Supporting Information

A Small-Molecule Probe of the Histone Methyltransferase G9a Induces Cellular Senescence in Pancreatic Adenocarcinoma

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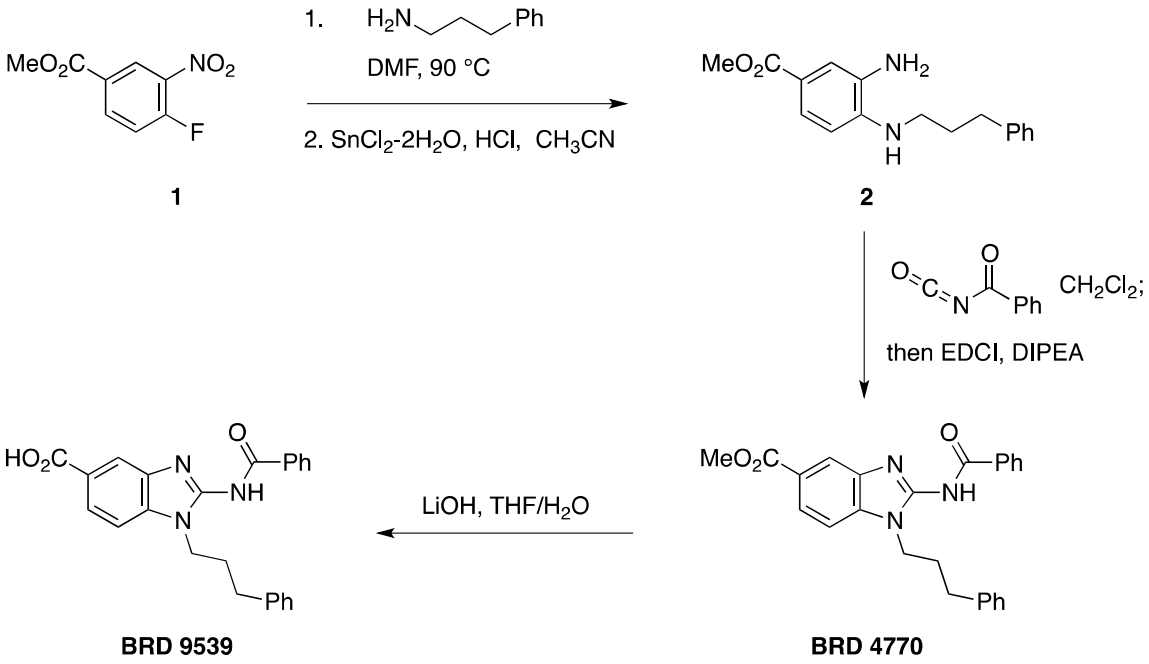
Supporting Methods

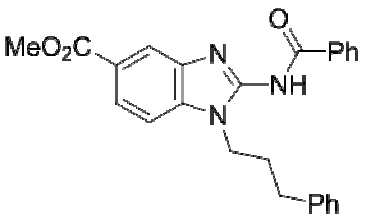
Experimental protocol for the synthesis of BRD4770 and BRD9539.



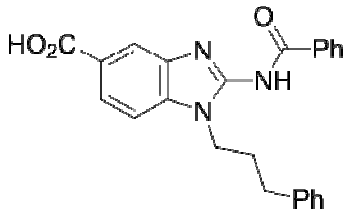
To a solution of methyl 4-fluoro-3-nitrobenzoate **1** (0.4 g, 2.0 mmol, 1.0 equiv) in DMF (4mL), was added phenylpropylamine (0.35 mL, 2.4 mmol, 1.2 equiv). The reaction mixture was heated at 90 ˚C for 3 h, then cooled down to room temperature, and concentrated down under reduced pressure. To a solution of the obtained yellow crude residue in CH_3CN (10mL), was added $SnCl_2$-$2H_2O$ (2.3 g, 10.0 mmol, 5.0 equiv) followed by HCl solution (2.5 mL, 4 M in H_2O, 10.0 mmol, 5.0 equiv). The reaction mixture was allowed to stir at room temperature for 42 h until starting material was consumed, then was added 1M NaOH aqueous solution to adjust the pH to 8, and was further diluted with EtOAc. The aqueous layer was separated and extracted with EtOAc. The combined organic layers were washed with brine and dried over Na_2SO_4. The dried solution was filtered and concentrated under reduced pressure. The resulting residue was further purified by silica gel chromatography, giving diamine as an off-white solid.

To a solution of diamine **2** (57 mg, 0.2 mmol, 1.0 equiv) in CH_2Cl_2 (5 mL), was added benzoyl isocyanate (30 uL, 0.22 mmol, 1.1 equiv). After the reaction mixture stirred at room temperature for 80 min, *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (57 mg, 1.3 mmol, 1.5 equiv) and diisopropylethylamine (0.16 mL, 0.9 mmol, 4.5 equiv) were added and the reaction mixture was left stirring at room temperature for another 14 h. Then, the reaction mixture was diluted with EtOAc and washed with 1 M HCl. The separated organic layer was washed with saturated $NaHCO_3$ aqueous solution, brine and dried over Na_2SO_4. The dried solution was filtered and the filtrate was concentrated under reduced pressure. The solid residue was washed with CH_2Cl_2 and filtered, providing BRD 4770 as an off-white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 12.45 (1H), 8.32-8.29 (m, 2H), 8.01-7.97 (m, 2H), 7.77-7.53 (m, 3H), 7.34-7.15 (m, 6H), 4.30 (t, *J* = 7.5 Hz, 2H,), 3.94 (s, 3H), 2.80 (t, *J* = 7.5 Hz, 2H), 2.28 (tt, *J* = 7.5, 7.5 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 176.9, 166.6, 154.8, 140.4, 137.6, 133.1, 129.3, 128.5, 128.3, 128.2, 128.0, 126.3, 125.2, 125.0, 112.4 108.6, 52.3, 41.9, 32.9, 29.3 ppm.

To a solution of BRD 4770 (11 mg, 0.026 mmol, 1.0 equiv) in THF/H2O (2.0 mL/0.5 mL), was added LiOH-H2O (11 mg, 0.26 mmol, 10.0 equiv). The reaction mixture was allowed to stir at room temperature for 45 h and diluted with EtOAc. The aqueous layer was collected and acidified to pH 5 with 1M HCl solution. The acidified aqueous layer was back extracted with EtOAc and the collected EtOAc extract was dried over Na_2SO_4. The dried solution was filtered and concentrated under reduced pressure, providing BRD9539 as a white solid. ^{1}H NMR (300 MHz, $(CD_3)_2SO$) δ 12.87 (1H), 8.14-8.09 (m, 3H), 7.87 (d, *J* = 9.0 Hz, 1H), 7.58 (d, *J* = 9.0 Hz, 1H), 7.53-7.43(m, 3H), 7.32-7.20 (m, 5H), 4.29 (t, *J* = 7.5 Hz, 2H,), 2.73 (t, *J* = 7.5 Hz, 2H), 2.13 (tt, *J* = 7.5, 7.5 Hz, 2H), 2.08 (br, 1H); ^{13}C NMR (75 MHz, $(CD_3)_2SO$) δ 167.3, 141.0, 131.2, 128.7, 128.3, 128.0, 125.9, 124.3, 109.6, 41.4, 32.2, 29.2 ppm.



BRD 4770



BRD 9539

Protocol for compound purity and identity test on LC-MS.

Compound purity and identity were determined by LC-MS (Waters). All compound tested were at least 90% pure measured by UV absorbance at 210 nm. Identity was determined on a SQ mass spectrometer by positive electrospray ionization. Mobile phase A consisted of 0.1%(w/v) ammonium hydroxide; mobile phase B consisted of 0.1%(w/v) ammonium hydroxide in acetonitrile. The gradient ran from 5%(v/v) to 95%(v/v) moble phase B over 0.8min at 0.45mL/min. An Acquity BEH C18, 1.7uM, 1.0X50-mm column was used with column temperature maintained at 65C. Compounds were dissociated in DMSO at the concentration of 1mg/ml and 0.25 uL of this solution was injected.

Enzymatic assays . The HDAC biochemical assays were run according to the protocol described by Bradner et al. (*1*). All HDACs were purchased from BPS Bioscience, Inc. Trypsin was purchased from Worthington Biochemical Corp. The substrate used for HDAC 1, 2, 3, and 6 was an acetyl-Leu-Gly-acetyl-Lys tripeptide; while a trifluoro-acetylated lysine substrate was used for HDAC 4, 5, 7, 8, and 9. The substrate concentration for each HDAC was run at their corresponding Km values. A 50 mM HEPES, 100 mM KCl, 0.001% (v/v) Tween-20, 0.05% (w/v) BSA, pH 7.4 buffer was used for the assay. 20 µl of substrate was added to a 384 well plate, 100 nl of compound was pinned into each reaction well, followed by the addition of 10 µl of HDAC and trypin, for a final reaction volume of 30 µl. The change of fluorescence was monitored using a plate reader (Envision, Perkin-Elmer). Fluorescence was plotted over time, and the linear portion of the slope was used to obtain the activity. Percent activity was determined from normalization to the DMSO controls. MS275 and SAHA were used as positive

controls. The profiling data for SUV39H2, MLL1, SET8, SET7/9, PRMT1, PRMT3, PRMT5 were obtained from BPS Bioscience. The profiling of kinases was performed by Millipore.

Western blotting. The following antibodies were used at 1:1000 dilution: trimethyl-histone H3 (Lys4) rabbit IgG (Cell Signaling), trimethyl-histone H3 (Lys9) rabbit IgG (Abcam), trimethyl-histone H3 (Lys27) rabbit IgG (Millipore), trimethyl-histone H3 (Lys36) rabbit IgG (Abcam), trimethyl-histone H3 (Lys79) rabbit IgG (Abcam), phospho-Chk1 (Ser345) rabbit IgG (Cell Signaling), phospho-Chk2 (Thr68) rabbit IgG (Cell Signaling), cdc25C (5H9) rabbit mAb (Cell Signaling). Tubulin mAb (Sigma-Aldrich) was used at 1:10,000 dilution. Secondary horseradish peroxidase-conjugated goat anti-mouse antibody and goat anti-rabbit antibody were used at 1:10,000 dilution.

RNA isolation and real time-PCR. Total RNA was extracted from cells using RNeasy Plus Mini Kit (Qiagen). Reverse transcription was performed using High Capacity cDNA Reverse Transcription Kit (ABI). qPCR reactions were detected in 384-well format with Applied Biosystems 7900HT. Primers were from Qiagen: EHMT1 (QT00084602) and G9a (QT00088627). Control primers were from Operon: hGAPDH forward: ACCACGTCCATGCCATCAC; hGAPDH reverse: TCCACCACCCTGTTGCTGTA; hActin forward: CTGGAACGGTGAAGGTGACA; hActin reverse: AAGGGACTTCCTGTAACAATGCA.

Comet assay. Treated cells were washed twice with ice-cold PBS, counted, and combined with agarose in a pre-warmed 96-well plate (OxiSelect^{TM} 96-well Comet Assay Kit, Cell Biolabs) Images were captured and analyzed with an Axiovert 200M microscope at 200X magnification.

DNA double-stranded break assay. Treated cells were fixed, stained using H2A.X Phosphorylation Assay Kit (Upstate Biotech), and analyzed with a BD LSR II flow cytometer. For immunofluorescent staining of γ-H2AX, we used anti-phospho-histone H2A.X (Ser139) (Cell Biolabs), Cy2-labeled secondary antibody (Jackson ImmunoResearch), and Hoechst 33342. Images were captured with an Axiovert 200M fluorescence microscope at 200X magnification.

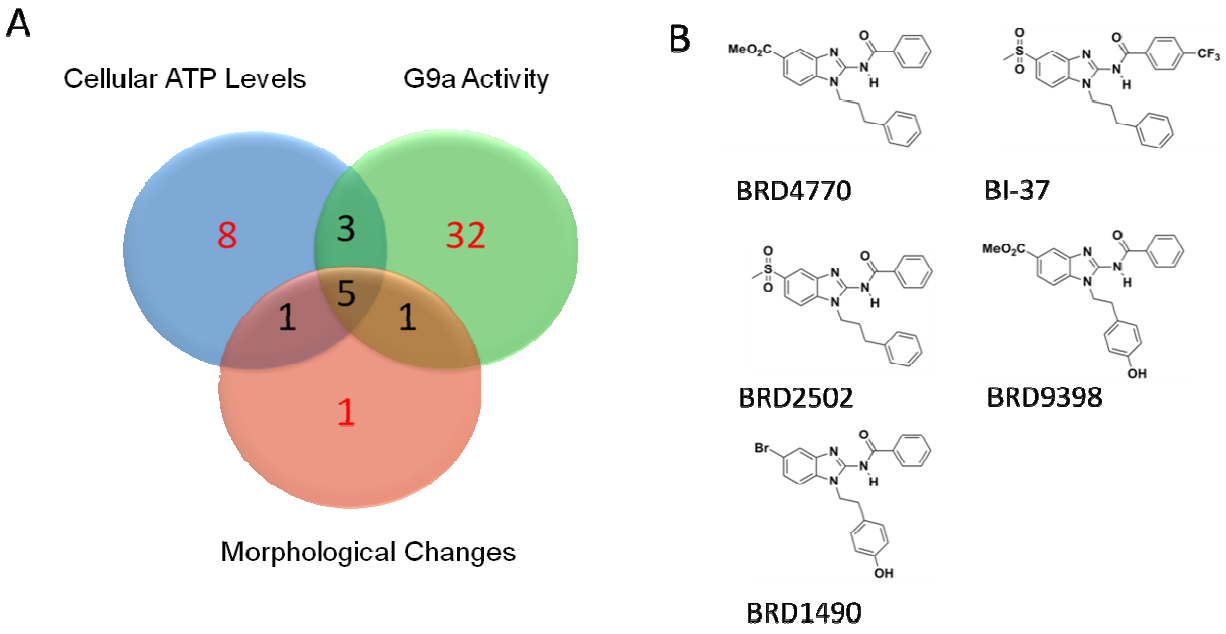


Figure S1. a) A focused library of SAM mimetics was tested in a biochemical assay against G9a activity, and two phenotypic assays for cellular ATP levels and visual examination of the morphological changes induced by compounds. b) Five compounds, BRD4770, BRD2502, BRD9398, BRD1490 and BI-37, were identified as hits in all three assays. BRD9539 (Figure 1, main text) is the carboxylic acid form of BRD4770, which was more efficient in inhibiting G9a biochemically but had no cellular activity.

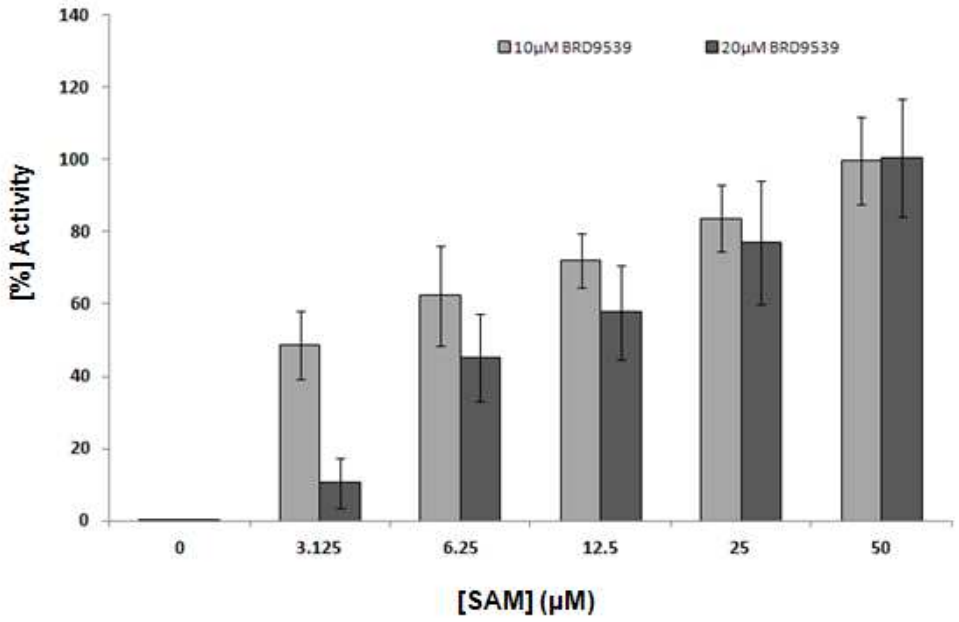


Figure S2. Biochemical activities of G9a following treatment with 10 μM and 20 μM BRD9539 with different SAM concentrations as indicated. Data represent the mean and standard error of eight independent reactions.

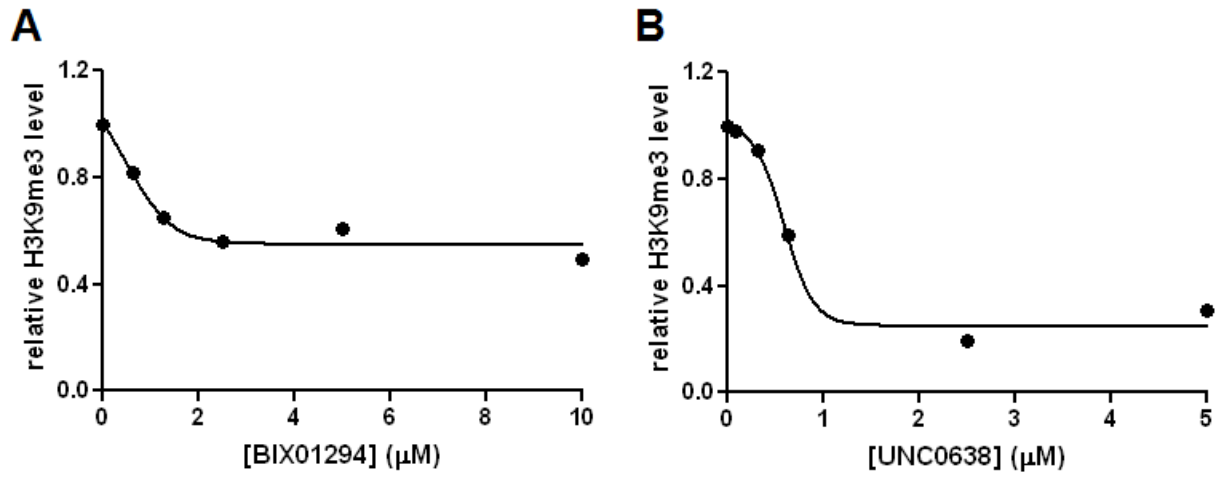


Figure S3. Western blot analysis of relative trimethylation levels of histone H3 lysine 9 in PANC-1 cells after 24-hour treatment with the indicated concentrations of a) BIX-01294 and b) UNC0638. Data was normalized to actin control and analyzed by ImageJ.

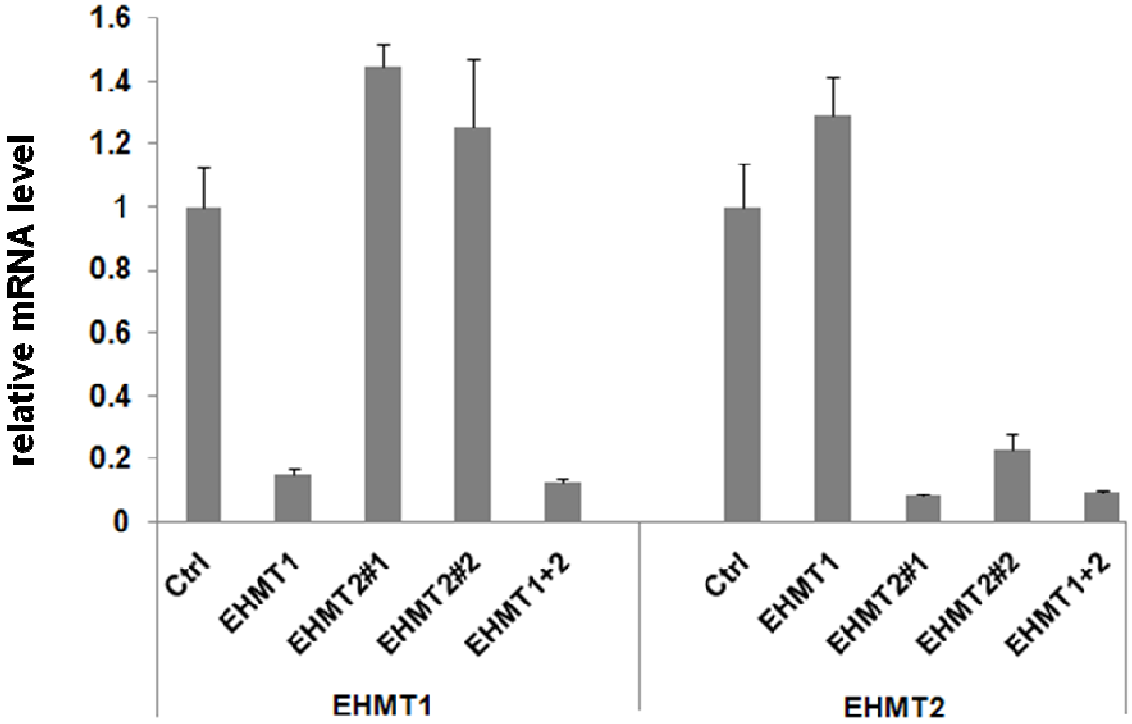


Figure S4. Quantification of the knockdown efficiency of EHMT1 (GLP) and EHMT2 (G9a) by real-time PCR. PANC-1 cells were transfected with siRNA against EHMT1 and EHMT2 for 72 hours. The average of GAPDH and actin was used as internal control for normalization.

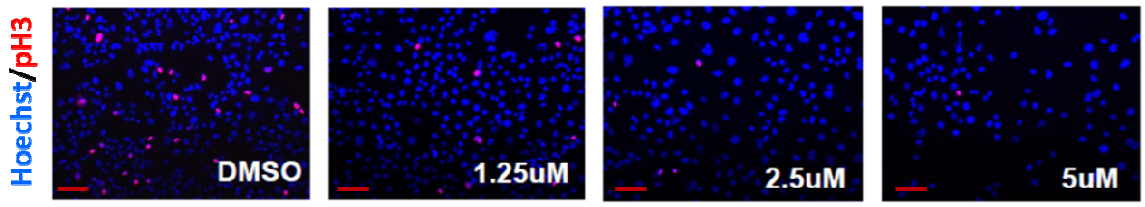


Figure S5. Assessment of BRD4770 effects on cell proliferation. PANC-1 cells were treated for 72 hours with DMSO or the indicated concentrations of BRD4770. The cells were then fixed and stained for phosphorylated Ser10 on histone H3. Scale bars = 100 μm.

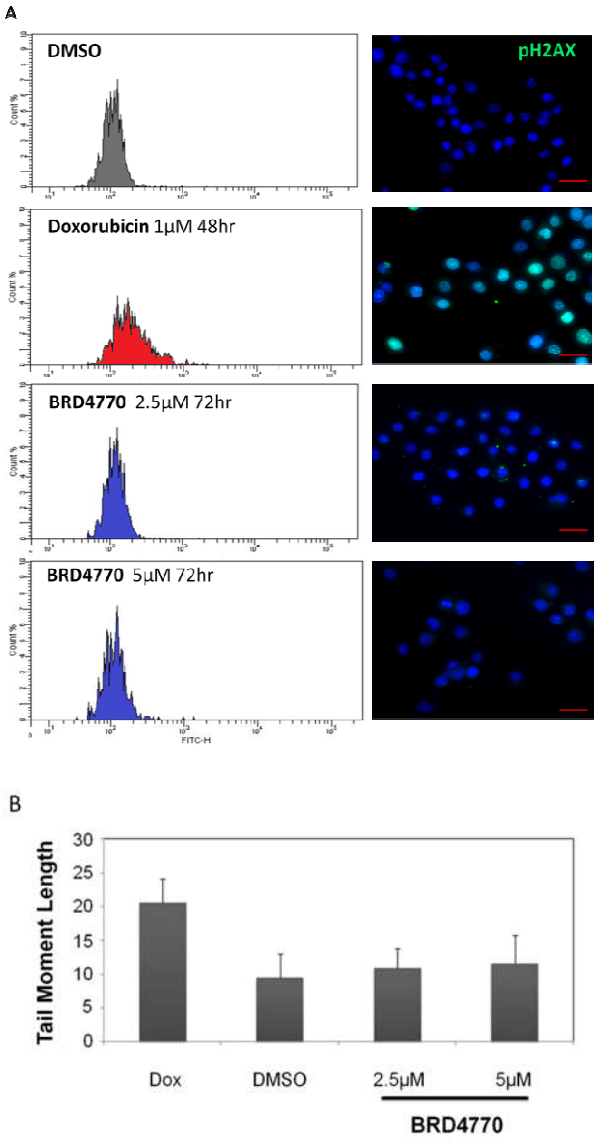


Figure S6. Effects of BRD4770 on DNA damage in PANC-1 cells. a) PANC-1 cells were treated for 72 hours with 2.5 μM or 5 μM BRD4770, or DMSO. The cells were fixed and stained for phosphorylated H2AX, and analyzed by flow cytometry and fluorescent microscopy. Doxorubicin was used as a positive control. Scale bars = 100μm. b) PANC-1 cells were treated as described in (a) for the DNA comet assay (see Methods). Data represent the mean and standard error of 50 cells from 4 independent reactions.

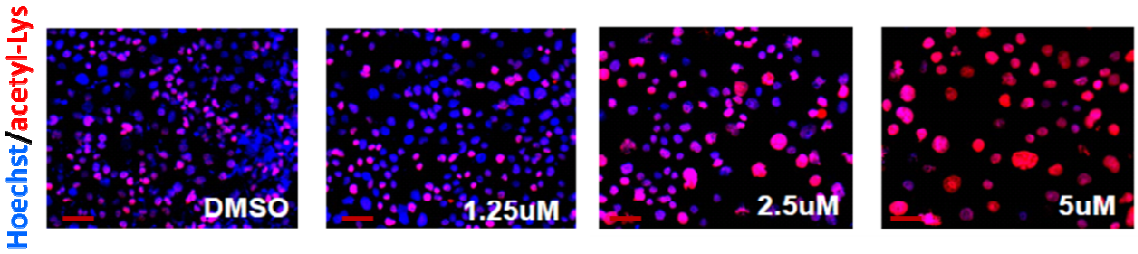


Figure S7. BRD4770 treatment increases the total acetylated lysine levels in a dose-dependent manner. PANC-1 cells were treated for 72 hours with DMSO or the indicated concentrations of BRD4770. The cells were then fixed and stained for total acetylated lysine. Scale bars = 100μm.

Enzyme Name	Remaining Activity %
G9a	54
PRC2	43
SUV39H1	102
SUV39H2	100
MLL1	98
SET7/9	95
SET8	100
PRMT1	99
PRMT3	96
PRMT5	100
NSD2	114
DNMT1	104
HDAC1	88 (86)
HDAC2	89 (85)
HDAC3	91 (83)
HDAC4	90 (83)
HDAC5(CD)	91 (87)
HDAC6	90 (84)
HDAC7	89 (89)
HDAC8	92 (91)
HDAC9	90 (93)

Table S1. The activities of 21 histone modifying enzyme were tested for BRD9539 at 10 μM. The activities of HDACs were also tested for BRD4770 at 10 μM (listed in brackets). The percentage remaining activities were calculated based on the average of at least two replicates.

Enzyme Name	Remaining Activity %	Enzyme Name	Remaining Activity %	Enzyme Name	Remaining Activity %	Enzyme Name	Remaining Activity %
Met(h)	71	PKG1β(h)	100	ROCK-II(h)	95	CDK1/cyclinB(h)	107
CK1γ2(h)	75	SGK(h)	100	Lck(h)	96	MST2(h)	107
CK1γ3(h)	80	Abl(h)	101	CaMKIV(h)	97	PKBβ(h)	107
Fms(h)	80	CaMKIδ(h)	101	CSK(h)	97	Src(1-530)(h)	107
Rsk4(h)	80	CK2α2(h)	101	Flt3(h)	97	ALK4(h)	108
Ret(h)	82	Abl(T315I)(h)	102	Syk(h)	97	CaMKI(h)	108
GSK3β(h)	83	CDK7/cyclinH/MAT1(h)	102	Arg(h)	98	HIPK2(h)	108
Rsk1(h)	83	MEK1(h)	102	CaMKIIγ(h)	98	JAK2(h)	108
RIPK2(h)	84	PKCγ(h)	102	CDK3/cyclinE(h)	98	MAPK1(h)	109
Rsk2(h)	84	Aurora-B(h)	103	CDK5/p35(h)	98	AMPKα2(h)	110
CK1δ(h)	85	CDK9/cyclin T1(h)	103	EGFR(h)	98	CaMKIIδ(h)	110
PKA(h)	85	CHK1(h)	103	EphB3(h)	98	CDK5/p25(h)	110
SGK2(h)	85	c-RAF(h)	103	MST1(h)	98	mTOR/FKBP12(h)	110
MAPKAP-K2(h)	86	DAPK1(h)	103	SAPK2a(h)	98	IKKα(h)	111
AMPKα1(h)	87	PKCβI(h)	103	EphB4(h)	99	PKCα(h)	111
mTOR(h)	89	IGF-1R(h), activated	104	JAK3(h)	99	CDK2/cyclinA(h)	112
CK1γ1(h)	91	Rsk3(h)	104	JNK2α2(h)	99	Tec(h) activated	112
Aurora-A(h)	93	SAPK2b(h)	104	JNK3(h)	99	CDK2/cyclinE(h)	113
PKBγ(h)	93	CDK6/cyclinD3(h)	105	KDR(h)	99	FAK(h)	113
PKG1α(h)	93	EphB2(h)	105	PKBα(h)	99	IGF-1R(h)	114
Plk1(h)	93	ErbB4(h)	105	CaMKIIβ(h)	100	CK2(h)	117
Flt4(h)	94	Flt1(h)	105	DAPK2(h)	100	SGK3(h)	120
ALK(h)	95	MAPK2(h)	105	IKKβ(h)	100	ASK1(h)	124
Pim-2(h)	95	PDK1(h)	105	JNK1α1(h)	100	TAK1(h)	127
PKCβII(h)	95	ROCK-I(h)	106	Pim-1(h)	100	Lck(h) activated	130

Table S2. 100 kinases that related to cell cycle control and cancer cell survival were selected and tested against BRD9539 at 5 μM**.** The percentage remaining activities were calculated based on the average of at least two replicates.

Name listed	Aliases
GLP	EHMT1, GLP1, KMT1D, H3-K9-HMTase 5
G9a	EHMT2, KMT1C, H3-K9-HMTase 3
SUV39H1	KMT1A, H3-K9-HMTase 1
SUV39H2	KMT1B, H3-K9-HMTase 2
MLL1	KMT2A
SET8	KMT5A
PRC2	EZH2, KMT6A
PRMT1	HRMT1L2
PRMT3	HRMT1L3
PRMT5	HRMT1L5

Table S3. Common names of Histone methyltransferases (see (*2*) for a discussion of nomenclature for various lysine methyltransferases, demethylases, and acetylases).

Supplementary references:

1. Bradner, J. E., West, N., Grachan, M. L., Greenberg, E. F., Haggarty, S. J., Warnow, T., and Mazitschek, R. (2010) Chemical phylogenetics of histone deacetylases, *Nat Chem Biol 6*, 238-243.
2. Allis, C. D., Berger, S. L., Cote, J., Dent, S., Jenuwien, T., Kouzarides, T., Pillus, L., Reinberg, D., Shi, Y., Shiekhattar, R., Shilatifard, A., Workman, J., and Zhang, Y. (2007) New nomenclature for chromatin-modifying enzymes, *Cell 131*, 633-636.