Supplementary methods

Targeting BRD4 and PI3K signaling pathways for the treatment of medulloblastoma

Bharti Sethi, Virender Kumar, Thilina D. Jayasinghe, Yuxiang Dong, Donald R. Ronning, Haizhen A. Zhong, Donald W. Coulter and Ram I. Mahato.

Reagents and cell culture

SF2523 (HY-101146) and LY294002 (HY-10108) were purchased from MedChem Express LLC. Cell culture media EMEM was purchased from the ATCC. DMEM was purchased from Hyclone Laboratories. FBS was purchased from BioTechne, antibiotic solution Anti-Anti was obtained from Sigma-Aldrich. Primary antibodies were obtained from Abcam, Biomatik, Cell signaling technology (CST), and Santacruz Biotech. Horseradish peroxidase (HRP) conjugated and florescent labeled secondary antibody were purchased from the Invitrogen (A16096) and Li-COR Biosciences (926-68070), respectively. All other reagents were obtained from Fisher Scientific.

Cell culture conditions

DAOY, HD-MB03, and ONS-76 MB cells were cultured in EMEM, DMEM, and RPMI, respectively, with 10% FBS and 1% Anti-Anti. All cells were maintained in an incubator at 37 °C temperature with relative humidity between 90 and 95% in the presence of 5% CO_2.

Synthesis and characterization of 8-(2,3-dihydrobenzo[b][1,4] dioxin-6-yl)-2-morpholino-4H-chromen-4-one (MDP5).

Synthesis step1. Phosphorous chloride ($POCl_3$; 6 mL, 64 mmol) was added to a mixture of 3-bromo-2-hydroxybenzoic acid (3.48 g, 16 mmol) and N-acetylmorpholine (4.12 g, 32 mmol) in chloroform ($CHCl_3$; 10 mL). The resulting mixture was stirred at room temperature (RT) for 1 h and then heated at 70 ºC for 24 h. After completion, reaction mixture was cooled to RT and quenched with water (50 mL), $CHCl_3$ (100 mL), and sodium acetate (NaOAc; 25 g, 305 mmol). The organic layer was separated, washed with 100

mL of brine, followed by the mixture of 50 mL of brine and 20 mL of 10% aqueous sodium hydroxide (NaOH), dried over magnesium sulfate ($MgSO_4$), filtered, and concentrated to obtain the chromenone intermediate (945 mg, 19%, yellowish solid), which was purified by crystallization from methyl tert-butyl ether (MTBE).

The chromenone intermediate was characterized by ^{1}H NMR as: ^{1}H NMR (500 MHz, $CDCl_3$) 8.10 (d, *J* = 8.0 Hz, 1H), 7.77 (d, *J* = 8.0 Hz, 1H), 7.23 (t, *J* = 8.0 Hz, 1H), 5.50 (s, 1H), 3.86 (t, *J* = 5.0 Hz, 4H), 3.59 (t, *J* = 5.0 Hz, 4H).

Synthesis step 2. 1,1'-Bis(diphenylphosphino)ferrocene)palladium(II) dichloride ($PdCl_2$ (DPPF), 40 mg, 0.055 mmol) was added to a mixture of the intermediate chromenone (ArBr, 310 mg, 1 mmol), 1,4-benzodioxane-6-boronic acid ($ArB(OH)_2$, 360 mg, 2 mmol), and triethanolamine (TEA, 303 mg, 3 mmol) in dioxane (10 mL) and water (4 mL) under N_2. The resulting mixture was heated at 100 ºC for 6 h. After completion, mixture was cooled to RT and 50mL of water was added. Then mixture was extracted three times with ethyl acetate (EtOAc, 20 mL). The organic layer was washed with brine (50 mL), dried over $MgSO_4$, filtered, and concentrated to provide crude product, which was purified by column chromatography (silica gel, EtOAc/EtOH = 3:1) followed by crystallization from ether to afford the desired product **MDP5** (281 mg, 77%) as a yellowish solid with melting point 182–184 ºC.

Step 2. MDP5 was characterized by ^{1}H NMR as: ^{1}H NMR (500 MHz, $CDCl_3$) 8.13 (d, *J* = 8.0 Hz, 1H), 7.53 (d, *J* = 8.0 Hz, 1H), 7.38 (t, *J* = 7.5 Hz, 1H), 7.05 (d, *J* = 1.5 Hz, 1H), 7.00 (dd, *J* = 7.5, 1.5 Hz, 1H), 6.94 (d, *J* = 8.0 Hz, 1H), 5.51 (s, 1H), 4.29–4.33 (m, 4H), 3.76 (t, *J* = 5.0 Hz, 4H), 3.40 (t, *J* = 5.0 Hz, 4H); ^{13}C NMR (125.7 MHz, $CDCl_3$) 177.28,

162.52, 150.53, 143.47, 143.45, 133.25, 129.71, 129.46, 124.68, 124.67, 123.35, 122.47, 118.37, 117.08, 86.96, 65.94, 64.49, 64.36, 44.78.

Molecular docking

The minimized proteins were imported to Maestro (Maestro and Glide dock reference) using the OPLS_2005 force field with backbone atoms restrained to reduce steric repulsion and, in the meantime, to minimize the impact on the backbone atoms. Structures of LY294002, quercetin, SF2523, SF2535, ABBV_744, IBET_762, and MDP1-MDP5 were built and minimized with MMFF force field using MOE program. The minimized structures were used to prepare the Grid files that identify and describe the binding pocket using the Glide Grid Generation protocol with the bound ligand as the centroid.

Three model proteins as defined by the grid file were used to dock the ligand structures of LY294002, quercetin, SF2523, SF2535, ABBV_744, IBET_762, and MDP1-MDP5. To allow some receptor flexibility during docking and extra-precision approach was applied and the scaling factor for receptor van der Waals for the nonpolar atoms was set to 0.8. The remaining parameters were used unchanged. The protein/ligand complexes were expressed as docking scores, where higher negative docking score indicate stronger ligand binding. The PyMOL program *(PYMOL Schrödinger,* New York*)* was used to generate protein/ligand interactions figures.

BRD2-BD2/BRD4-BD2 molecular cloning, expression, and purification

For Large-scale production of proteins, transformed E. coli were grown in LB media supplemented with carbenicillin (264 mM). Cells were incubated at 37 °C with constant

shaking (100 RPM) until the OD_{600nm} reached to 0.6. Next, 1 mM isopropyl β-D-1-thiogalactopyranoside was added to the cultures (16 °C), to induce the protein expression. Cells were harvested after 20 hours of induction. The resulting cell pellet was resuspended in a lysis buffer containing 50 mM Tris pH 7.5, 200 mM NaCl, 0.5 mM imidazole, and 0.3 mM tris(2-carboxyethyl) phosphine (TCEP). The cell suspension was treated with DNase-I and lysozyme, placed on ice for 30 min, and then sonicated to lyse the cells. Clarification of the lysate was accomplished by centrifugation at 12,000 rpm for 45 minutes. A metal affinity cobalt column of 5 mL capacity was pre-equilibrated with lysis buffer and then used to separate the supernatant. Then, unbound *E. coli* proteins were eliminated by passing 25 column volumes of lysis buffer. Elution buffer containing 50 mM Tris pH 7.5, 200 mM NaCl, 150 mM imidazole, and 0.3 mM TCEP was used to elute recombinant protein, isocratically. The N-terminal histidine tag of the recombinant protein was cleaved using the rhinovirus 3C protease, and the sample was dialyzed against a buffer containing 50 mM Tris pH 7.5, 100 mM NaCl and 0.3 mM TCEP. After 12 hours, the protein solution was run through a 5 mL cobalt column that had been pre-equilibrated with dialysis buffer. The flowthrough constituted the recombinant protein without the His-tag was collected.

Protein thermal shift assay

Protein Thermal Shift Dye (ThermoFisher) was also added to 1X concentration. Under these conditions, 3 protein melting curves with each respective BD and 100 mM final concentration of ligand were performed from 25 to 95 °C with a ramp rate of 0.5 °C/min. Evaluation of the derivative of the fluorescence signal employed the Applied

Bioscience Protein Thermal Shift Software version 1.3 to determine the average melting temperature (T_m) for each sample and the standard deviation(s) of those T_m values.

Protein crystallization and X-ray diffraction experiments

All complexes were incubated on ice for 4 hours prior to crystallization. For crystallization, the hanging drop vapor diffusion technique was used, and drops were made by combining 2 µL of reservoir well solution with 2 µL of the protein-inhibitor complexes. The 100 µL well solution of BRD2-BD1/inhibitor complex consisted of 0.2 M sodium tartrate dibasic dihydrate pH 7.5 and 20 % w/v PEG 1000. The solution containing 0.1 M Bis-Tris of pH 6.5, 16 % w/v PEG 3350, and 0.1 M HEPES of pH 7.5 was used for the BRD2-BD2/inhibitor and BRD4-BD2/inhibitor complexes, respectively. The resulting crystals were observed after four days. For cryoprotection, 2 µL of 50 % w/v PEG-3350 was added to the crystallization drop. In preparation for X-ray diffraction experiments, cryoprotected crystals were harvested and flash-cooled in liquid nitrogen. The ligand-free BRD4-BD2 crystals were prepared by mixing 2 µL of 5 mg/mL protein and 2 µL of well solution (0.1 M HEPES pH 7.5, 28 % w/v PEG 3350). The ligand-free BRD4-BD2 crystals were cryoprotected and flashed-cooled according to the procedure described above.

Molecular replacement used the previously published BRD2-BD2/HWV complex structure (RCSB accession number 6E6J)EJ Faivre, KF McDaniel, DH Albert, SR Mantena, JP Plotnik, D Wilcox, L Zhang, MH Bui, GS Sheppard, L Wang, et al. [1], and BRD4-BD2/89J complex structure (RCSB accession number 5UF0) [2] as the search models for phasing the BRD2-BD2/MDP5, SF2523, and BRD4-BD2/ SF2523 and ligand-free BRD4-BD2, respectively, using Phaser [3] in PHENIX [4]. All refinements were performed using PHENIX. COOT was used for visualization and manual refinement of

the structures [5]. Structure validation was performed in PHENIX using MolProbity and by RCSB following submission of coordinates and phases [6].

Cell viability and migration assays

Briefly, 3×10^3 cells/well were grown for overnight in 96 well plate and then treated for 48 h with different drug concentrations. MTT (Biosynth Carbosynth; T-2450) reagent was added to the treated cells and incubated for 4 h. The media was removed carefully without disturbing the crystals and added 200µL DMSO to dissolve formazan crystals. The absorbance was measured at 560 nm using a microplate reader (Molecular Devices Me5). The IC_{50} was determined by nonlinear least square regression by performing each experiment in triplicate.

For transwells migration assay, cells were trypsized and approximately 1×10^5 cells were suspended in serum free media and added into apical chamber of insert while media containing FBS was placed in the basolateral chamber of a 24 wells plate. After 24 h, cells from apical side were washed with PBS thrice and removed with an cotton tipped applicator. Next, migrated cells on the dorsal side of the insert membrane were fixed and stained for 30 minutes with formalin and 0.5% crystal violet, respectively. The images were captured using light microscope at 20× magnification. After that, 10% acetic acid solution was used to dissove stained cells and optical density (OD) was measured at 590nm.

For wound healing/scratch assay, both MB cells were grown in 6 well plates for overnight to form a monolayer. Sterile cell scraper was used to create scratch or wound in the center of the wells, washed twice with PBS to remove unattached cells, and then

monolayer were treated with SF2523, MDP1, and MDP5 with concentration of 5.0 µM. The images were taken at 0 and 24 h with light microscope at 4× magnification.

Effect of MDP5 on colony formation, cell cycle, and apoptosis

For colony formation assay, approximately 500 DAOY and HD-MB03 cells were grown on 6 well plates for overnight and treated with drugs at 5.0 µM concentration. After 48 hours of treatment, the medium was changed to drug-free media, and allowed the cells to continue grow for 12–14 days. Cells were rinsed thrice with PBS, fixed with 10% formalin, and stained with a 0.5% solution of crystal violet dye (Fisher Scientific; C581-25) in 20% methanol. After taking pictures, the colonies were dissolved in 10% acetic acid solution to measure the optical density (OD).

Cell cycle distribution was determined in proliferating MB cells. Cells (0.2×10^6) were seeded in a 6 wells plate, and growth inhibition was induced by adding drug solutions at the concentration of 5.0 µM for 48 h. Cells were harvested with 0.25% EDTA-free trypsin (Gibco, USA) and washed twice in ice-cold PBS. Control and treated cells (0.1×10^6) were stained with FxCycle PI/RNase Staining Solution (ThermoFisher Scientific) and analyzed for DNA content via flow cytometry. For apoptosis assay, the cells were collected as above and stained in dark for 5 min with 5 µL Annexin V-FITC and 10µL propidium iodide (PI) in 500 µL of 1 × binding buffer. Flow cytometry (FACS Calibur cytometer, BD Biosciences) was performed to quantify and calculate the cell apoptosis rate.

RT-qPCR and Western blot analysis

The cells were washed three times with cold PBS before total RNA was isolated with RNA extraction kit (RNeasy, Qiagen). cDNA was synthesized by reverse transcription using TaqMan Reverse Transcription Reagents (applied biosystems; N8080234). Next, RT-qPCR were run using above synthesized cDNA, primers, SYGR green, and RNase free water (Roche Light cycler 480 SYBR Green I Master; 04707516001). The gene expression levels were calculated using $2^{-\Delta\Delta Cq}$ method with β-actin as the internal reference gene.

To detect the target protein expression in MB cells, treatments were performed as mentioned above. Protein was extracted from cells with RIPA buffer in the presence of protease and phosphatase inhibitors. The concentration of protein was quantified by BCA assay. Then Laemmli were added to protein and boiled for 5 min to denature the protein. Next, 40µg of lysate protein was run on SDS page. Next, protein was transferred from gel to polyvinylidene difluoride (PVDF) membrane. Membrane was incubated with 5% solution of non-fat dry milk in Tris-buffered saline plus Tween 20 for 1 h, following four-five washing for 5 min each. Membrane was probed against several primary antibodies such as GLI1, GLI2, MYCN, p-MYCN, CyclinD1, p-PI3K, PI3K, p-Akt, Akt, and β-actin for overnight in 4°C. Next, membrane was washed for five times with TBST and incubated with secondary antibodies conjugated with HRP and labeled with IR dye (1:10000) at room temperature for 1 h. Before scanning (iBright FL1000; Invitrogen), membrane was washed with TBST four times.

Tumor spheroid assay

For tumor spheroids assay, 1500 and 500 DAOY and HD-MB03 cells were suspended in 100µL media and seeded in ultra-low attachment (ULA) 96-well plate for a

week and 50µL of media was replaced by fresh media every third day and spheroid size was track under fluorescent microscope. When tumor spheroid size reached to 200 µm, then spheroids were treated with SF2523, MDP1, and MDP5 with concentration of 5.0 µM. After 7 days of treatment period, spheroids were stained with Calcein AM (2µM) and ethidium homodimer1 (3µM) (LIVE/DEAD^{TM}; L3224) to check the live/dead cells. Images were taken by confocal laser microscope (Zesis LSM 710) with 10X magnification at 40µm depth.

Animal studies

All animal experiments were conducted according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Nebraska Medical Center and met federal guidelines. We generated subcutaneous and orthotopic MB mouse models in this study. For establishing the subcutaneous model, DAOY cells (1×10^6) and HD-MB03 cells (2×10^6) were injected into the right and left flank, respectively, of 8-10 weeks old male and female mice. Tumor growth was measured non-invasively by using vernier calipers. For both measurements, tumor volumes were calculated by using the following formula: Volume = (Length × Width^2)/2

To establish orthotopic MB model, stably luciferase expressing DAOY cells (0.1×10^6) were used to generate the orthotopic MB tumor in NSG mice. Bioluminescence imaging using IVIS (Spectrum) was done to monitor the tumor growth weekly after implantation surgery.

References

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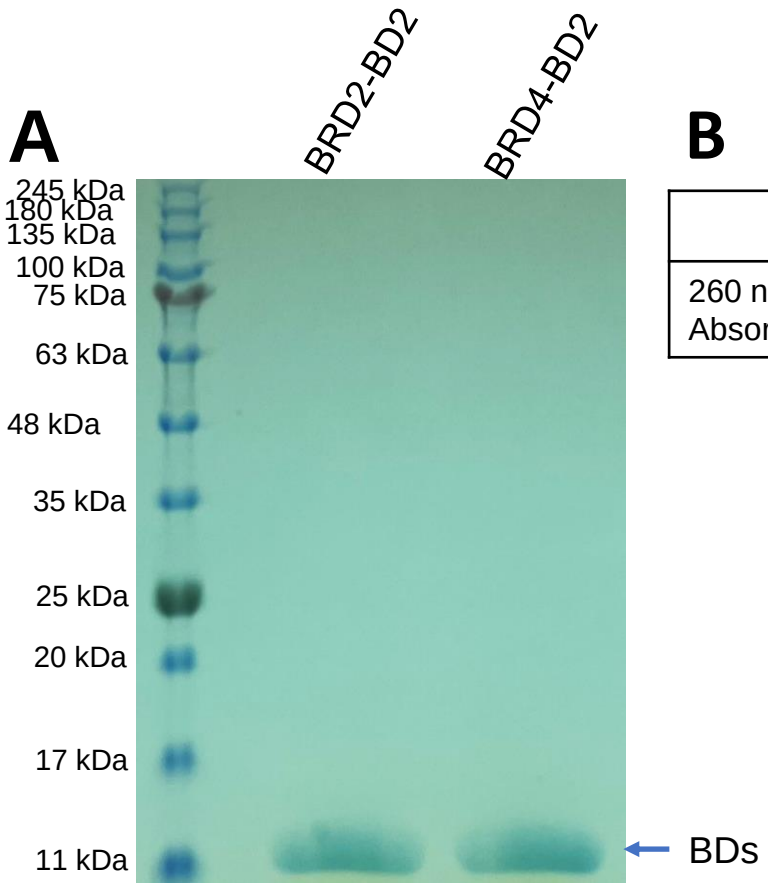
Supplement Tables: S1 to S5

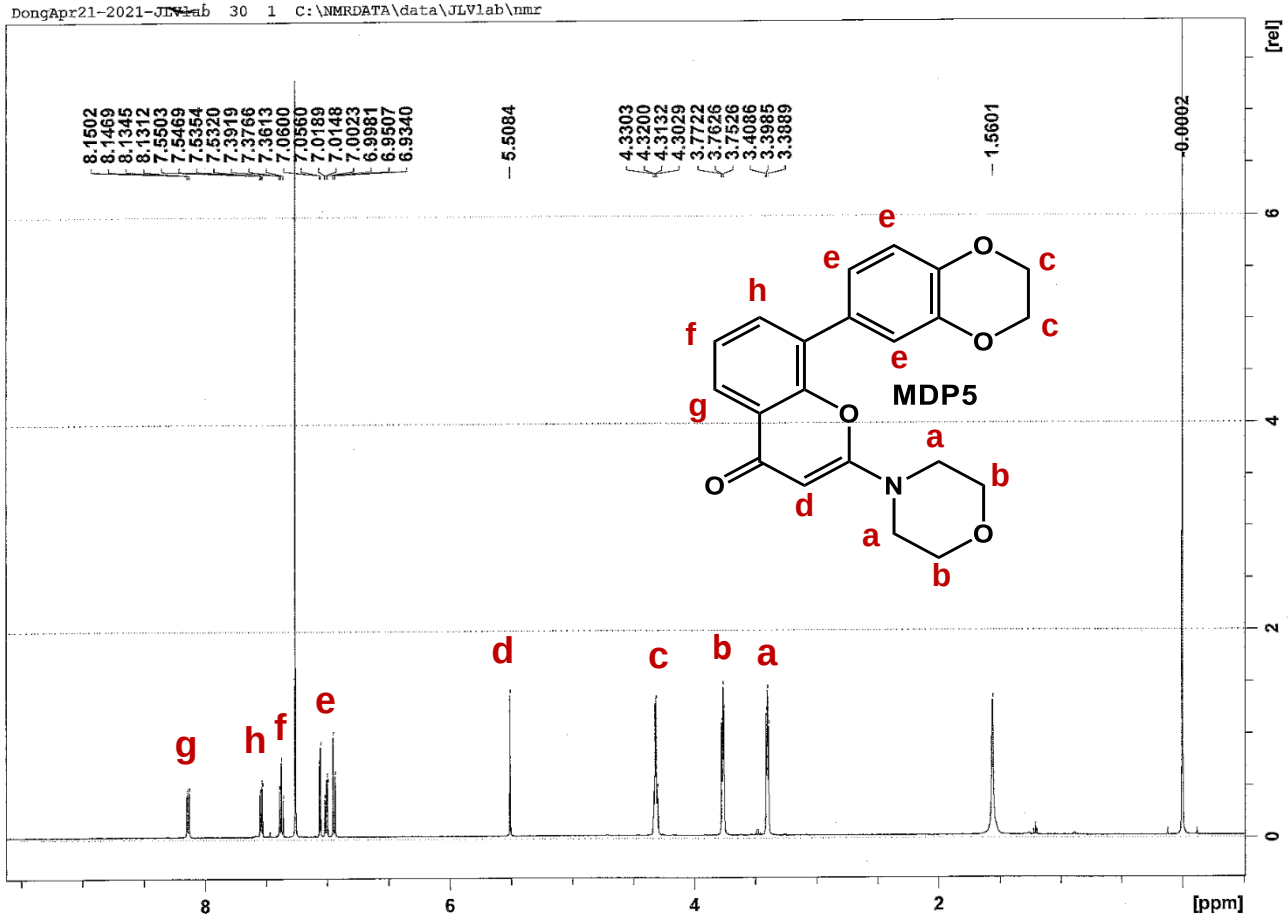


B

	BRD2-BD2	BRD4-BD2
260 nm/280 nm Absorbance ratio	0.64	0.69

Figure S1. The purity assessment of BRD2-BD2 and BRD4-BD2. **A**. SDS-PAGE gel image of purified BRD2-BD2 and BRD4-BD2. **B**. The 260/280 nm wavelength absorbance ratio of each purified proteins illustrating a lack of nucleic acid contamination.

a **1H-NMR**



b **Mass spectra**

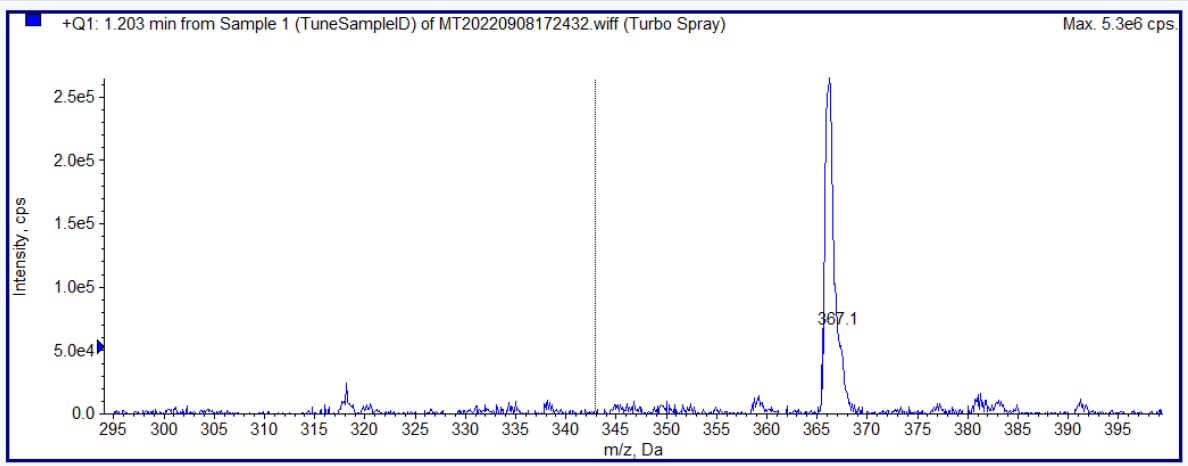
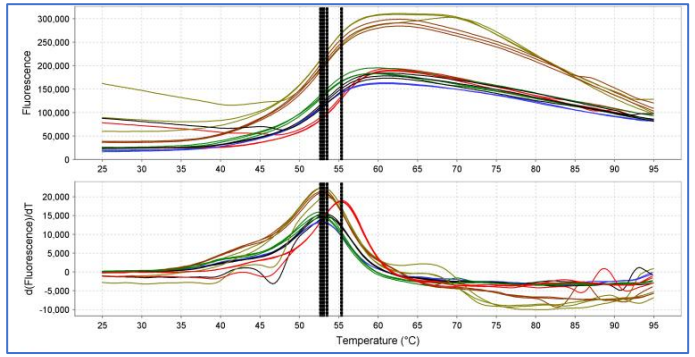


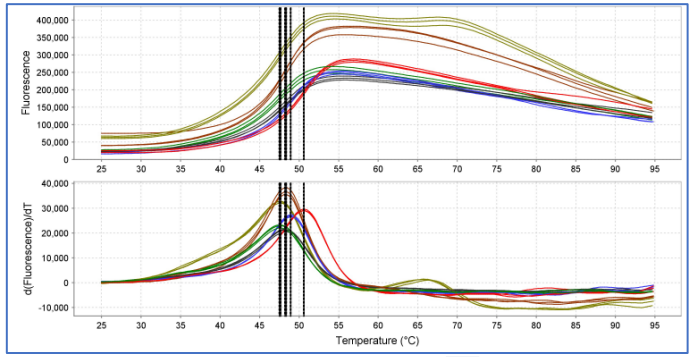
Figure S2. Characterization of MDP5. MDP5 structure was characterized by **a** ^{1}H NMR. **b** Mass spectrometer.

a



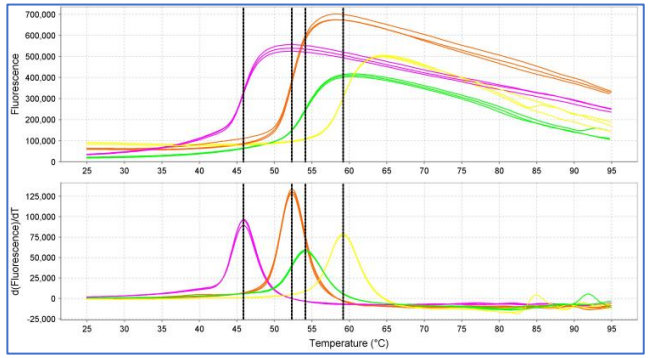
BRD2-BD2 (3) BRD2-BD2-MDP1 (3) BRD2-BD2-MDP2 (3)
BRD2-BD2-MDP3 (3) BRD2-BD2-MDP4 (3)
BRD2-BD2-MDP5 (3)

b



BRD4-BD2 (3) BRD4-BD2-MDP1 (3) BRD4-BD2-MDP2 (3)
BRD4-BD2-MDP3 (3) BRD4-BD2-MDP4 (3)
BRD4-BD2-MDP5 (3)

c



BRD2-BD2 (3) BRD2-BD2-SF2523 (3)
BRD4-BD2 (3) BRD4-BD2-SF2523(3)

d

($T_m \pm SD$) °C							
	Protein Only	MDP1	MDP2	MDP3	MDP4	MDP5	SF2523
BRD2-BD2	52.9 ± 0.1	53.1 ± 0.3	52.9 ± 0.1	52.7 ± 0.1	53.3 ± 0.2	55.3 ± 0.1	59.2 ± 0.0
BRD4-BD2	48.9 ± 0.0	47.6 ± 0.1	48.3 ± 0.1	47.6 ± 0.1	48.3 ± 0.1	50.6 ± 0.0	52.3 ± 0.0

Figure S3. Protein thermal shift analysis results of MDP series and SF2523. **a** BRD2-BD2 with MDP series. **b** BRD4-BD2 with MDP series. **c** BRD2-BD2 and BRD4-BD2 with SF2523. **d** Summary of the protein thermal shift results.

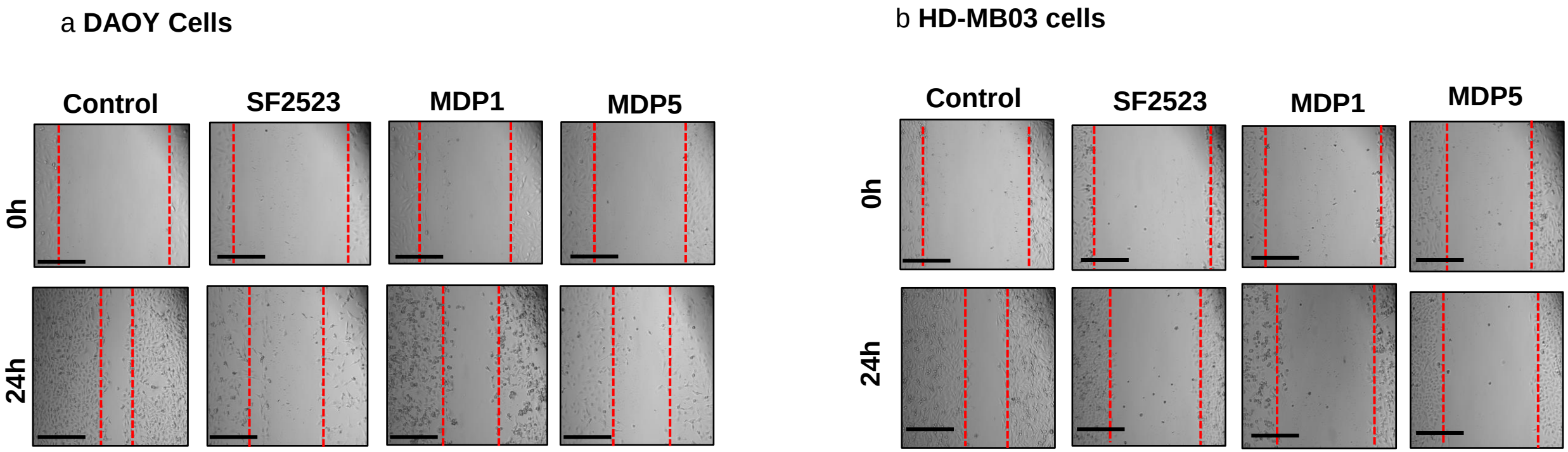
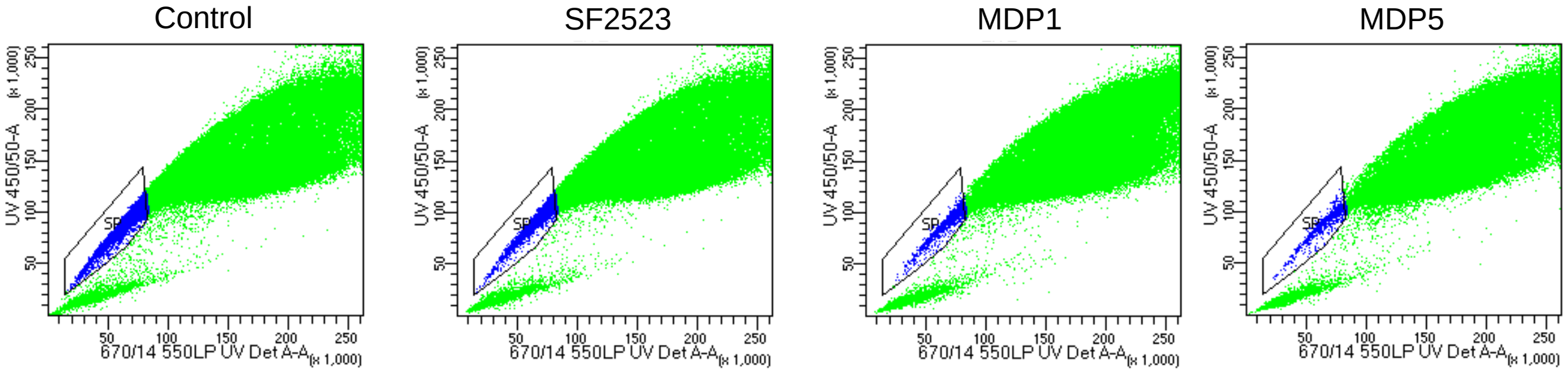


Figure S4. In vitro wound-healing/scratch assay. **a, b** DAOY and HD-MB03 cells were treated with 5.0 μM of indicated drugs. Photomicrographs were obtained at the indicated time points using a 4X objective on an inverted microscope. Scale bar = 100 μm.

a **Flow cytometry plots**



b **Side population**

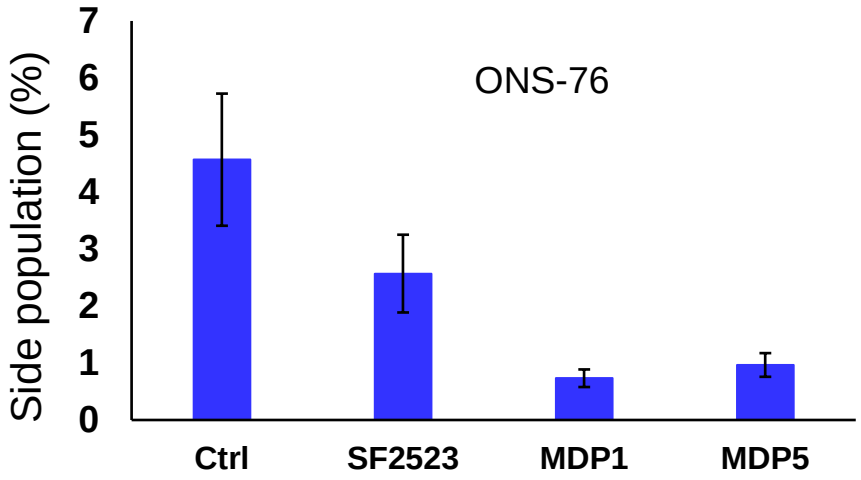


Figure S5. Effect of treatments on stem cell like side population in ONS-76 MB cells. **a** Representative flow cytometry plot. **b** Quantitation of % side population after treatment in ONS-76 cells.

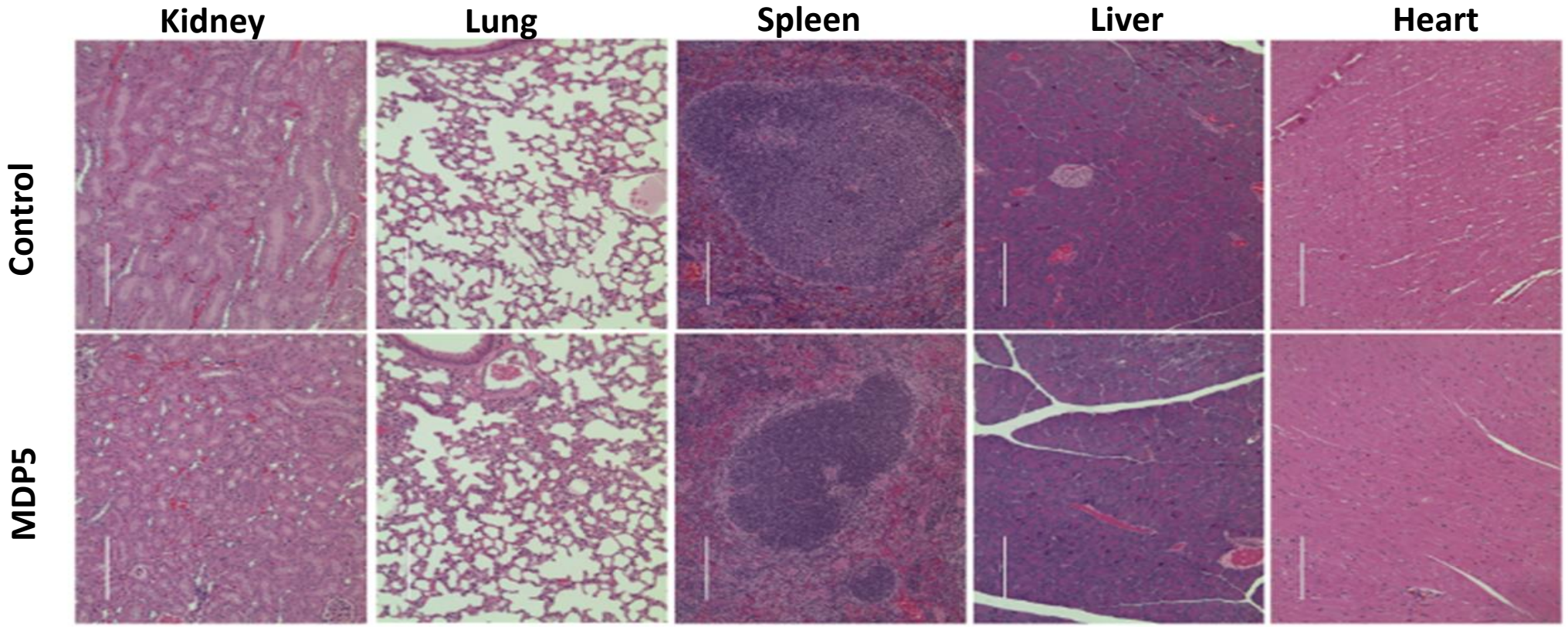


Figure S6. Histology examination of major organs including heart, liver, spleen, lung and kidney in mice of all treatment groups was performed.

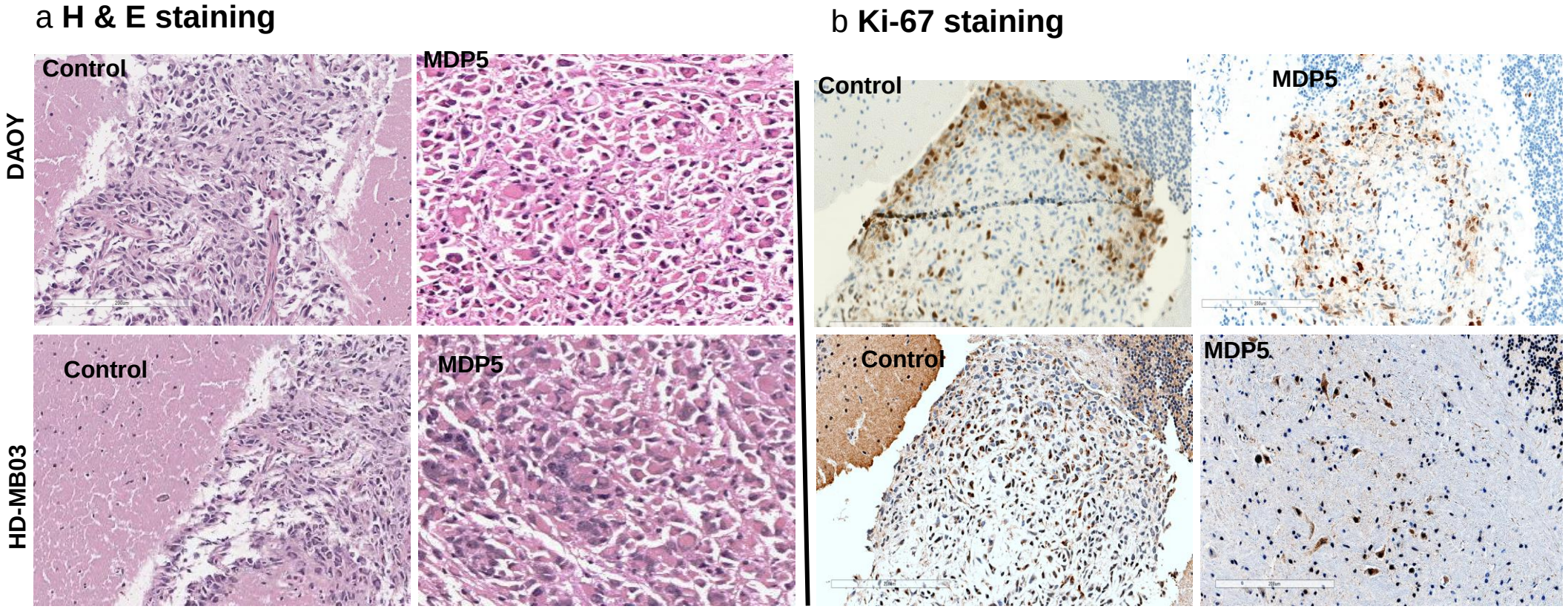


Figure S7. Immunohistochemical (IHC) staining of xenograft tumors. **a** H&E staining and **b** IHC staining of Ki67 of section of mice brain show the tumor and proliferating cells.

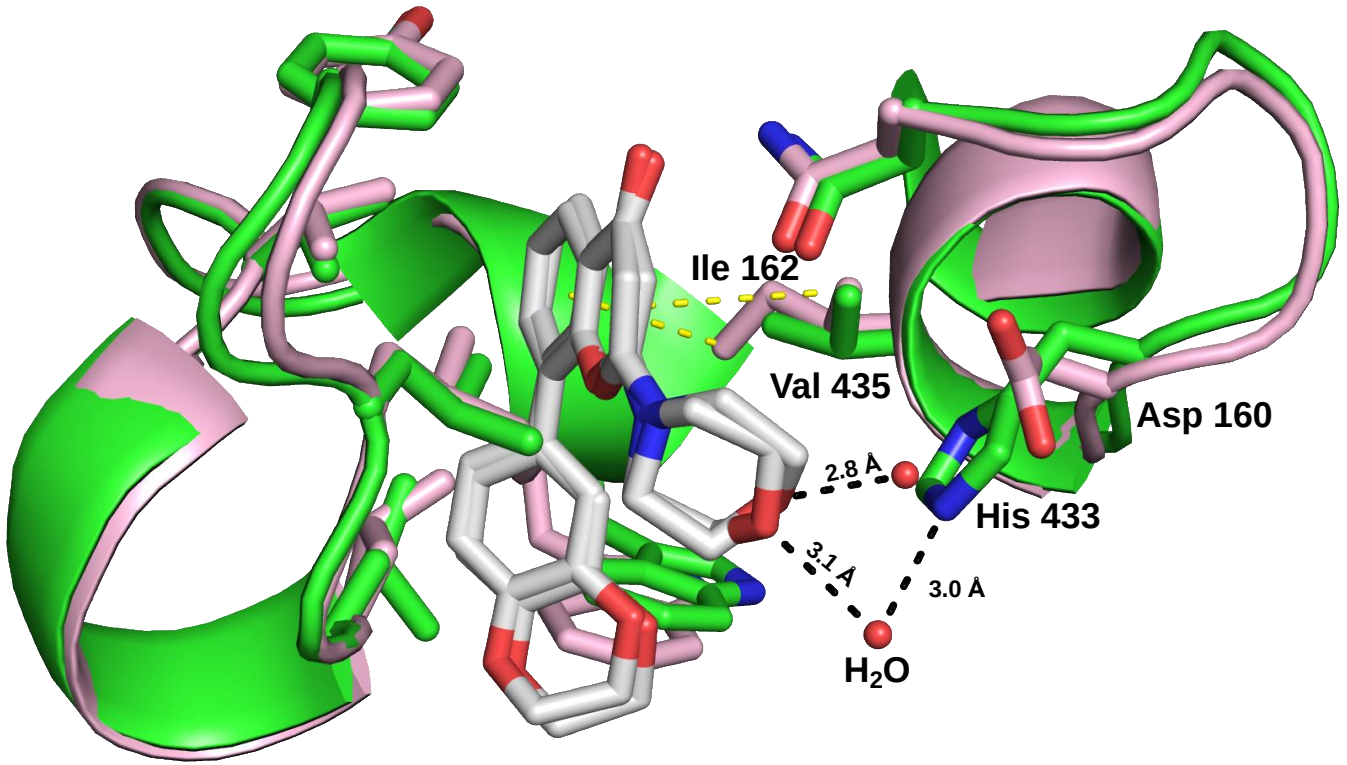


Figure S8. Superimposed acetyllysine binding pockets of BRD2-BD1(pink carbon atoms)/MDP5 and BRD2-BD2 (green carbon atoms)/MDP5 complexes.

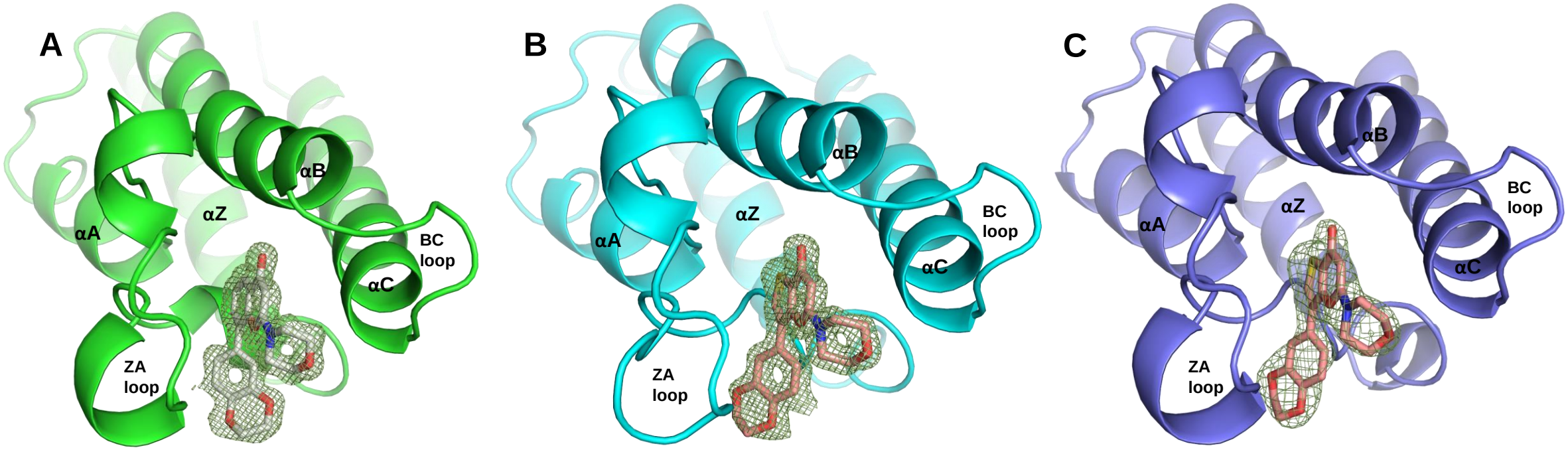


Figure S9. MDP5 and SF2523 bound acetyllysine binding pockets of BRD2-BD2 and BRD4-BD2. **A** BRD2-BD2/MDP5 complex structure. **B** BRD2-BD2/SF2523 complex structure. **C** BRD4-BD2/SF2523 complex structure. The $2F_O - F_C$ composite omit maps of all three structures are contoured at 1.5σ.

Supplementary Tables

Table S1. Reagents for RT-PCR.

a

Gene	Forward	Reverse
Cyclin D1	AGCAACAGCAACGCAAAG	AATAGCCAGGAGAGGAGGA
BCL2	ATTCGATCCACCCTTCAAG	TTCCAGTTTCTCACCGTGTCACCT
β-actin	TTAGTTGCGTTACACCCTTTCTTG	CTGTCACCTTCACCGTTCCAGTTT

b

Reagent	Supplier	Catalog
RNA extraction kit (RNeasy)	Qiagen	74004
TaqMan Reverse Transcription Reagents	Applied Biosystems	N8080234
SYBR Green, I Master	Roche	04707516001

a Sequence of forward and reverse primers used. **b** Reagents used for reverse transcriptase and polymerization chain reaction.

Table S2. Reagents used for Western blot analysis**.**

a

Reagent	Supplier	Catalog
Protease inhibitors	Sigma-Aldrich	P8340
Phosphotase inhibitors	Sigma-Aldrich	P0044
Peirce BCA assay kit	Thermo Scientific	23227
Laemmli	Alfa Aesar	J61337
SDS page	BIO-RAD	4561084
Polyvinylidene difluoride (PVDF)	Invitrogen	IB401001
Blotto, non-fat dry milk	ChemCruz	sc2324
Tris-buffered saline	Thermo Scientific	28358
Tween 20	Sigma Aldrich	P5927
ECL™ start western blotting detection reagent	Cytiva Amersham	RPN3244

b

Primary antibodies	Dilution factor	Supplier	Catalog
GLI1	1:1000	Abcam	ab134906
GLI2	1:1000	EMD Millipore Corp	ABN2241
MYCN	1:1000	Santacruz Biotech;	sc-53993
p-MYCN	1:1000	Bethyl	A300-206A
CyclinD1	1:1000	Abcam	ab16663
PI3K	1:1000	Cell Signalling Technology (CST)	13666S
p-PI3K	1:500	Biomatik	CAF11012
Akt	1:1000	CST	9272S
p-Akt	1:500	CST	4060S
β-actin	1:1000	Santacruz Biotech	sc-69879

a Material used during Western blot. **b** Primary antibodies with dilution factor.

Table S3. Docking scores (kcal/mol) and errors (ΔΔG, kcal/mol) between docking predicted and observed binding affinity of known BD1 and BD2 inhibitors.

Compound	BD1_Kd (nM)	BD2_Kd (nM)	RD0_d	BD1_d	BD2_d	BD1_O	BD2_O	ΔΔG_BD1	ΔΔG_BD2
ABBV_744	2006	4	-7.04	-7.34	-11.64	-7.77	-11.46	-0.43	0.19
IBET_762	545	158	-3.79	-7.26	-6.99	-8.54	-9.28	-1.29	-2.29
SF2523	150	710	-8.36	-6.88	-8.33	-9.31	-8.39	-2.42	-0.06
SF2535	277	688	-7.40	-6.16	-8.74	-8.95	-8.41	-2.78	0.34
							mean	-1.73	-0.46

Note: ΔΔG = ΔG (observed, BD1_O, or BD2_O) – ΔG (predicted, BD1_d, BD2_d) , where BD1_d, and BD2_2 were measured by the docking scores obtained from the Glide Dock, and the ΔG (observed) = $1.987*298.15*\ln (Kd*10^{-9})/1000$, where Kd in nM concentration.

Table S4. Docking scores (kcal/mol) and predicted binding affinity (Kd, μM) of synthesized BD1 and BD2 inhibitors.

Compound	Glide_RD0 (kcal/mol)	Glide_BD1 (kcal/mol)	Glide_BD2 (kcal/mol)	PI3K_Pred (IC50 or KD, μM)	BD1_Pred (IC50 or KD, μM)	BD2_Pred (IC50 or KD, μM)
LY294002	-6.12	-7.42	-9.20	32.41	3.64	0.18
MDP1	-8.41	-6.93	-7.75	0.68	8.28	2.08
MDP2	-7.24	-7.34	-3.63	4.94	4.16	2167.63
MDP3	-4.95	-6.77	-5.21	234.31	10.87	152.10
MDP4	-6.26	-6.36	-6.86	25.85	21.72	9.34
MDP5	-7.31	-7.91	-9.29	4.38	1.59	0.16
Quercetin	-8.42	-9.48	-8.83	0.67	0.11	0.33

Please note the predicted IC_{50} or Kd is based on docking score conversion and due to the errors as seen in Table 1, they may not be exactly the same as what you have from experimentally. If the trends turn out to be right, it would be good. Note: Predicted Kd = $e^{((Glide\_score * 1000)/(1.987 * 298.15)} * 10^6$, Kd unit: μM.

Table S5. X-ray and crystallographic statistics.

a

	BRD2-BD1/MDP5	BRD2-BD2/MDP5	BRD2-BD2/SF2523	BRD4-BD2/SF2523	BRD4-BD2
X-ray diffraction					
Wavelength (Å)	0.98	0.98	0.98	0.98	0.98
Space group	P 1 21 1	P 21 21 2	P 21 21 2	P 43 21 2	P 21 21 2
Unit cell dimensions					
a (Å)	63.810	52.560	52.345	77.625	55.785
b (Å)	149.872	72.277	71.732	77.625	73.054
c (Å)	63.819	32.213	32.096	100.645	32.309
α	90°	90°	90°	90°	90°
β	112.3°	90°	90°	90°	90°
ϒ	90°	90°	90°	90°	90°
Resolution (Å)	35.56 - 2.5	19.29-1.20	29.59 - 1.27	32.82 - 2.08	19.45 - 1.22
R_{pim}	0.048 (0.402)	0.039 (0.385)	0.041 (0.380)	0.055 (0.643)	0.036 (0.239)
R_{merge}	0.126 (1.065)	0.106 (1.083)	0.111 (0.753)	0.192 (1.075)	0.077 (0.452)
$CC_{1/2}$	0.978 (0.834)	0.991 (0.762)	0.990 (0.699)	0.999 (0.627)	0.999 (0.832)
I/σI	24.6 (2.0)	28.7 (3.4)	28.8 (1.3)	15.1 (1.2)	37.9 (3.6)
Completeness (%)	99.69 (99.64)	91.85 (90.65)	98.76 (93.20)	99.55 (99.84)	95.77 (99.86)
Refinement					
Total no. of reflections	1368277	832780	842847	3050708	439225
No. of unique reflections	38336 (3873)	35994 (3501)	32284 (2984)	19034 (1874)	41150 (4228)
R_{work}/R_{free}	0.2015/0.2497	0.1772/0.1973	0.1674/0.1913	0.2116/0.2654	0.2124/0.2305
No. of atoms					
Protein	5537	926	941	1777	882
Ligand/ion	376	36	50	52	24
Water	75	249	195	117	188
B-factors (Å²)					
Protein	54.42	12.72	17.38	52.01	18.36
Ligand/ion	65.76	16.82	28.56	53.38	31.73
Water	53.00	23.46	29.99	52.99	28.59
R.m.s deviations					
Bond length (Å)	0.009	0.007	0.011	0.009	0.006
Bond angels (°)	1.00	1.00	1.06	1.05	1.54
Ramachandran					
Favored (%)	98.17	100.00	100.00	99.07	100.00
Outliers (%)	0.00	0.00	0.00	0.00	0.00

b

	BRD2-BD2/MDP5	BRD2-BD2/SF2523	BRD4-BD2/SF2523	BRD4-BD2
Data collection				
Wavelength (Å)	0.98	0.98	0.98	0.98
Space group	P 21 21 2	P 21 21 2	P 43 21 2	P 21 21 2
Unit cell dimensions				
a (Å)	52.560	52.345	77.625	55.785
b (Å)	72.277	71.732	77.625	73.054
c (Å)	32.213	32.096	100.645	32.309
α	90º	90º	90º	90º
β	90º	90º	90º	90º
ϒ	90º	90º	90º	90º
Resolution (Å)	19.29-1.20	29.59 - 1.27	32.82 - 2.08	19.45 - 1.22
R_{pim}	0.039 (0.385)	0.041 (0.380)	0.055 (0.643)	0.036(0.239)
R_{merge}	0.106 (1.083)	0.111 (0.753)	0.192 (1.075)	0.077 (0.452)
$CC_{1/2}$	0.991 (0.762)	0.990 (0.699)	0.999 (0.627)	0.999 (0.832)
I/σI	28.7 (3.4)	28.8 (1.3)	15.1 (1.2)	37.9 (3.6)
Completeness (%)	91.85 (90.65)	98.76(93.20)	99.55 (99.84)	95.77 (99.86)
Refinement				
Total no. of reflections	832780	842847	3050708	439225
No. of unique reflections	35994 (3501)	32284 (2984)	19034 (1874)	41150 (4228)
R_{work}/R_{free}	0.1772/ 0.1973	0.1674/ 0.1913	0.2116/0.2654	0.2124/0.2305
No. of atoms				
Protein	926	941	1777	882
Ligand/ion	36	50	52	24
Water	249	195	117	188
B-factors (Å²)				
Protein	12.72	17.38	52.01	18.36
Ligand/ion	16.82	28.56	53.38	31.73
Water	23.46	29.99	52.99	28.59
R.m.s deviations				
Bond length (Å)	0.007	0.011	0.009	0.006
Bond angels (°)	1.00	1.06	1.05	1.54
Ramachandran				
Favored (%)	100.00	100.00	99.07	100.00
Outliers (%)	0.00	0.00	0.00	0.00

a X-ray diffraction and crystallographic refinement statistics for the four described structures. **b** Crystallographic data table of BRD2-BD2/**MDP5**, BRD2-BD2/**SF2523**, BRD4-BD2/**SF2523.** and BRD4-BD2/Apo structures. Values for the highest resolution shell are