collected at 450 nm using SpectraMax Plus Microplate Reader (SoftMax® Pro 6). DMSO-treated cells were used for normalizing the readings. GraphPad Prism 8 software was used for calculating EC_{50} by nonlinear regression analysis.

Immunoblotting

Cells were suspended in 1 mL of culture medium in 12-well cell culture plate, which were treated with compound at various concentrations. After the indicated time, cells were lysed with 2X SDS sample buffer (50 mM Tris-HCl (pH6.8), 2% (W/V) SDS, 0.1% (W/V) Bromophenol Blue, 10% (V/V) Glycerin and 1% (W/V) 2-

Mercaptoethanol in ddH2O) and heated at 100℃ for 15 min. 10 μL of the cell lysate were loaded onto 10% SDS-PAGE gel for the protein band separation, and the gel then electrotransfered to PVDF membranes at 4℃, 220 mA for 1.5 h. Incubated with primary antibody at 4 ℃, overnight. Primary antibodies: MELK (Rabbit mAb, ab108529, 1: 1000) was purchased from abcam, β-actin (Rabbit mAb, #4970, 1: 2500) from CST, β-tubulin (Mouse mAb, 66240-1-Ig, 1: 2500) from Proteintech, mTOR (Mouse mAb, 66888-1-Ig, 1: 1000) from Proteintech, p-mTOR (Mouse mAb, 67778-1-Ig, 1: 1000) from Proteintech. Rabbit (Invitrogen, Prod#31460, 1: 4000)/mouse (Invitrogen, Prod#31430, 1: 4000) secondary antibodies were incubated at room temperature for 1 hours. Blots were imaged with M5 Hiper ECL Western HRP Substrate (MF074-05) on Tanon 5200 instrument. For grayscale analysis, we use the imageJ 1.50i. GraphPad Prism 8 software was used for calculating DC_{50} by nonlinear regression analysis.

Apoptosis assay

Flow cytometry (FCM) assays were performed to investigate the apoptosis of RAMOS cells induced by DMSO or MGP-39. RAMOS cells cultures in 6-well cell cultures plate (1×10^6 cells/well) were treated with indicated concentrations of MGP-39 or DMSO for 24 hours. Cells were harvested and washed with cold PBS. Cells were resuspend with 1 × Annexin V Binding Buffer (Pricella) and stained with 2.5 μL Annexin V-FITC and 2.5 μL propidium iodide (Pricella). Cell apoptosis was analyzed with a flow cytometer (BD Biosciences) and the results were analyzed using FlowJo software.

Cell cycle analysis

RAMOS cells cultures in 6-well cell cultures plate were treated with indicated concentrations of MGP-39 or DMSO for 24 hours. After 24 hours, cells were harvested and washed with cold PBS. Cells were fixed overnight with 70% ethanol. Subsequently, the cells were stained with 500 μL propidium iodide staining working solution (MedChemExpress) at 37℃ for 30 min in the dark. Cell cycle stage was

determined by flow cytometer (BD Biosciences) and analyzed by Modifit software.

Calcein AM/PI staining assay

The ratio was detected using a calcein-AM/propidium iodide kit (Beyotime, C2015S). In brief, RAMOS cells cultures in 12-well cell cultures plate (2×10^5 cells/well) were treated with indicated concentrations of MGP-39 or DMSO for 24 hours. After 24 hours, cells were harvested and washed with cold PBS. Then the cells were incubated with 500 uL calcein-AM/propidium iodide staining working solution (Beyotime, C2015S) at 37℃ for 30 min in the dark. The cells were observed under a fluorescence microscope (Zeiss LSM980 NLO).

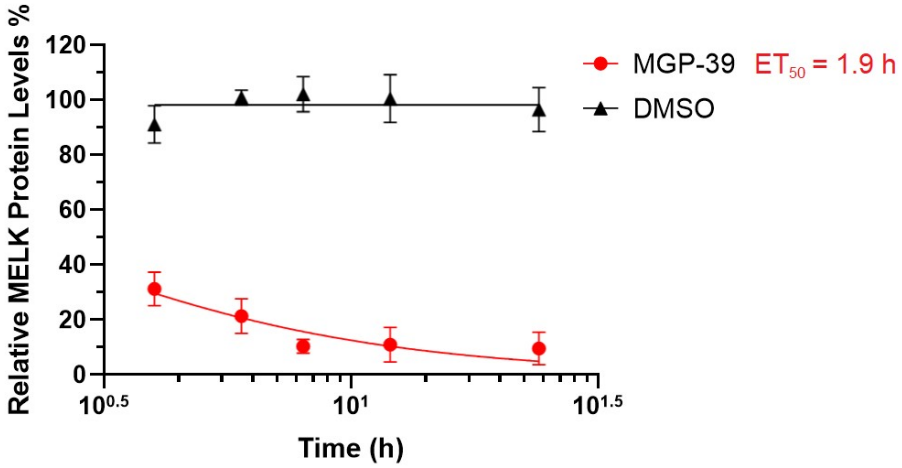
Tube formation assay

Matrigel (BD Biocoat, 356234) was thawed overnight at 4 ℃, then each well of a prechilled 24-well cell cultures plate was coated with 160 μL Matrigel and incubated at 37℃ for 4 hours. HUVEC cells (3×10^4 cells/well) were added in 600 μL medium with indicated concentrations compounds. After 4 hours, the endothelial cell tube formation was assessed and imaged under an optical microscope (OLYMPUS BX53). The tube formation were analyzed by ImageJ software in three independent repeated experiments.

RNA extraction and qPCR

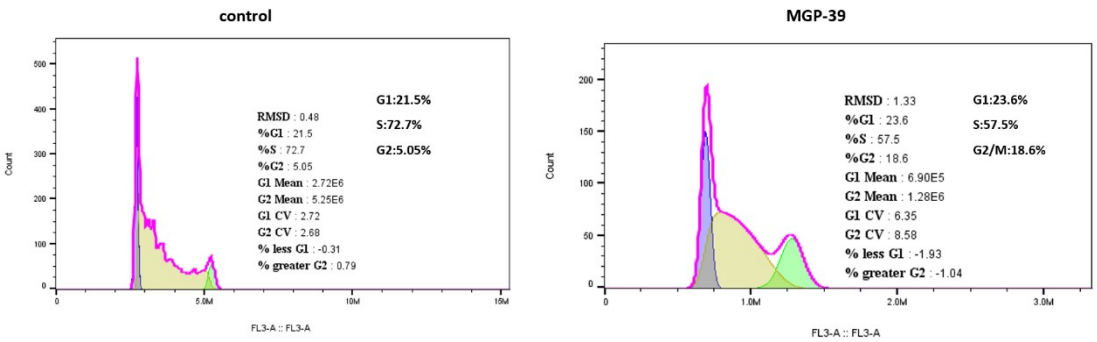
RAMOS cells cultures in 6-well cell cultures plate were treated with indicated concentrations of MGP-39 20 μM or DMSO for 24 hours. After 24 hours, cell were harvested and total RNAs were extracted from RAMOS cells according to protocols using TRIzol reagent (Thermo Fisher). cDNA synthesis was finished using a reverse transcriptase kit (LABLEAD, F0202) followed by the protocols. Subsequently, quantitative real-time PCR was performed using a Tap Pro Universal SYBR qPCR Master Mix kit (Vazyme, Q712) and a 7500 real-time PCR system (Applied Biosystems, USA). MELK primer sequences used are listed below: MELK: forward primer: 5'-CCGCCCCTCAGGTTCTTTT-3', reverse primer: 5'-TGATCCGGGGCAAATCACTC-3'. Actin was used as an internal control. Relative

mRNA expression was calculated by the $2^{-\Delta\Delta Ct}$ method and normalized to 18S mRNA levels. The experiments were performed in triplicate.



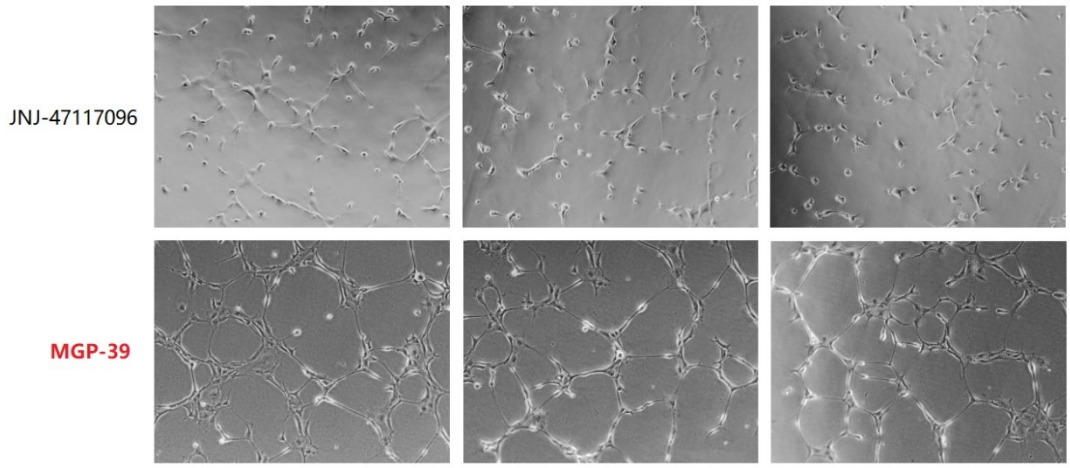
Supplementary information, Fig S1. Degradation efficiency of MGP-39.

Immunoblot for MELK and *β*-actin after indicated times treatment on RAMOS cells with 20 μM of compound **MGP-39**. [a*] 2×10^5 cells in each hole (12-well plate). The final ET_{50} was calculated by ImageJ and GraphPad Prism 8 software.



Supplementary information, Fig. S2. Cell cycle assay.

MELK degrader **MGP-39** induced G2/M phase arrest in RAMOS cells. Cells were treated with 10 μM of compound for 24 h and stained with propidium iodide. The cell cycle phases were determined by flow cytometry.



Supplementary information, Fig. S3. Tube formation assay.

Tube formation assay on HUVEC cells treated with the indicated compounds 4 μM for 24 h.

References

1 Liu, J.; Wu, F.; Xie, A.; Liu, C.; Bao, H. J. A.; chemistry, b. Preparation of nonconjugated fluorescent polymer nanoparticles for use as a fluorescent probe for detection of 2,4,6-trinitrophenol. **2020,** *412* (5), 1235.

2 Lu, J.; Qian, Y.; Altieri, M.; Dong, H.; Wang, J.; Raina, K.; Hines, J.; Winkler, J.; Crew, A.; Coleman, K.et al. Hijacking the E3 Ubiquitin Ligase Cereblon to Efficiently Target BRD4. **2015,** *22* (6), 755.

3 Risseeuw, M.; De Clercq, D.; Lievens, S.; Hillaert, U.; Sinnaeve, D.; Van den Broeck, F.; Martins, J.; Tavernier, J.; Van Calenbergh, S. J. C. A "clickable" MTX reagent as a practical tool for profiling small-molecule-intracellular target interactions via MASPIT. **2013,** *8* (3), 521.

4 Suzuki, N.; Koyama, S.; Koike, R.; Ebara, N.; Arai, R.; Takeoka, Y.; Rikukawa, M.; Tsai, F. J. P. Palladium-Catalyzed Mizoroki-Heck and Copper-Free Sonogashira Coupling Reactions in Water Using Thermoresponsive Polymer Micelles. **2021,** *13* (16).

5 Yoon, H.; Lee, D.; Lim, D.; Koo, H.; Kim, K. J. A. m. Copper-free Click Chemistry: Applications in Drug Delivery, Cell Tracking and Tissue Engineering. **2021**, DOI:10.1002/adma.202107192 10.1002/adma.202107192, e2107192.