Supplementary Information

Menin-MLL Inhibitors Reverse Oncogenic Activity of MLL Fusion Proteins in Leukemia

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Supplementary Results

Assessment of the interaction of small molecule inhibitors with menin by thermal shift binding assay

Binding of MI-1 and its analogues to menin was also validated by the thermal shift binding assay (Cummings et al., 2006; Matulis et al., 2005). In this assay, the ligand-dependent changes in the thermal stability of a target protein are measured as a change in T_m (a midpoint in the thermal ramp that represents the temperature where the free energy of the native and non-native forms are equivalent) (Matulis et al., 2005), which reflects direct ligand binding to the protein and is proportional to the ligand binding affinity. First, we tested the binding of MBM1 (MLL_{4-15}) peptide to the wild-type menin, and as expected, it resulted in a significant increase in T_m (ΔT_m = 5.3 °C), Supplementary Fig. 3 and Supplementary Table 4. Then, we have tested small molecule inhibitors of the menin-MLL interaction and found that compounds with low IC_{50} values lead to a substantial stabilization of menin (Supplementary Table 4). The largest increase in T_m was observed for the most potent menin-MLL inhibitors MI-2 and MI-3 (ΔT_m = 3.4-3.7 °C, Supplementary Fig. 3 and Supplementary Table 4), indicative of their direct binding to the protein. ΔT_m for menin correlates very well with the *in vitro* inhibition of the menin-MLL interaction, as moderate effect was observed for MI-1 (ΔT_m = 1.7 °C) and no effect was seen for MI-nc and MI-7 that are very weak inhibitors of this interaction.

We have recently solved the crystal structure of menin homolog from *Nematostella vectensis* and identified point mutations in human menin which disrupt binding with MLL (Murai et al., 2011). To further verify specificity of the interactions of small molecule compounds and menin we have carried out thermal shift assay for the two menin mutants (M278K and Y232K) which do not bind to MLL (Murai et al., 2011). No, or marginal increase

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in ΔT_m for both mutants was observed upon addition of MBM1 or menin-MLL inhibitors indicating that they do not interact with menin mutants lacking MBM1 binding (Supplementary Table 4). Overall, the thermal shift assay clearly demonstrates that our compounds bind directly and specifically to menin in the MLL MBM1 binding site.

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Supplementary Table 1. Small molecule screening data for HTS performed to identify menin-MLL inhibitors.

Category	Parameter	Description
Assay	Type of assay	*In vitro* fluorescence polarization (FP) competition assay
	Target	Menin (inhibition of the menin-MLL interaction)
	Primary measurement	Detection of fluorescence polarization signal
	Key reagents	Menin, FLSN-MBM1 (MLL_{4-15}) peptide, FP buffer: 50mM TRIS, 50mM NaCl, 1mM DTT, pH=7.5
	Assay protocol	Assay protocol is described in the Supplementary Method Section
Library	Library size	49,000
	Library composition	Maybridge hit finder, Chembridge custom collection of small molecules, ChemDiv custom collection of small molecules
	Source	Center for Chemical Genomics (CCG), University of Michigan
Screen	Format	384-well, Corning 3676
	Concentration tested	20 μM, 1% DMSO
	Plate controls	NC: Menin, FLSN-MBM1, PC: Menin, FLSN-MBM1, MBM1
	Reagent/ compound dispensing system	Biomek FX, Beckman
	Detection instrument and software	PHERAstar, BMG
	Assay validation/QC	Z' = 0.92; Mean FP for NC= 197.6, SD = 2.8; Mean FP for PC = 36.95, SD = 1.7
	Normalization	% inhibition = 100 × (average of negative control – sample result)/(average of negative control – average of positive control)
Post-HTS analysis	Hit criteria	Change in FP signal > 3 standard deviations from mean
	Hit rate	0.37%
	Additional assay(s)	FP with Texas Red labeled MBM1, NMR STD to validate binding of compounds to menin
	Confirmation of hit purity and structure	Compounds were repurchased from ChemBridge and ChemDiv and verified analytically

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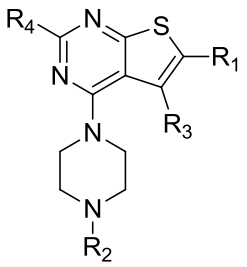
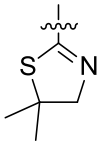
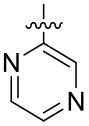
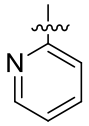
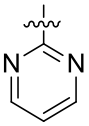
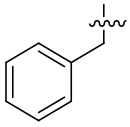
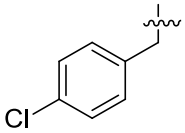
Supplementary Table 2. SAR for thienopyrimidine compounds inhibiting the menin-MLL interaction. IC_{50} values were measured by FP. ND – not determined due to limited solubility of the compound. $\Delta_{75\mu M}$, $\Delta_{250\mu M}$ and $\Delta_{1000\mu M}$ indicate percent inhibition at 75 μM, 250 μM and 1,000 μM, respectively.



Entry	R1	R2	R3	R4	IC_{50} (μM)
MI-7 (5)	H	H	H	H	>1,000 $\Delta_{1,000\mu M}$ = 30%
MI-8 (6)	R_1-R_3= Cyclohexyl	H	R_1-R_3= Cyclohexyl	H	428 ± 159
MI-1 (1)	R_1-R_3= Cyclohexyl		R_1-R_3= Cyclohexyl	H	1.9 ± 0.35
MI-9 (7)	R_1-R_3= Cyclohexyl		R_1-R_3= Cyclohexyl	H	ND $\Delta_{250\mu M}$ = 17%
MI-10 (8)	-CH_2CH_3		H	H	58 ± 3.5
MI-11 (9)	-CH_2CH_3		H	H	87 ± 19
MI-4 (10)	-CH_2CH_3		H	H	52 ± 13
MI-12 (11)	-CH_2CH_3		H	H	14 ± 1.4

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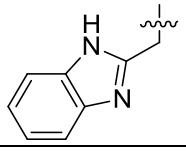
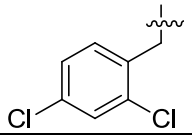
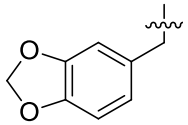
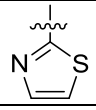
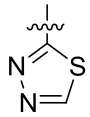
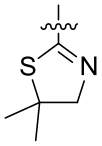
MI-13 (12)	$-CH_2CH_3$		H	H	201 ± 30
MI-14 (13)	$-CH_2CH_3$		H	H	ND $\Delta_{75\mu M}$ = 30%
MI-15 (14)	$-CH_2CH_3$		H	H	54 ± 9.2
MI-16 (15)	$-CH_2CH_3$		H	H	46 ± 5.6
MI-6 (MI-nc) (4)	$-CH_2CH_3$		H	H	193 ± 11
MI-17 (16)	$-CH_2CH_2CH_3$		H	$-CH_3$	2.7 ± 0.1

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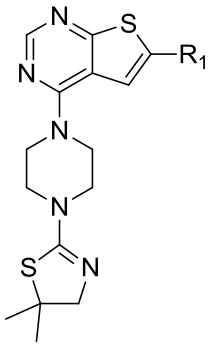
Supplementary Table 3. SAR for thienopyrimidine compounds with modifications at R1.

IC_{50} values for inhibition of the menin-MLL interaction were measured by FP.



Entry	R1	IC_{50} (μM)
MI-18 (17)	-Br	12 ± 1.4
MI-19 (18)	$-CH_2CH_3$	1.2 ± 0.07
MI-2 (2)	$-CH_2CH_2CH_3$	0.45 ± 0.028
MI-3 (3)	$-CH(CH_3)_2$	0.65 ± 0.025
MI-20 (19)	$-CH_2CH_2CH_2CH_3$	0.75 ± 0.20
MI-21 (20)	$-CH(CH_3)CH_2CH_3$	2.8 ± 0.14
MI-22 (21)	-Ph	14 ± 1.4
MI-23 (22)	$-CH_2Ph$	4.0 ± 0.85
MI-24 (23)	$-CH_2CH_2Ph$	1.7 ± 0.28
MI-25 (24)	$-CH_2CH_2CH_2Ph$	1.4 ± 0.35
MI-26 (25)	$-CH_2CH_2CH_2CH_2Ph$	1.9 ± 0.14

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Supplementary Table 4. Binding of small molecule compounds to menin assessed by thermal shift assay. Compounds which bind to menin induce thermal stabilization of the protein as reflected in increase in T_m. Only MI-1, MI-2 and MI-3 bind to menin and result in increase in ΔT_m. No protein stabilization is observed for weak inhibitors (MI-nc and MI-7) as well as for all the compounds and menin mutants (M278K and Y323K) which lack MLL binding. The MLL MBM1 peptide has been included for comparison.

T_m values were measured for menin and two menin mutants M278K and Y323K. ΔT_m were calculated as a T_m difference between protein mixed with the 50 μM compound and protein mixed with DMSO. The IC_{50} values determined by FP are also reported for menin inhibitors and wild-type menin.

Compound	T_m (°C) Menin WT	ΔT_m (°C) Menin WT	IC_{50} (μM) Menin WT	T_m (°C) Menin M278K	ΔT_m (°C) Menin M278K	T_m (°C) Menin Y323K	ΔT_m (°C) Menin Y323K
DMSO	41.8 ± 0.03	-		43.4 ± 0.03	-	40.9 ± 0.03	-
MI-nc	41.9 ± 0.08	0.1	193	43.3 ± 0.07	-0.1	40.6 ± 0.03	-0.3
MI-7	41.7 ± 0.06	-0.1	>1,000	43.2 ± 0.06	-0.2	40.8 ± 0.05	-0.1
MI-1	43.5 ± 0.09	1.7	1.9	43.3 ± 0.03	-0.1	40.9 ± 0.02	0.0
MI-3	45.2 ± 0.02	3.4	0.65	43.5 ± 0.08	0.1	40.7 ± 0.03	0.2
MI-2	45.5 ± 0.04	3.7	0.45	44.0 ± 0.08	0.6	40.8 ± 0.04	0.1
MBM1	47.1 ± 0.05	5.3	0.23	43.6 ± 0.06	0.2	41.3 ± 0.04	0.4

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Supplementary Figure 1

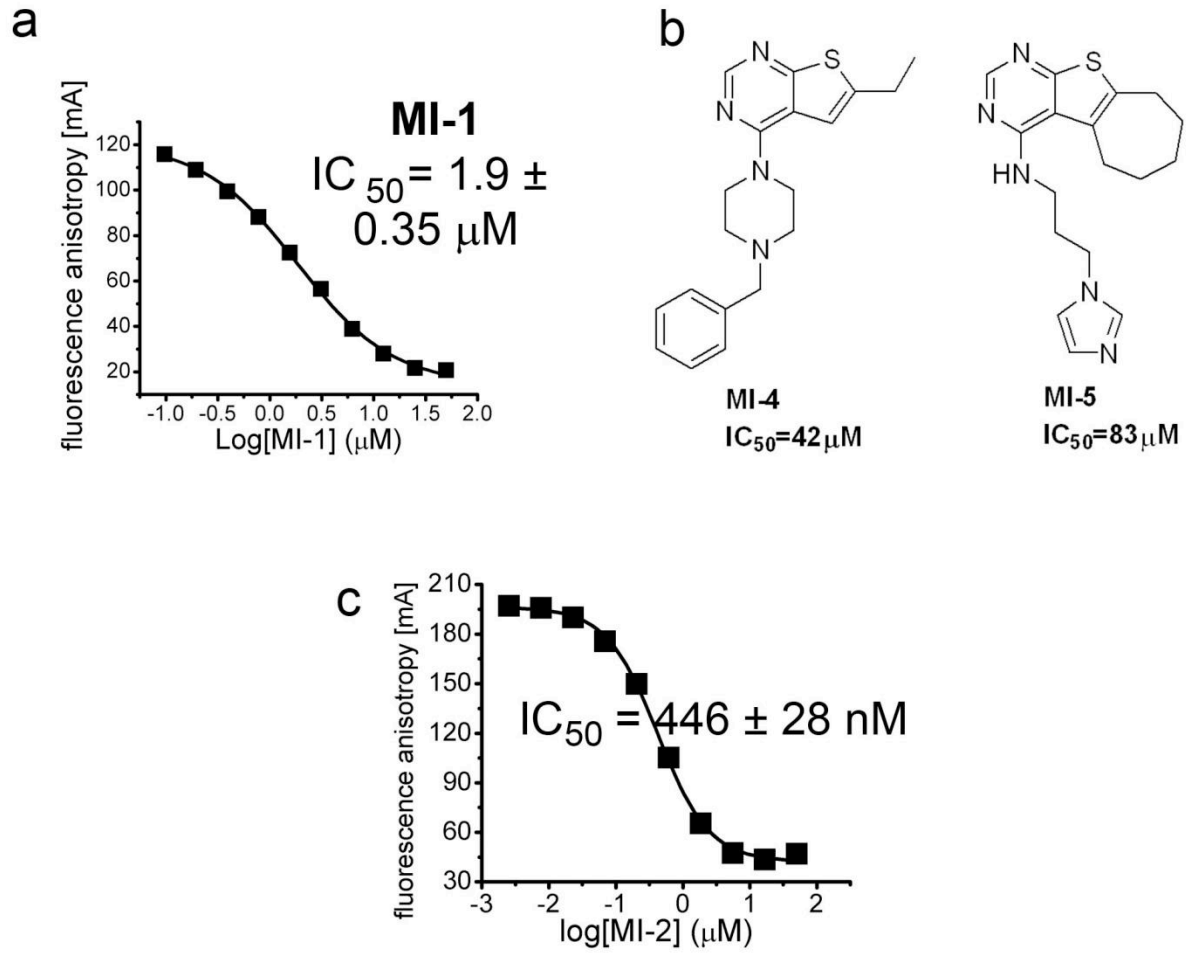

Figure 1. IC_{50} values and structures for thienopyrimidine compounds. a) Competition FP experiment between MI-1 and FLSN-MBM1 for binding to menin. b) Structures of HTS hits MI-4 and MI-5 together with the IC_{50} values for these compounds measured by competition FP experiment. c) Competitive displacement of FLSN-MBM1 peptide (15 nM) from menin (150 nM) by MI-2 measured by FP.

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Supplementary Figure 2

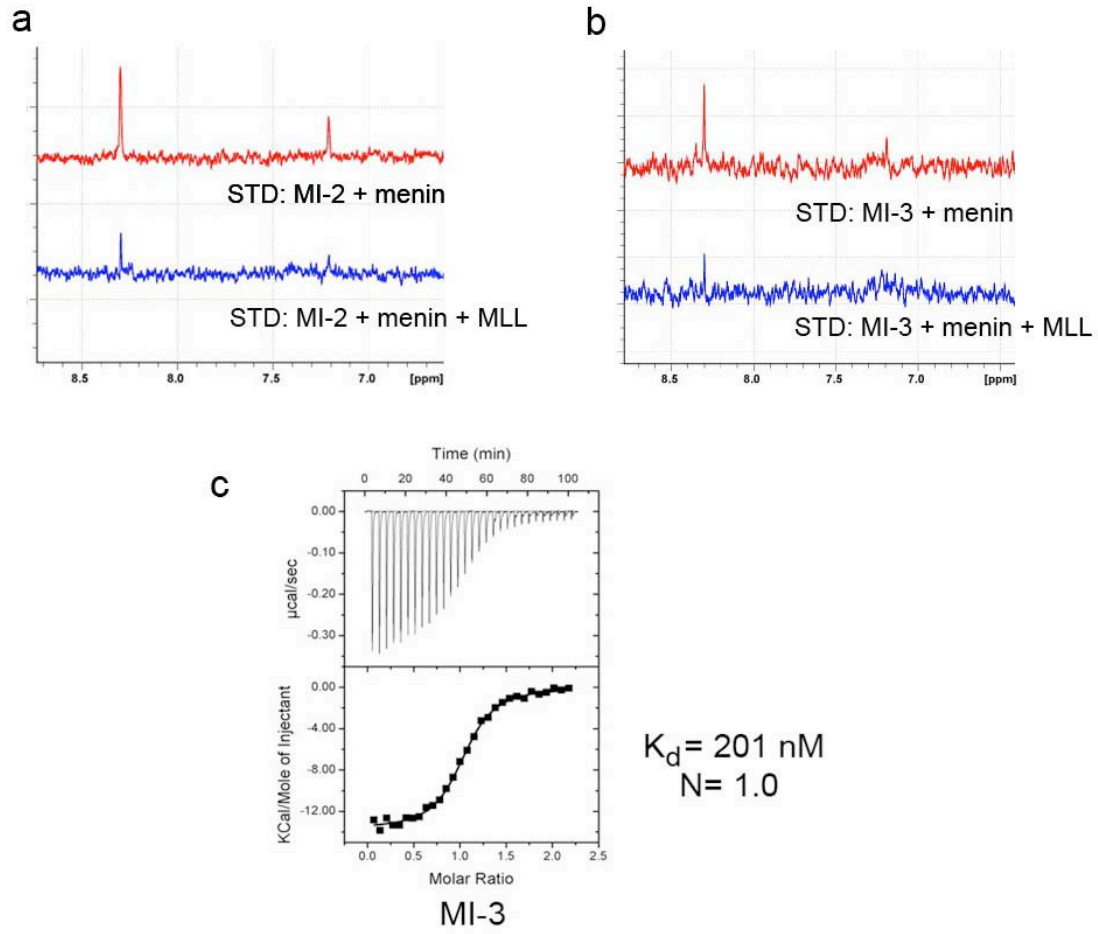

Figure 2. NMR STD (a,b) and ITC (c) experiments demonstrating direct and specific binding of MI-2 and MI-3 to menin.

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Supplementary Figure 3

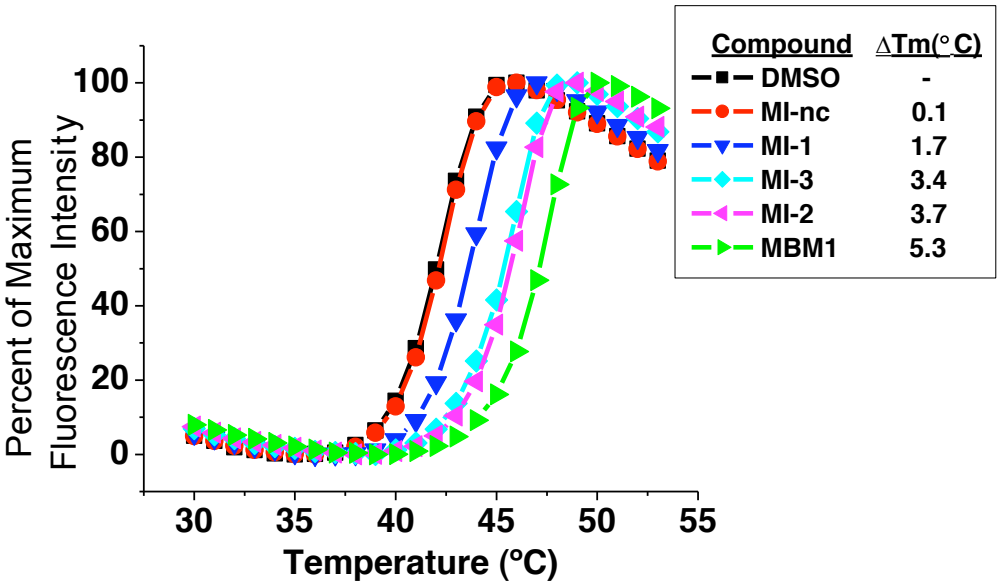

Figure 3. Change in thermodynamic stability of menin upon binding of small molecule inhibitors and MBM1 (MLL_{4-15}) peptide. ΔT_m has been calculated as a difference to the protein sample with DMSO.

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Supplementary Figure 4

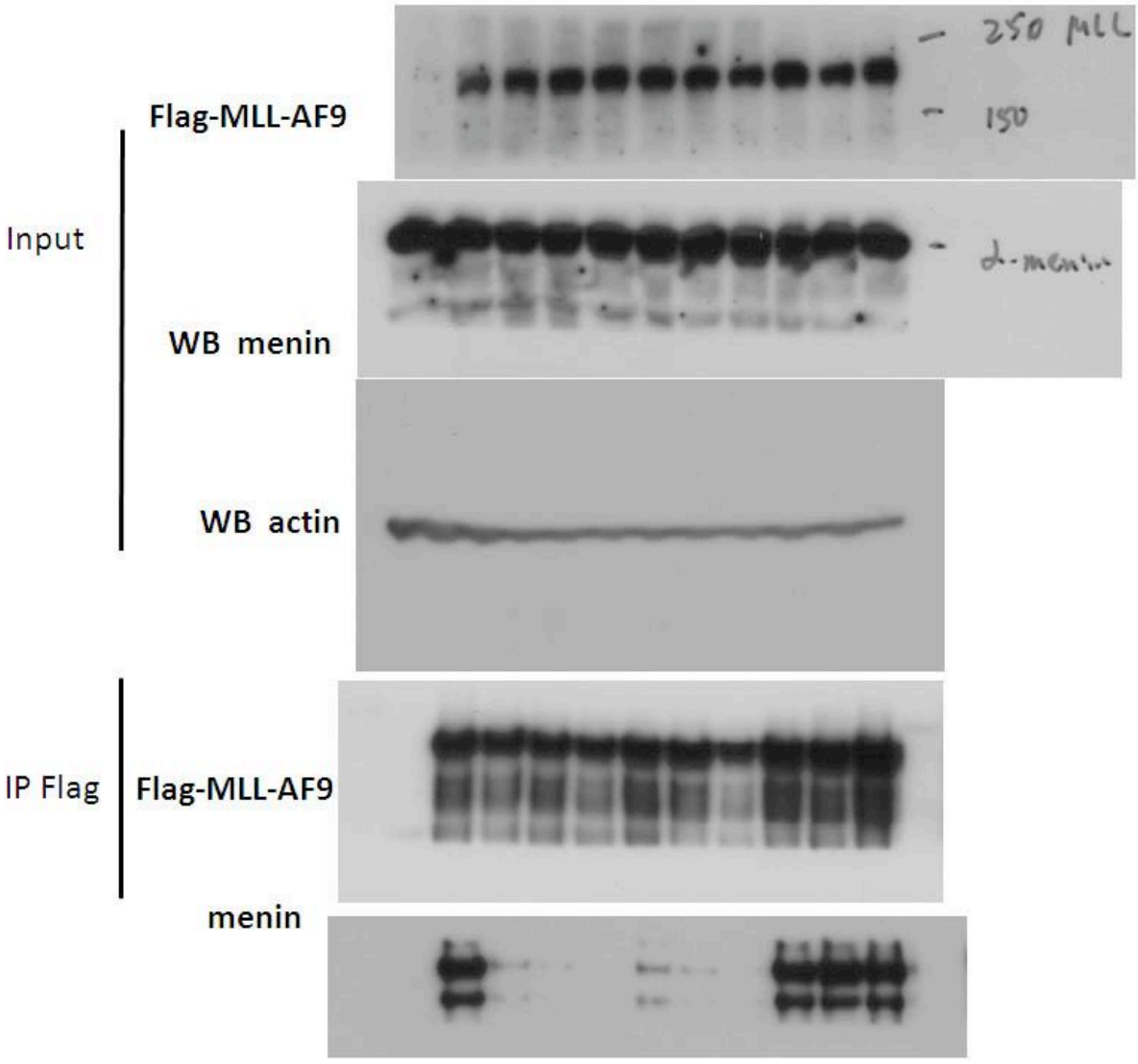

Figure 4. Full gel images for the co-IP experiment performed in HEK293 cells transfected with MLL-AF9. This figure supports Fig. 1e from the paper. Two bands for menin at the bottom gel correspond to the full length menin (top band) and the degradation product of menin (bottom band). WB - Western Blot.

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Supplementary Figure 5

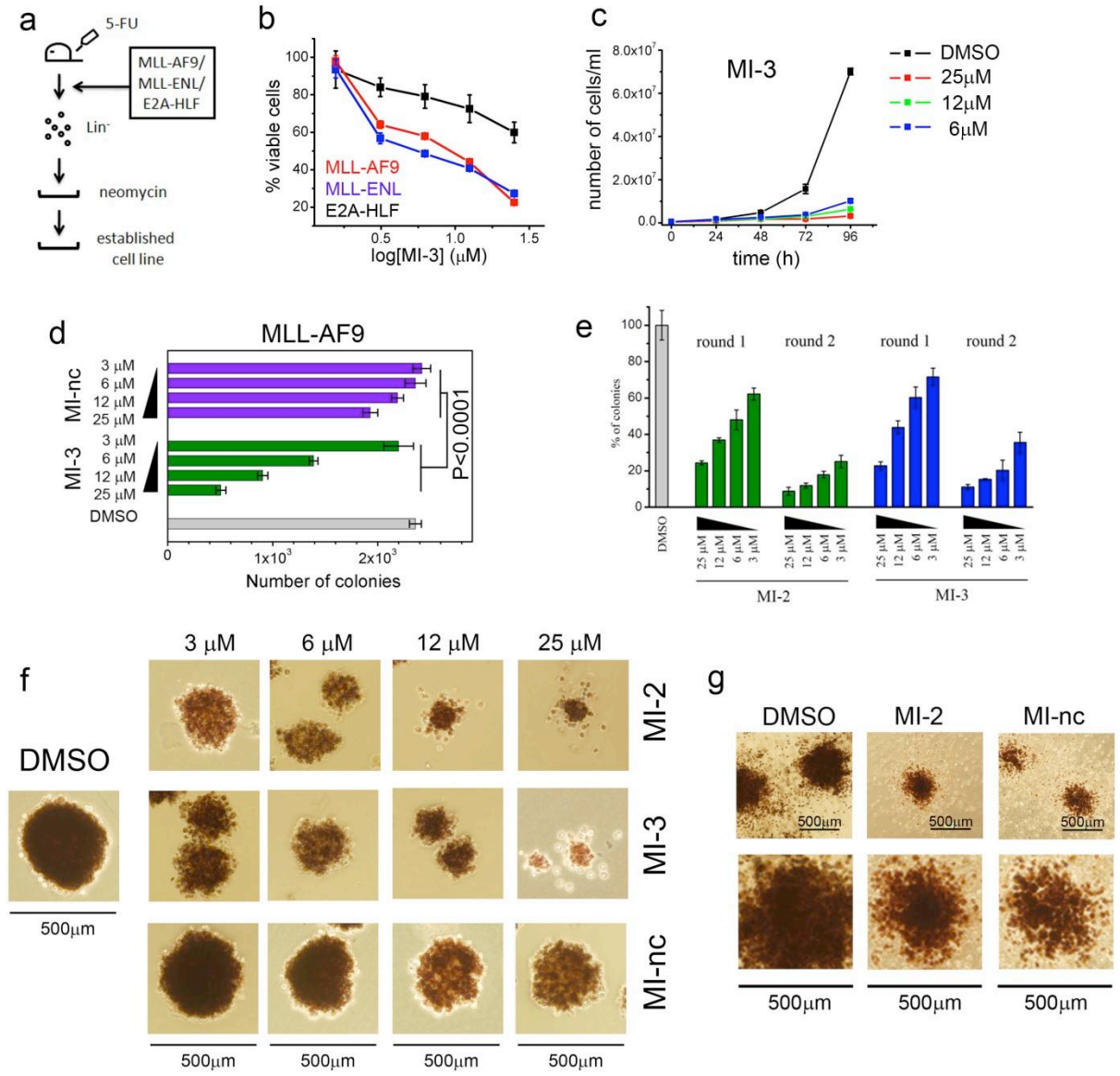

Figure 5. Effects of MI-2 and MI-3 in MLL fusion transduced BMC. a) Schematics for preparing the MLL-AF9, MLL-ENL and E2A-HLF transduced murine bone marrow cell lines. b) MI-3 effectively inhibits proliferation of mouse BMC transduced with the MLL-AF9, MLL-ENL but not E2A-HLF. c) Growth curves for MI-3 treated MLL-AF9 transduced BMC grown in liquid culture. d) Colony counts for methylcellulose colony assay performed with MLL-AF9

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transduced BMC treated for 7 days with MI-3 and MI-nc. e) Colony counts for methylcellulose colony assay performed with MLL-AF9 transduced BMC treated for 7 days with MI-2 and MI-nc (round 1) and then replated and treated for additional 7 days (round 2). Number of colonies has been normalized to DMSO. Error bars indicate SD from duplicate experiments. f) Representative colonies shown for DMSO, MI-2, MI-3 and MI-nc treated MLL-AF9 transduced BMC plated on methylcellulose. g) Representative colonies for DMSO, MI-2 (25 μM), and MI-nc (25 μM) treated E2A-HLF transduced bone marrow cells.

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Supplementary Figure 6

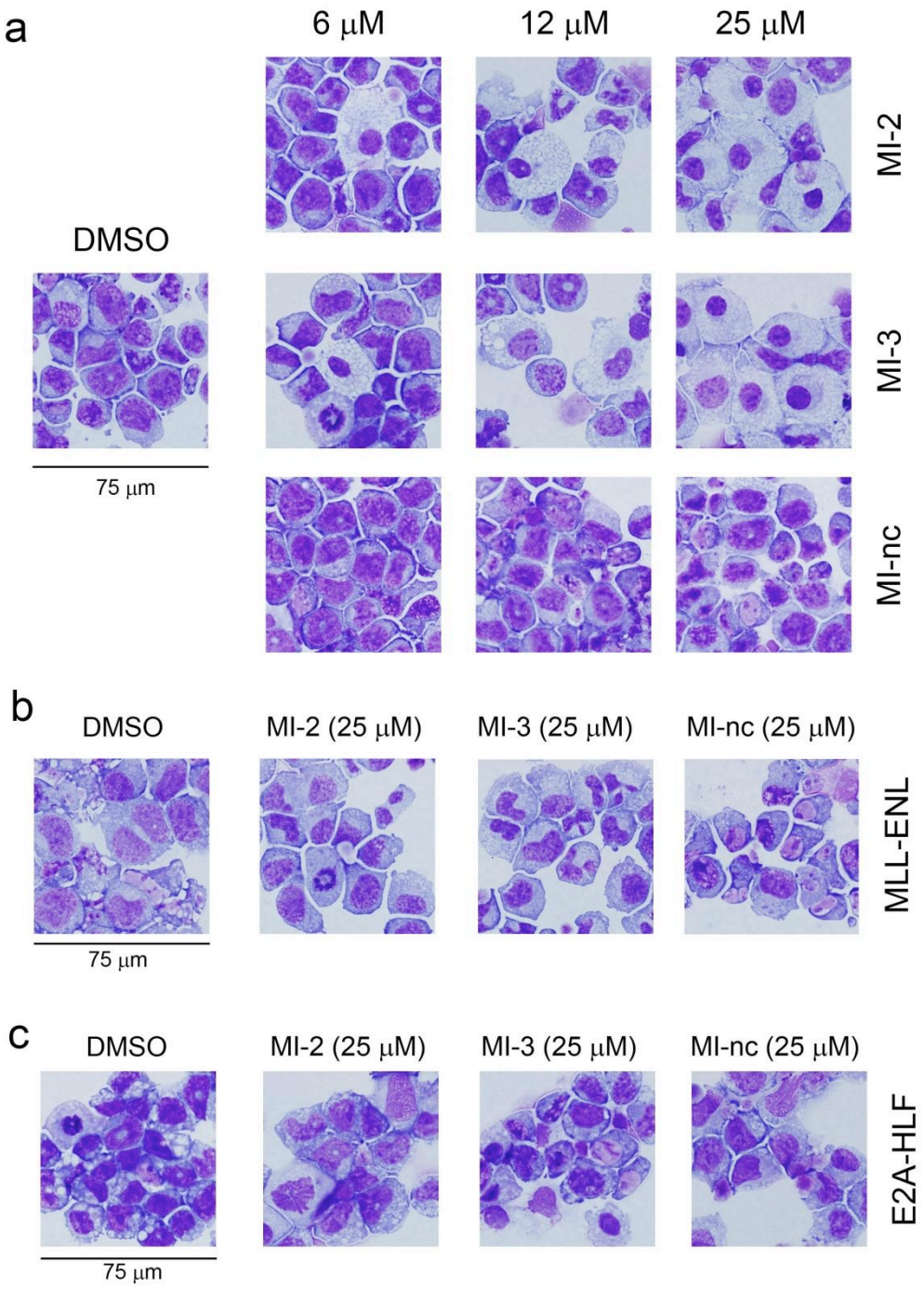

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Figure 6. MLL fusion protein transformed bone marrow cells differentiate after treatment with the menin-MLL inhibitors.

a) Wright-Giemsa stained cytospins on MLL-AF9 transformed bone marrow cells after 10 days of treatment with MI-2, MI-3, MI-nc, and DMSO, demonstrating hematopoietic differentiation of cells induced by menin-MLL inhibitors. b) Wright-Giemsa stained cytospins on MLL-ENL transformed bone marrow cells after 10 days of treatment with MI-2, MI-3, MI-nc, and DMSO, demonstrating hematopoietic differentiation of cells induced by MI-2 and MI-3, but not MI-nc. c) Wright-Giemsa stained cytospins on E2A-HLF transformed bone marrow cells after 10 days of treatment with MI-2, MI-3, MI-nc, and DMSO, showing no effect of compounds on differentiation of these cells.

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Supplementary Figure 7

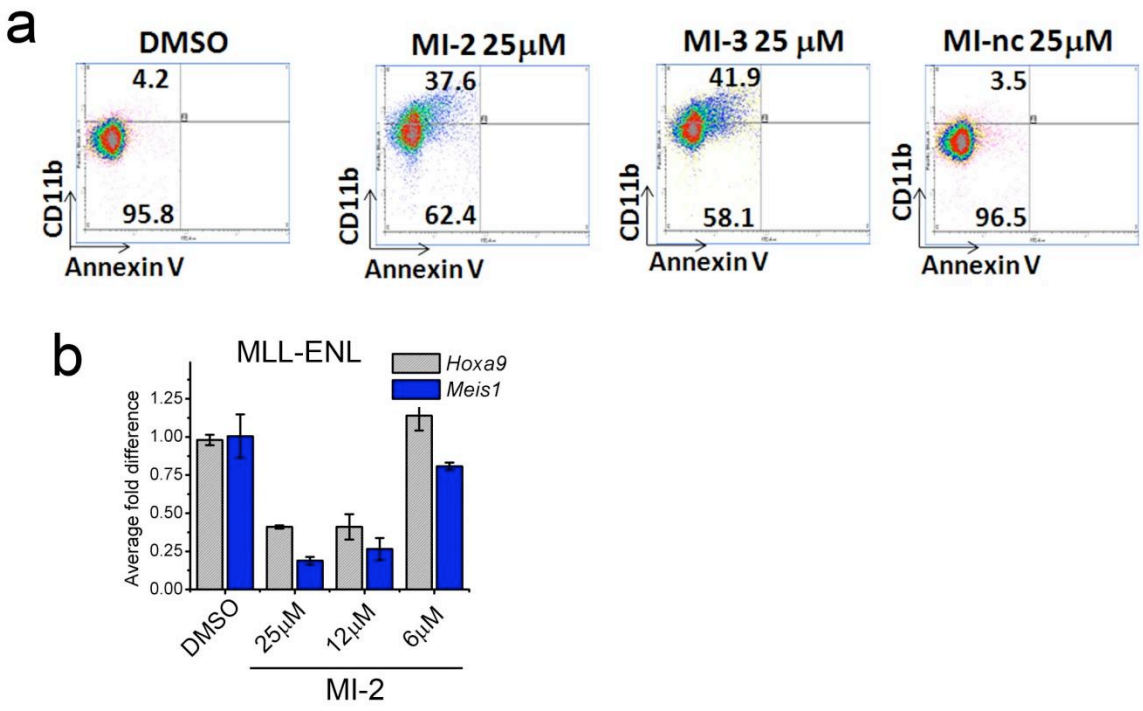

Figure 7. Menin-MLL inhibitors induce differentiation and downregulate MLL fusions downstream targets. a) Flow cytometry histograms showing increased expression of CD11b in MLL-AF9 transduced bone marrow cells upon 7 days of treatment with MI-2 and MI-3. No increase in CD11b expression was observed for MI-nc compound. b) Quantitative real-time PCR showing the expression of *Hoxa9* and *Meis1* in MLL-ENL transduced bone marrow cells upon 6 days of treatment with MI-2. Expression of *Hoxa9* and *Meis1* has been normalized to β-actin and is referenced to the DMSO treated cells.

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Supplementary Figure 8

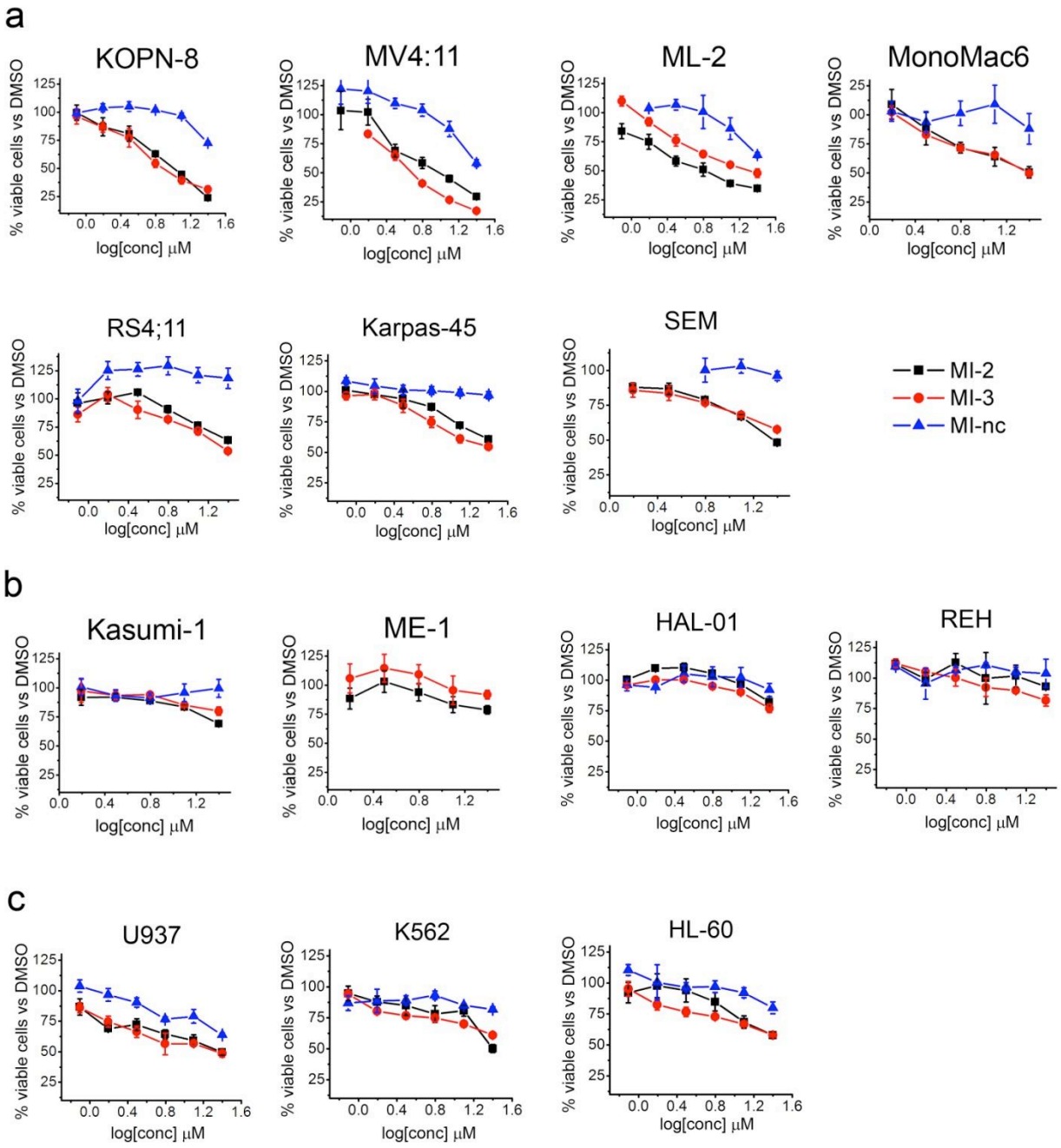

Figure 8. MI-2 and MI-3 selectively inhibit cell growth in the MLL leukemia cells. a) Inhibition of cell proliferation in different MLL leukemia cells after 72h treatment, as detected by the MTT cell viability assay. MI-nc showed no effect in these cell lines. Representative experiments are shown with mean values ± SD for four samples per condition. b) Effect of

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menin-MLL inhibitors on proliferation of non-MLL human acute leukemia cells with low expression of *HOXA9* and/or *MEIS1*. c) Effect of menin-MLL inhibitors on growth of non-MLL leukemia cell with high or moderate expression of *HOXA9* and/or *MEIS1*.

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Supplementary Figure 9

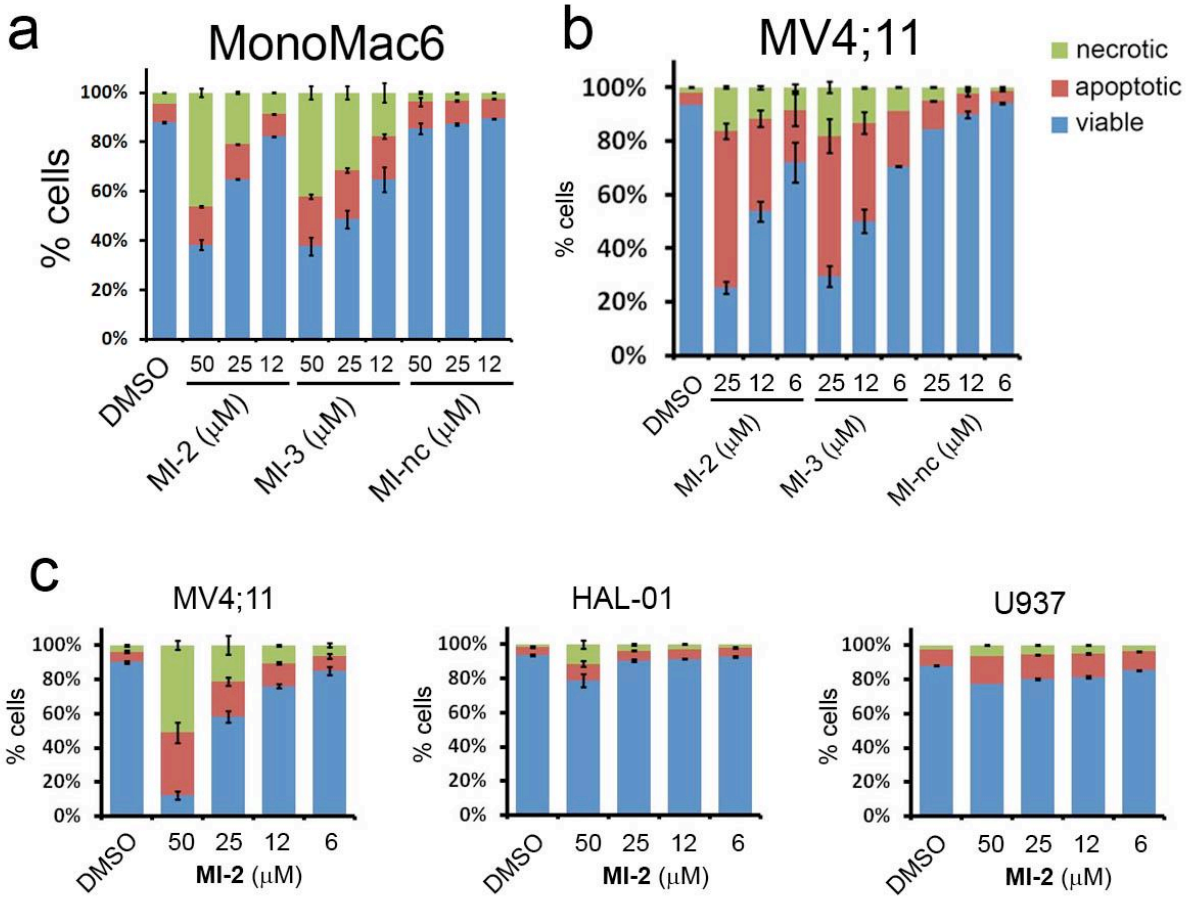

Figure 9. MI-2 selectively induces apoptosis in MLL-leukemia cells. a) Apoptosis and cell death induced by MI-2 and MI-3 in MonoMac (MLL-AF9) leukemia cells after 48h incubation with compounds as detected by flow cytometry using AnnexinV/propidium iodide (PI) staining. MI-nc served as a negative control. b) Apoptosis in MV4;11 cells induced after 7 days treatment with MI-2, MI-3 and MI-nc. c) Quantification of apoptosis induced by MI-2 in MV4;11, HAL-01 and U937 cells after 48h treatment assessed by flow cytometry using Annexin V and Propidium Iodide staining. The apoptosis in cells without MLL translocations (HAL-01 and U937) is very limited.

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Supplementary Figure 10

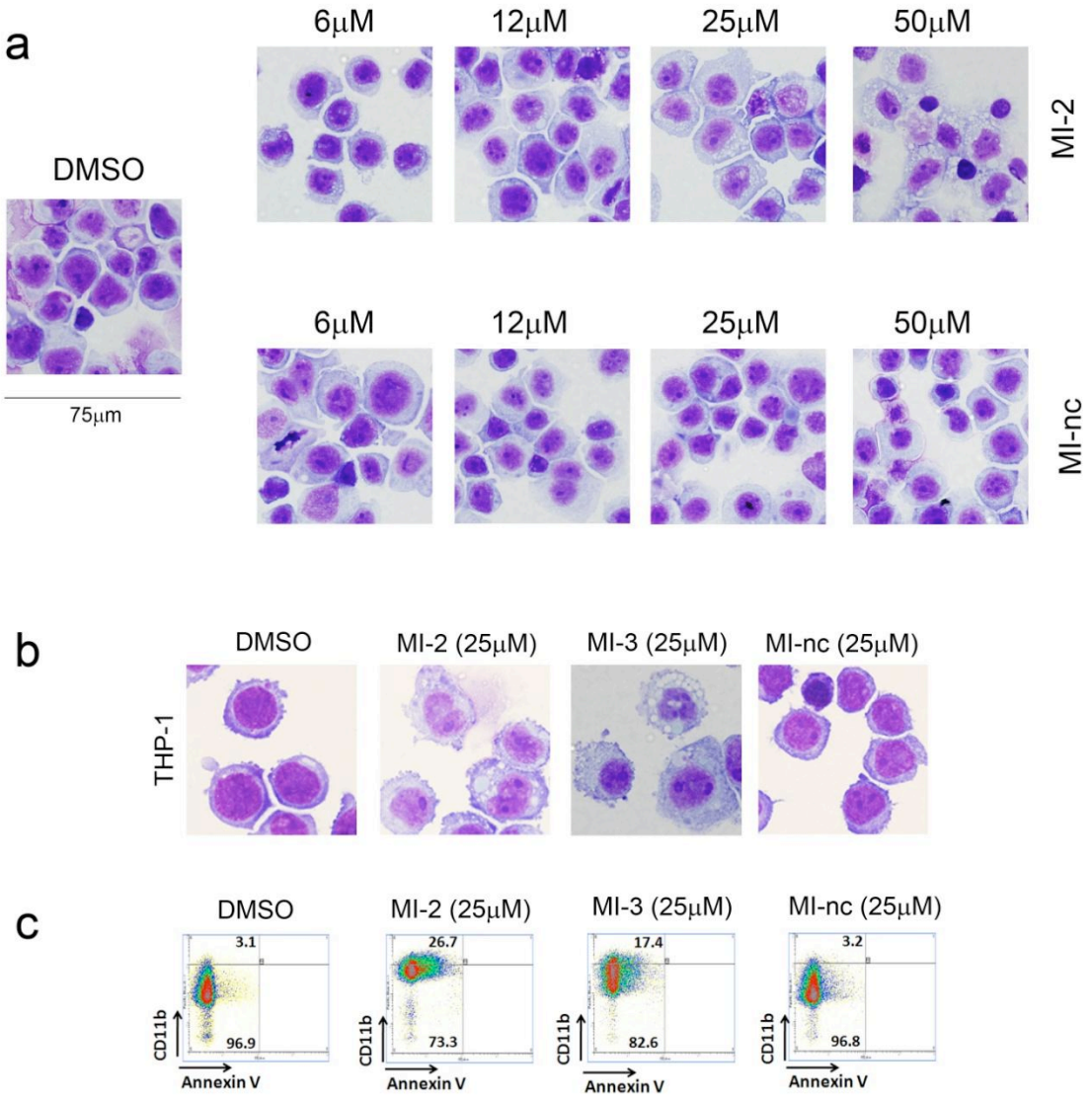

Figure 10. MLL leukemia cells differentiate after treatment with the menin-MLL inhibitors. a) Wright-Giemsa stained cytospins on THP-1 cells (harboring MLL-AF9) after 10 days of treatment with MI-2, MI-nc, and DMSO, demonstrating dose dependent hematopoietic differentiation of cells induced by menin-MLL inhibitor MI-2. No effect on differentiation was observed for the negative control compound MI-nc. b) Comparison of Wright-Giemsa stained cytospins on THP-1 cells after 10 days of treatment with DMSO, MI-2, MI-3 and MI-nc,

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demonstrating hematopoietic differentiation of these cells induced by MI-2 and MI-3. c) Histograms from flow cytometry experiments showing shift in cell population resulting from increased expression of CD11b in THP-1 cells after 6 days of treatment with MI-2 and MI-3. No increase in CD11b expression was detected for MI-nc.

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Supplementary Figure 11

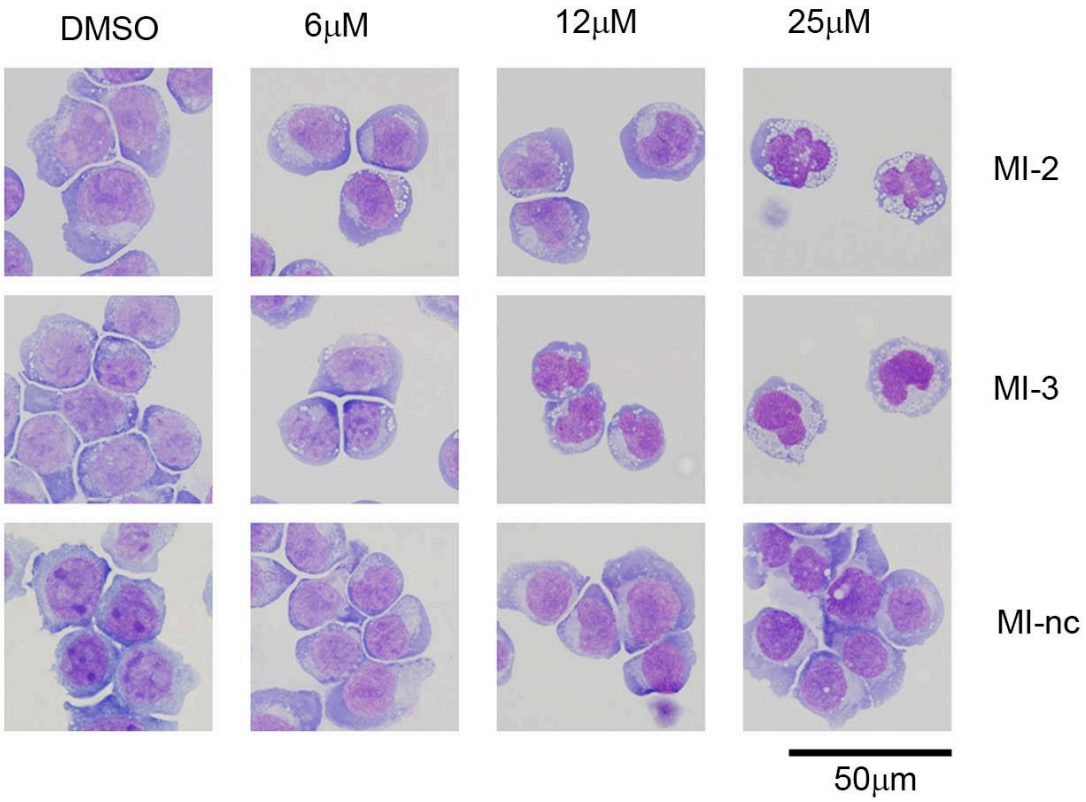

Figure 11. MI-2 and MI-3 induce differentiation in MV4;11 cells. Wright-Giemsa stained cytospins on MV4;11 cells (harboring MLL-AF4) after 10 days of treatment with DMSO, MI-2, MI-3 and MI-nc, demonstrating hematopoietic differentiation of these cells induced by MI-2 and MI-3 but not by MI-nc.

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Supplementary Figure 12

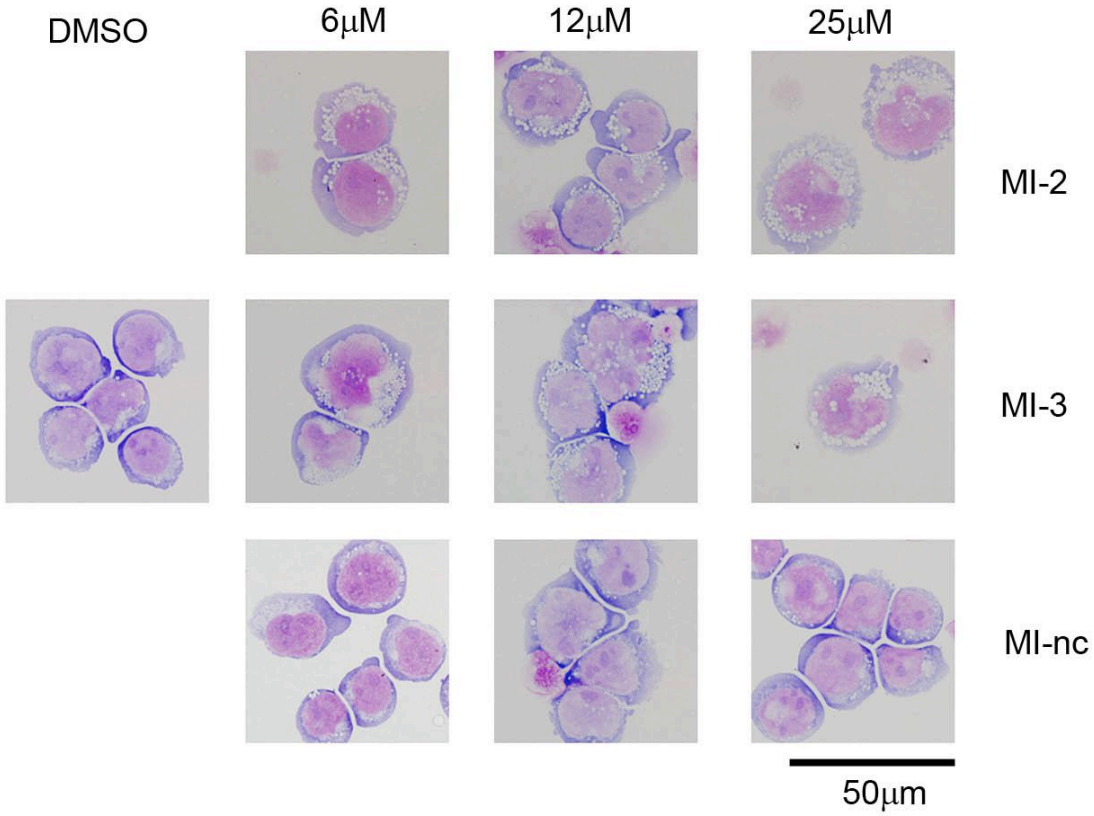

Figure 12. MI-2 and MI-3 induce differentiation in KOPN-8 cells. Wright-Giemsa stained cytospins on KOPN-8 cells (harboring MLL-ENL) after 10 days of treatment with DMSO, MI-2, MI-3 and MI-nc, demonstrating hematopoietic differentiation of these cells induced by MI-2 and MI-3 but not by MI-nc.

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Supplementary Figure 13

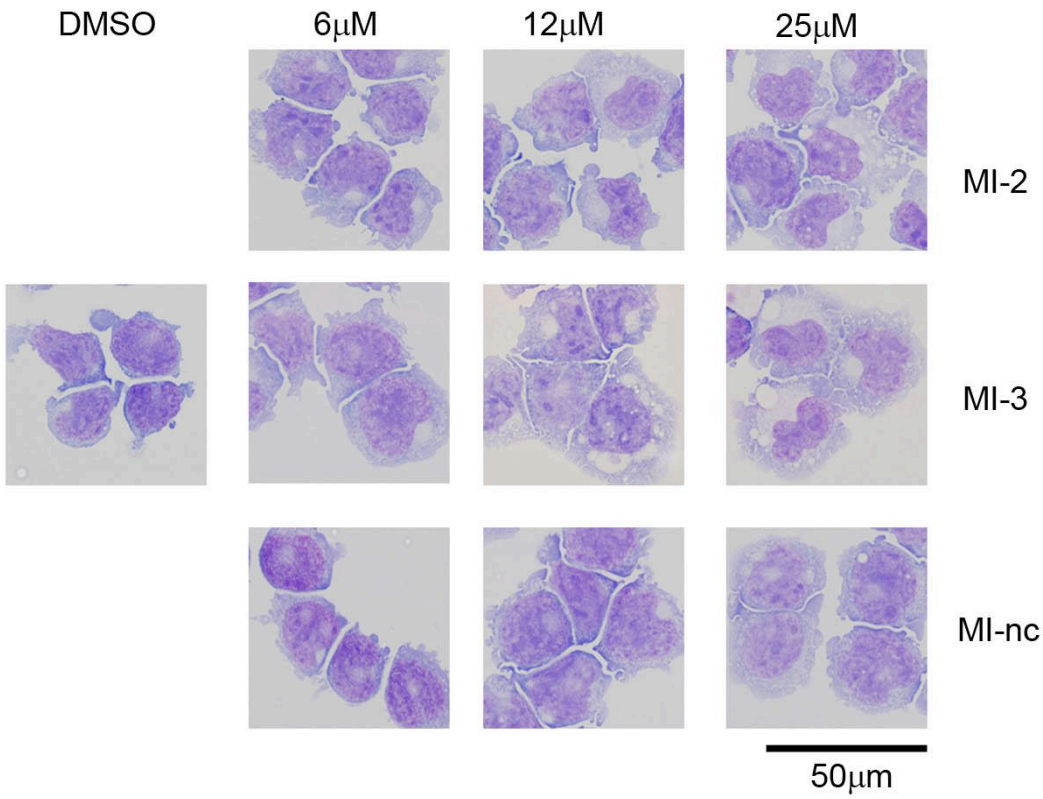

Figure 13. MI-2 and MI-3 induce differentiation in ML-2 cells. Wright-Giemsa stained cytospins on ML-2 cells (harboring MLL-AF6) after 10 days of treatment with DMSO, MI-2, MI-3 and MI-nc, demonstrating hematopoietic differentiation of these cells induced by MI-2 and MI-3 by not by MI-nc.

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Supplementary Figure 14

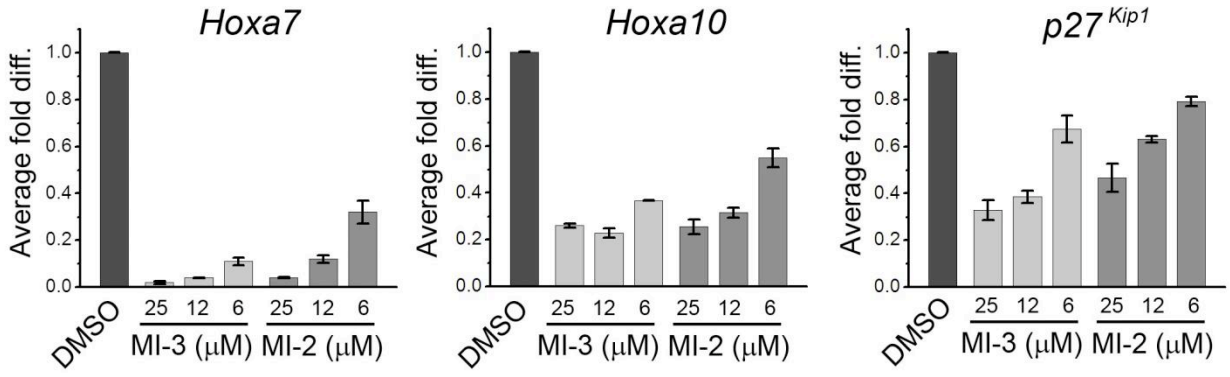

Figure 14. MI-2 and MI-3 downregulate expression of menin-MLL target genes.

Expression of the *HOXA7*, *HOXA10* and $p27^{Kip1}$ genes normalized to 18S rRNA determined by qRT-PCR in THP-1 cells treated for 6 days with MI-2 and MI-3. Data are presented as mean ± SD.

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Supplementary Methods

FP assays for inhibition of menin-MLL interaction

Fluorescein labeled MLL-derived peptide, FITC-MBM1 (FITC-MLL_{4-15}) was obtained from GenScript (>95% purity). FITC-MBM1 at 15 nM and menin at 150 nM in the FP buffer (50mM TRIS, 50mM NaCl, 1mM DTT, 0.01% BSA, pH = 7.5) were incubated in the dark for 1h at room temperature. A 95 μL of the aliquot of the protein-peptide mixture and 5 μl of DMSO solutions of the compounds were mixed on 96-well black COSTAR plates and incubated at room temperature for 1h. After incubation, change in fluorescence polarization and anisotropy were monitored at 525 nm after excitations at 495 nm using the PHERAstar microplate reader (BMG) and applied to assess inhibition (IC_{50} determination) for compounds with the Origin 7.0 program.

NMR spectroscopy

NMR samples for the saturation transfer difference (STD) experiments contained 5μM menin solution in 50 mM phosphate buffer, 50 mM NaCl, 1mM DTT, pH= 7.5, with 5% of D_2O. The compounds were added as stock solutions in DMSO to final 100 μM concentration and 5% DMSO. All NMR experiments were recorded using 600 MHz Bruker Avance III spectrometer at 25°C. For the STD experiments we used 2s irradiation using a train of 50ms gaussian pulses centered at 0 ppm using published pulse sequence (Mayer and Meyer, 2001). Samples for the competition STD experiments contained additional MBM1 peptide at final concentration of 50 μM or 100 μM and the measurements were carried out is the same way as described above for STD experiments.

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Isothermal Titration Calorimetry

Menin was extensively dialyzed at 4°C against ITC buffer (50 mM phosphate, pH 7.5, 50 mM NaCl, 1mM β-mercaptoethanol) and degassed prior to measurement. Compounds were dissolved in DMSO and diluted with the ITC buffer to final concentrations (50-100 μM, 5% DMSO). Protein solution was adjusted to contain 5% DMSO final concentration. The titrations were performed using a VP-ITC titration calorimetric system (MicroCal) at 25°C. The calorimetric cell, containing menin (concentrations in the range 5 μM – 10 μM), was titrated with the compounds (50 μM – 100 μM) injected in 10 μl aliquots. Data was analyzed using Origin 7.0 (OriginLab) to obtain K_d and stoichiometry.

Thermal shift binding assay

Thermal shift binding experiments were carried out using Thermofluor 384 ELS system. Protein unfolding was examined by monitoring the fluorescence of ANS (1-anilinonaphthalene-8-sulfonic acid) by increasing the temperature from 20 to 60 °C. All samples were prepared in quadruplicates and contained proteins at 5 μM concentrations in 50 mM TRIS, 50 mM NaCl, 1 mM DTT, pH = 7.5; 50 μM ligand (2.5% final concentration of DMSO) and 50 μM ANS. To limit evaporation, the samples were covered with mineral oil.

Analysis of compounds inhibition in HEK293 cells by Co-immunoprecipitation

4×10^5 HEK293 cells were plated in 6-well plates and transfected with 1μg of β-actin Flag-MLL-AF9 plasmid using Fugene 6 (Roche Indianapolis, IN). 48h after transfection cells were treated with compounds (0.25% final DMSO concentration) or 0.25% DMSO for 12h. Whole cell lysates were prepared using BC-300 lysis buffer (20 mM Tris-HCl, 300 mM KCl, 1mM EDTA,

10% glycerol, 0.1% NP-40, pH = 8.0) containing compounds at corresponding concentrations, centrifuged and immunoprecipitated with ANTI-FLAG M-2 Magnetic beads (Sigma-Aldrich, St. Louis, MO) at 4°C for 2h. After extensive PBS washing, the immunoprecipitation samples were applied to SDS-PAGE electrophoresis and Western blotting. Antibodies used include rabbit anti-menin (A300-115A, Bethyl Laboratries, Inc. Montgomery, TX), rabbit DYKDDDK tag antibody (Cell Signaling Technology Inc. Danvers, MA), mouse monoclonal specific for N-MLL antibody from Millipore (Temecula, CA 92590), mouse anti-β-actin monoclonal antibody (Genscript USA Inc, Piscataway, NJ), goat anti-mouse HRP (Genscript USA Inc, Piscataway, NJ) and goat anti-rabbit HRP (Genscript USA Inc, Piscataway, NJ).

Inhibitor effects on proliferation of MLL leukemia cells

Cells were plated in 90 μL of culture medium in 96-well flat bottom microtiter plates (Fisher Sceintific, Newark, DE, USA) at concentrations of 1×10^5 cells/ml. Cells were treated with 0.25% sterile DMSO (Sigma, St. Louis, MO, USA) or serial dilutions of compounds from 10 mM stock solutions in DMSO (0.25% final concentration of DMSO). Cells were incubated in a 5% CO_2 incubator at 37 °C for 72h. A Vybrant MTT cell proliferation assay kit (Molecular Probes, Eugene, OR, USA) was employed. Plates were read for absorbance at 570 nm using a PHERAstar BMG microplate reader. The experiments were performed three times in quadruplicated with calculation of mean and standard deviation for each condition.

For growth curves, 5×10^5 cells/ml MLL-AF9 transduced murine bone marrow cells were plated (1 ml/well) and treated with compounds or 0.25% DMSO. Media were changed every 48h with viable cell concentration restored to 5×10^5 cells/ml and compound re-supplied. At designated time point, cell culture samples were mixed with trypan blue solution (GIBCO,

Invitrogen, Carlsbad, CA) and filled into a hemocytometer. Dye excluded cells were counted as viable cells under the microscope.

Cytospin/Wrigth-Giemsa staining

THP-1, MV4;11, KOPN-8, ML-2 and mouse bone marrow cells transduced with MLL-AF9 or E2A-HLF were plated in 12-well plates (1ml/well) at an initial concentration of 5×10^5 cells/ml, treated with compounds (0.25% final DMSO concentration) or 0.25% DMSO control and incubated at 37 °C in a 5% CO_2 incubator. At designated time point, 1×10^5 cells were harvested and placed in Shandon EZ Single Cytofunnel (Thermo Electron Corporation, Pittsburgh, PA). Samples were centrifuged at 600 rpm for 5 min. The slides were air-dried before staining with Hema-3 kit (Fisher Scientific, Pittsburgh, PA).

Chromatin immunoprecipitation

ChIP was performed in MLL-AF9 BMC treated with 12.5 μM of MI-2 or DMSO for 48h. Primary antibodies specific for Menin (Bethyl), AF9, histone H3, H3K4 trimethylation, and H3K79 dimethylation (Abcam) were used. Briefly, paraformaldehyde cross-linked cell pellets were lysed in SDS lysis buffer and DNA fragmented using a Bioruptor XL water bath sonicator set at 300W for 15 minutes with 30 second on/off pulses. Lysate was diluted 10 fold and immunoprecipitated overnight using the above antibodies. IP's were washed with low salt, high salt, LiCl and TE buffers according to Millipore recipes. Elutions were carried out at 42 °C in elution buffer (0.6 ml 10% SDS +48 mg $NaHCO_3$ in 6 ml water). Cross links were reversed by NaCl treatment at 65 °C overnight. DNA fragments are recovered using Qiagen PCR purification kits. Quantitative real-time PCR was performed on the precipitated DNAs with TaqMan

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fluorescent labeling with primers and qPCR probes described below. Binding was quantified as follows: $\Delta C_T = C_T(\text{input}) - C_T(\text{Chromatin IP})$, $\%\text{total} = 2^{\Delta CT}$.

Hoxa9 Primer Probe Set 1

Probe: 5' - CCTGCGGTGGCAACCTCAGATCC – 3'

Forward: 5' – GCCATCAAGGCCTAATCGTG – 3'

Reverse: 5' – AAGACCCGAAGCTCCTCCTG – 3'

Hoxa9 Primer Probe Set 2

Probe: 5' – CCCACATCGAGGGCAGGAAACACT – 3'

Forward: 5' – CACCCGCGGCGTCTT – 3'

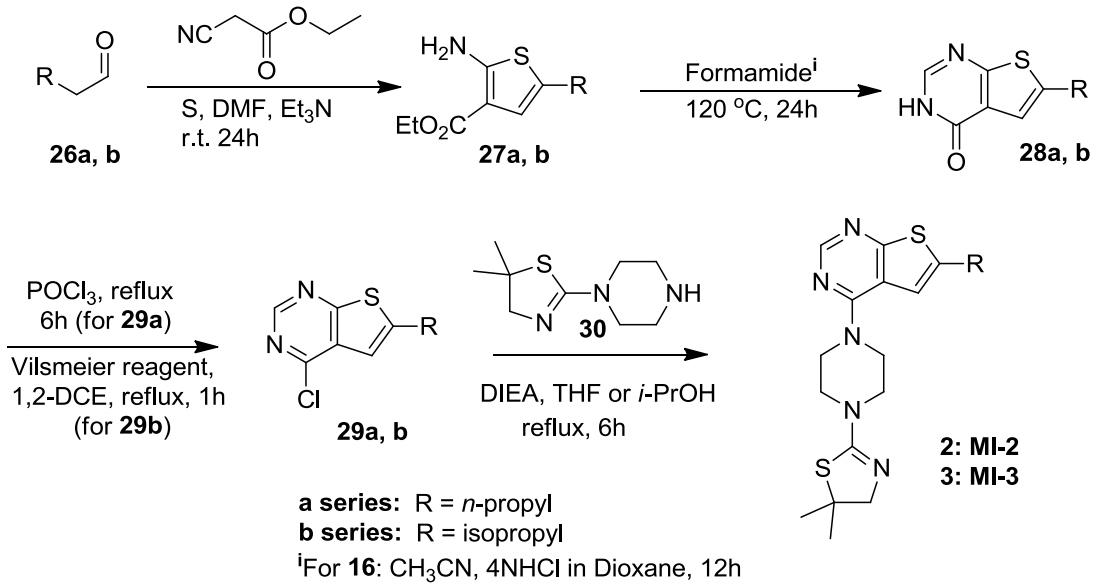
Reverse: 5' – CGAACCAATGGATCTGGCA – 3'

Inhibitor effects on cell cycle of MLL leukemia cells

5×10^5/ml MV4;11 cells were plated in 12-well plates (1ml/well) and treated with compounds (0.25% final concentration of DMSO for each condition) or 0.25% DMSO control and incubated for 48 h at 37 °C in a 5% CO_2 incubator. After incubation, 5×10^5 cells were harvested, washed in PBS buffer, resuspended in 1 ml PBS buffer and mixed with 9 ml of 70% ethanol. Cells were kept at -20°C for at least 24 h, and then washed and resuspended in FACS buffer followed by incubation with 100 μg/ml RNase (QIAGEN Inc. Valencia, CA) and 10 μg/ml propodium iodide at 37°C for 30 minutes before being processed by flow cytometry.

Chemical synthesis

Supplementary Scheme 1. Synthetic pathway to prepare MI-2 (2, R = *n*-propyl), MI-3 (3, R= isopropyl) and other compounds with varying R substituents.



MI-2 and MI-3 were synthesized as shown in Scheme 1. The condensation of aldehyde 26 and ethyl cyanoacetate in the presence of elemental sulfur and triethylamine in DMF gave product 27 in accordance with the Gewald reaction (Hesse et al., 2007; Hwang and Choi, 1991; Jang et al., 2010). Cyclization of 27 in formamide (Acetonitrile with 4N hydrochloride in dioxane for 16, MI-17) gave compound 28, (Dhanoa et al.), which was converted to chloride derivative 29 with phosphorus oxychloride or Vilsmeier reagent. Substitution of the chloride with 5,5-dimethyl-2-(piperazin-1-yl)-4,5-dihydrothiazole (30) under basic conditions yielded amine product 2 or 3, which was then converted to its monochydrochloride salt with one equivalent of HCl in ether, resulting in final products MI-2 and MI-3 in 50 % yield.

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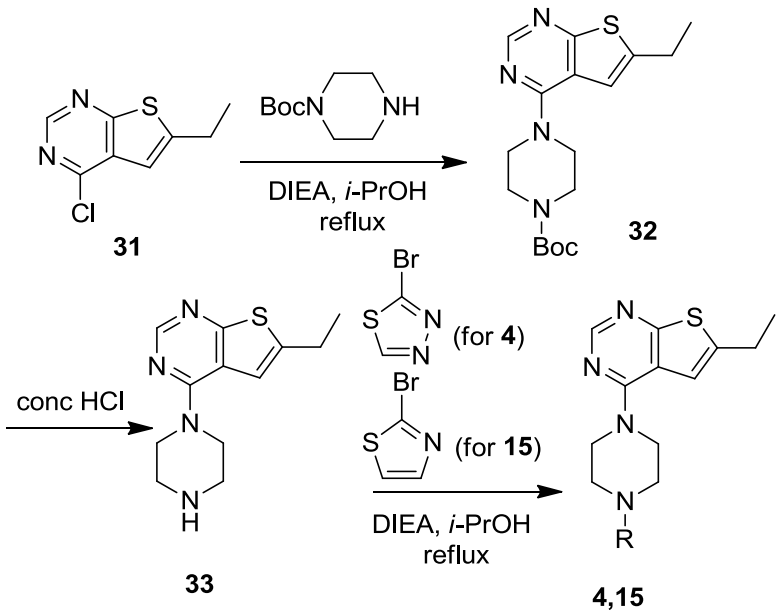
Supplementary Scheme 2. Synthetic pathway to prepare MI-nc (4) compound.



MI-nc was synthesized as shown in Scheme 2. Substitution of commercially available chloride 31 with *N*-Boc-piperazine under basic conditions provided product 32, which was then deprotected with concentrated HCl to give amine 33. A second substitution reaction was then carried out by condensation of 33 with 2-bromo-1,3,4-thiadiazole to provide target compound 4 (MI-nc) or 15.

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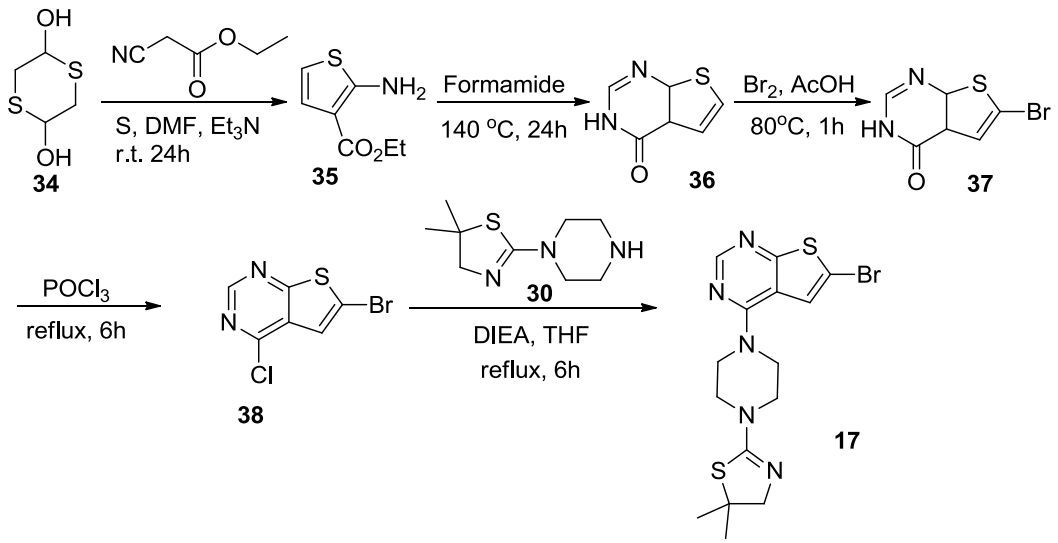
Supplementary Scheme 3. Synthetic pathway to prepare MI-18 (compound 19).



17 (MI-18) was synthesized as shown in Scheme 3. The condensation of 34 and ethyl cyanoacetate in the presence of elemental sulfur and triethylamine in DMF gave product 35, which was treated with formamide to yield compound 36. Bromination of 36 with bromine in acetic acid provided 37, which was then converted to chloride 38 with phosphorus oxychloride. Substitution of the chloride with 5,5-dimethyl-2-(piperazin-1-yl)-4,5-dihydrothiazole (30) under basic conditions yielded amine product 17.

Experimental section for chemical synthesis

General. Glassware was oven-dried before use for reactions run under anhydrous conditions. Melting points were determined in open capillary tubes on a Thomas Hoover UniMelt capillary melting point apparatus. The NMR spectra were recorded on a Bruker instrument at 500 MHz for ^{1}H and 125 MHz for ^{13}C spectra or on Varian instrument at 400 MHz for ^{1}H and 100 MHz for ^{13}C spectra. Chemical shift values are recorded in δ units (ppm). Mass spectra were recorded

on a Micromass TofSpec-2E Matrix-Assisted, Laser-Desorption, Time-of-Flight Mass Spectrometer in positive ESI mode unless otherwise noted. TLC was performed on Analtech silica gel HLF plates, 250 micron w/uv254. All reagents were commercially available and used as received.

MI-1 (1)

MI-1 was purchased from Labotest (cat # LT01870086) as a free amine and converted to its monohydrochloride salt by adding one equivalent of 1N HCl solution in diethyl ether followed by filtering: mp 154˚-156˚C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.54 (6H, s), 1.82 (2H, m), 1.93 (2H, m), 2.90 (4H, m), 3.42 (4H, *t*, *J*=5 Hz), 3.57 (4H, t, *J*=5 Hz), 3.75 (2H, s), 8.54 (1H, s); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 22.80, 22.96, 25.82, 26.76, 28.86 (2C), 47.84 (2C), 50.30 (2C), 60.07, 73.38, 121.61, 127.00, 135.65, 151.55, 162.13, 164.17, 168.53; Mass spec (HRMS): *m/z* 387.1564 (M^+).

Synthesis of MI-2 (2)

Ethyl 2-amino-5-propylthiophene-3-carboxylate (27a, R = *n*-propyl). To a solution of ethyl cyanoacetate (1.13 g, 10 mmol) and triethylamine (1.01 g, 10 mmol) in DMF (10 mL) valeraldehyde (0.86 g, 10 mmol) was added and the mixture was stirred for 10 min. Elemental sulfur (320 mg, 10 mmol) was added to the mixture. After stirring for 24 h, the suspension was partitioned between ethyl acetate and H_2O. The combined organic extracts were washed with brine, dried over $MgSO_4$ and concentrated to give orange oil. Purification by silica gel chromatography using hexane and ethyl acetate (9:1) as eluent gave 27a (2.01 g, 94.4%) as a

yellow oil; ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.93 (3H, t, *J*=7 Hz), 1.32 (3H, t, *J*=7 Hz), 1.57 (2H, m, *J*=7.4 Hz), 2.53 (2H, t, *J*=7 Hz), 4.24 (2H, q, *J*=7 Hz), 5.93(2H, broad s, N-H), 6.62 (1H, s); ^{13}C NMR (100 MHz, $CDCl_3$): δ 13.53, 14.54, 24.31, 31.76, 59.59, 106.00, 121.52, 126.52, 161.64, 165.47; Mass spec (ES^+): *m/z* 213.1 (M^++1).

6-Propylthieno[2, 3-*d*]pyrimidin-4(3*H*)-one (28a, R= *n*-propyl). A solution of 27a (2.87 g, 13.5 mmol) in formamide (20 mL) was heated at 140˚C for 24 h. After cooling, saturated sodium bicarbonate solution (20 mL) was added, and the mixture was partitioned between ethyl acetate and H_2O. The combined organic extracts were washed with brine, dried over $MgSO_4$ and concentrated to give a yellow solid. Purification by silica gel chromatography using dichloromethane and methanol (99:1) as eluent gave 3a (2.50 g, 95.5%) as an orange solid: mp 182˚C -185˚C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.01 (3H, q, *J*=7 Hz), 1.76 (2H, m, *J*=7 Hz), 2.86 (2H, t, *J*=7 Hz), 7.18 (1H, s), 8.04 (1H, s), 12.70 (1H, broad s, N-H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 13.76, 24.48, 32.83, 117.88, 125.13, 143.04, 144.96, 159.98, 164.67; Mass spec (ES^+): *m/z* 194.1 (M^++1).

4-Chloro-6-propylthieno[2, 3-*d*]pyrimidine (29a, R= *n*-propyl). A solution of 28a (1.87 g, 9.6 mmol) in phosphorus oxychloride (5 mL) was refluxed for 4 h. After cooling, the solvent was distilled off. The residue was diluted with saturated sodium bicarbonate solution and extracted with ethyl acetate. The combined organic extracts were washed with brine, dried over $MgSO_4$ and concentrated. The crude residue was purified by silica gel column chromatography using dichloromethane and methanol (80:2) as eluent to give 29a (1.22 g, 60.4%) as an orange oil; ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.04 (3H, t, *J*=7 Hz), 1.82(2H, m, *J*=7 Hz), 2.93 (2H, t, *J*=7 Hz), 7.08 (1H, s), 8.76 (1H, s); ^{13}C NMR (100 MHz, $CDCl_3$): δ 13.66, 24.10, 33.22, 115.99, 130.46, 149.33, 152.00, 153.19, 168.51; Mass spec (ES^+): *m/z* 212.1 (M^++1).

5,5-Dimethyl-2-(piperazin-1-yl)-4,5-dihydrothiazole (30). A solution of *tert* butyl-1-piperazinecarboxylate (0.5 g, 4.4 mmol) and methallyl isothiocyanate (822 mg, 4.4 mmol) in ethanol (5 mL) was stirred at room temperature for 1.5 h. The mixture was concentrated to give a crude yellow solid that was mixed with concentrated HCl (3mL) in a sealed tube. The mixture was stirred at 100˚C for 1.5 h. After cooling, the mixture was neutralized with aqueous ammonium hydroxide and extracted with ethyl acetate. The combined organic extracts were washed with brine, dried over $MgSO_4$ and concentrated to a residue. Silica gel chromatography using dichloromethane/methanol (97:3) as eluent gave 5 (0.46g, 52%) as a pale yellow solid; ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.53 (6H, s), 2.96 (4H, t, *J*=5 Hz), 3.49 (4H, t, *J*=5 Hz), 3.73 (2H, s); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 28.83 (2C), 45.76 (2C), 49.34 (2C), 59.52, 73.30, 164.16; mp 67˚C -70˚C; Mass spec (ES^+): *m/z* 199.2 (M^++1).

MI-2 (2)

4-(4-(5,5-Dimethyl-4,5-dihydrothiazol-2-yl)piperazin-1-yl)-6-propylthieno[2,3-*d*]pyrimidine (2, R = *n*-propyl). A solution of 29a (0.5 g, 2.4 mmol), 30 (0.56 g, 2.8 mmol), and *N,N*-diisopropylethylamine (0.91 g, 7.1 mmol) in THF (20 mL) was refluxed for 6 h. After cooling, the mixture was partitioned between ethyl acetate and H_2O. The combined organic extracts were washed with brine, dried over $MgSO_4$ and concentrated to a pale yellow solid. Purification by silica gel column chromatography using dichloromethane/methanol (97:3) as eluent gave 2 (0.82g, 91.1%) as a pale yellow solid. Its monohydrochloride salt was obtained by adding 1 equivalent of 1N HCl solution in diethyl ether to a solution of 2 in ethanol: mp 197˚C-200˚C ; HPLC >99.5% pure; ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.01 (3H, t, *J*=7 Hz), 1.57 (6H, s), 1.76 (2H, m, *J*=7.4 Hz), 2.86 (2H, t, *J*=7 Hz), 3.68 (4H, t, *J*=6.7 Hz), 3.79(2H, s), 3.93 (4H, t, *J*=6.7 Hz), 6.96 (1H, s), 8.46 (1H, s); ^{13}C NMR (100 MHz, $CDCl_3$): δ 13.74, 24.50, 28.76 (2C), 33.17,

46.57 (2C), 48.39 (2C), 59.79, 71.00, 116.48, 117.61, 143.01, 152.18, 157.91, 165.26, 168.94; (HRMS): *m/z* 375.1546 (M^+).

Synthesis of MI-3 (3)

Ethyl 2-amino-5-isopropylthiophene-3-carboxylate (**27b**, R = isopropyl). This compound was synthesized according to the procedure of Hwang and Choi (Hwang and Choi, 1991).

6-Isopropylthieno[2,3-*d*]pyrimidin-4(3*H*)-one (**28b**, R = isopropyl). This compound was synthesized according to a patent procedure (Dhanoa et al.).

4-Chloro-6-isopropylthieno[2,3-*d*]pyrimidine (**29b**, R = isopropyl). Oxalyl chloride (7.04 g, 54.4 mmol) was added drop-wise to a stirred solution of *N*,*N*-dimethylformamide (3.97 g, 54.4 mmol) in 1,2-dichloroethane (33 mL) at 0-5 °C. Following the addition the mixture was stirred at room temperature for 15 min, and then **28b** (4.8g, 24.7 mmol) was added in one portion. After 2 h at room temperature the mixture was heated to reflux for 1h, and then cooled. The mixture was diluted with 75 mL of ice water and extracted with dichloromethane. The combined extracts were washed with H_2O, and then brine, dried over $MgSO_4$, and concentrated to provide 5.1 g (97%) of **29b** as a caramel-colored solid: mp 39-40 °C; R_f 0.43 (hexane/ethyl acetate, 2:1); ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.87 (1H, s), 7.30 (1H, s), 3.50 - 3.27 (1H, m), 1.41 - 1.37 (6H, m); Mass spec (ES^+): *m/z* 213, 215 ($M^+ + 1$).

MI-3 (3)

4-(4-(5,5-Dimethyl-4,5-dihydrothiazol-2-yl)piperazin-1-yl)-6-isopropylthieno[2,3-*d*]pyrimidine (**3**, R = isopropyl). A solution of **29b** (91 mg, 0.43 mmol), **30** (85 mg, 0.43

mmol), and *N*,*N*-diisopropylethylamine (69 mg, 0.53 mmol) in isopropanol (3 mL) was refluxed briefly to effect solution, and then stirred at room temperature. After 20 h the mixture was concentrated and the residue partitioned between dichloromethane and H_2O. The combined organic extracts were washed with H_2O and then brine, dried over $MgSO_4$, and concentrated to a residue that was purified by silica gel chromatography utilizing gradient elution (3-4 column volumes ethyl acetate and then ethyl acetate/methanol/triethylamine, 83:15:2) to afford 108 mg (67 %) of **3** as a white solid: mp 139-140 °C; Mass spec (ES^+): *m/z* 376 ($M^+ + 1$). Its monohydrochloride salt was obtained by adding 1 equivalent of 1N HCl solution in diethyl ether to a solution of **3** in methanol and then concentrating the mixture to a glassy residue that was triturated in acetone/hexanes. The solid was collected to give **MI-3** (**3**) as a white powder: mp 212-213 °C (dec); HPLC >98% pure; ^{1}H NMR (500 MHz, DMSO-d_6): δ 10.61 (1H, s), 8.43 (1H, s), 7.35 (1H, s), 4.07 (6H, broad s), 3.96 (2H, broad s), 3.76 (2H, broad s), 3.28-3.25 (1H, m), 1.50 (6H, s), 1.38-1.35 (6H, m), ^{13}C NMR (150 MHz, DMSO-d_6): δ 24.10, 27.18, 30.09, 43.76, 45.22, 47.32, 50.31, 57.59, 61.07, 115.73, 116.30, 148.48, 151.78, 157.00, 167.16, 170.11; Mass spec (ES^+): *m/z* 376.5 ($M^+ + 1$).

Synthesis of MI-6 (4, MI-nc)

tert-Butyl 4-(6-ethylthieno[2,3-*d*]pyrimidin-4-yl)piperazine-1-carboxylate (**32**). 4-Chloro-6-ethylthieno[2,3-*d*]pyrimidine (obtained from Ukrorgsyntez Ltd., Kiev, Ukraine) (**31**; 1.33 g, 6.7 mmol) was added to a stirred solution of *N*,*N*-diisopropylethylamine (1.04 g, 8.05 mmol) and *N*-Boc-piperazine (1.25 g, 6.7 mmol) in isopropanol (13 mL) at room temperature and the mixture was heated at reflux for 2 min. After standing at room temperature for 20 h the mixture was

refrigerated, and then filtered. The collected solid was rinsed with cold isopropanol and dried to afford 1.8 g (77 %) of **32** as an off-white solid, homogeneous by tlc (ethyl acetate) : mp 129-130 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.37 (1H, s), 7.34 (1H, s), 4.00 – 3.75 (4H, m), 3.50 (4H, broad s), 2.91 (2H, dd, J = 14.9, 7.5 Hz), 1.43 (9H, s), 1.32 – 1.21 (3H, m); Mass spec (ES$^+$): *m/z* 349 ($M^+ + 1$).

6-Ethyl-4-(piperazin-1-yl)thieno[2,3-*d*]pyrimidine hydrochloride (**33**). A mixture of **32** (2.165 g, 6.2 mmol) and concentrated HCl (2.64 mL, 31 mmol) was stirred at room temperature. After 30 min, isopropanol (10-15 mL) was added and the formed off-white solid was collected and dried to leave 1.8 g (90 %) of **33**: mp 258°C (dec), homogenous by tlc (methanol/NH_4OH, 95:5); ^{1}H NMR (500 MHz, DMSO-d_6): δ 9.47 (2H, broad s), 8.51 (1H, s), 7.41 (1H, s), 4.11 – 3.98 (4H, m), 3.24 (4H, broad s), 2.93 (2H, q, J = 7.5 Hz), 1.31 (3H, t, J = 7.5 Hz); Mass spec (ES$^+$): *m/z* 249 ($M^+ + 1$).

MI-nc (4)

4-(4-(1,3,4-Thiadiazol-2-yl)piperazin-1-yl)-6-ethylthieno[2,3-*d*]pyrimidine (**4**). A solution of **33** (150 mg, 0.47 mmol), 2-bromo-1,3,4-thiadiazole (78 mg, 0.47 mmol; commercially available from Matrix Scientific, Columbia, SC), *N*,*N*-diisopropylethylamine (240 mg, 1.87 mmol) and isopropanol (1.9 mL) was stirred at room temperature for 20 h, and then at reflux for 24 h. Additional thiadiazole (30 mg) was added and after 5 h the mixture was cooled, concentrated, and the residue partitioned between dichloromethane and water. The combined organic extracts were washed with H_2O and then brine, dried over $MgSO_4$, and concentrated to a residue that was purified over silica gel chromatography with ethyl acetate as eluete to afford **4** (93 mg; 60 %) as a syrup: R_f 0.22 (ethyl acetate). Monohydrochloride salt formation on **4** was carried out as

described for 3. Following removal of methanol, trituration in dichloromethane/methanol (98:2) provided MI-nc (4) as an amorphous pink powder: HPLC 99% pure; ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.89 (1H, s), 8.58 (1H, s), 7.49 (1H, s), 4.13 (8H, s), 3.74 (4H, s), 2.98 – 2.93 (2H, m), 1.32 (3H, t, J = 7.4 Hz); ^{13}C NMR (150 MHz, DMSO-d_6): 15.92, 24.10, 45.94, 49.60, 117.00, 117.77, 143.62, 144.54, 151.98, 157.68, 172.09; Mass spec (ES$^+$): *m/z* 333.5 (M^+ + 1).

Synthesis of MI-18 (17)

ethyl 2-aminothiophene-3-carboxylate (35). To a solution of ethyl cyanoacetate (4.52 g, 40 mmol) and triethylamine (4.04 g, 40 mmol) in methanol (20 mL) was added 1,4-dithiane-2,5-diol (5.04 g, 20 mmol) and the mixture was stirred for 48 h, the solvent was removed and the residue was purified by silica gel chromatography using hexane and ethyl acetate (9:1) as eluent gave 35 (6 g, 87.5%) as a yellow oil; ^{1}H NMR (600 MHz, $CDCl_3$): δ 1.34 (3H, t, J=7 Hz), 4.27 (2H, q, J=7 Hz), 5.93(2H, broad s, N-H), 6.18 (1H, d, J=6Hz), 6.97(1H, d, J=6Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.2, 59.6, 107.0, 125.7, 162.6, 165.5, 171.2; Mass spec (ES$^+$): *m/z* 172.0 (M^++1).

6-bromothieno[2,3-d]pyrimidin-4(3H)-one (37). A solution of compound 35 (6g, 35mmol) in formamide (15ml) was heated up to 140°C for 72 hours. After cool down, the precipitate was filtered and washed with ethanol several times to give compound 36 (4g, 74%) as a gray solid. 36 was used for next step as a crude without further purification. To a suspension of compound 36 (20g, 131 mmol) in acetic acid (200mL) at room temperature, bromine (6.77mL, 131 mmol) was added dropwise. The mixture was stirred for 10 minutes and then heated up to 80°C for 1 hour. The precipitates were collected and washed with hexane and ethanol several times and dried to afford 19g (62.8%) of 37 as a brown solid; ^{1}H NMR (400 MHz, DMSO-d_6): δ_H 7.56

(1H, d, J=5Hz), 8.16 (1H, d, J=4Hz), 12.65 (1H, broad s); ^{13}C NMR (150 MHz, DMSO-d_6): δ 110.26, 124.52, 125.54, 146.54, 156.01, 164.94; Mass spec (ES^+): *m/z* 233.2 (M^++1).

6-bromo-4-chlorothieno[2,3-d]pyrimidine (38). A solution of 37 (9 g, 3.9 mmol) in phosphorus oxychloride (50 mL) was refluxed for 4 h. After cooling, the solvent was distilled off. The residue was diluted with saturated sodium bicarbonate solution and extracted with ethyl acetate. The combined organic extracts were washed with brine, dried over $MgSO_4$ and concentrated. The crude residue was purified by silica gel column chromatography using dichloromethane and methanol (80:2) as eluent to give 38 (6.42 g, 66.7%) as a brown solid; ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 7.49 (1H, d, J=1.6Hz), 8.82 (1H, s); ^{13}C NMR (150 MHz, $CDCl_3$): δ_C 118.76, 122.25, 130.54, 152.94, 153.33, 169.61; Mass spec (ES^+): *m/z* 250.1 (M^++1).

MI-18 (17)

6-bromo-4-(4-(5,5-dimethyl-4,5-dihydrothiazol-2-yl)piperazin-1-yl)thieno[2,3-d]pyrimidine (17) was synthesized according to the procedure as for compound 2 (MI-2): ^{1}H NMR (600 MHz, $CDCl_3$): δ_H 1.57 (6H, s), 3.60 (4H, t, J=5Hz), 3.75 (2H, s), 3.92 (4H, t, J=5Hz), 7.32 (1H, s), 8.45(1H, s); ^{13}C NMR (150 MHz, $CDCl_3$): δ_C 28.85, 46.48, 47.84, 60.35, 73.17, 111.94, 117.52, 122.73, 152.95, 157.30, 163.72, 170.25; (HRMS): *m/z* 411.0178 (M^+).

Characterization of chemical compounds

MI-7 (5)

4-(piperazin-1-yl)thieno[2,3-d]pyrimidine (MI-7) was purchased from Enamine (cat # T5832712): ^{1}H NMR (600 MHz, $CDCl_3$): δ_H 3.03 (4H, t, J=5Hz), 3.90 (4H, t, J=5Hz), 7.28 (1H,

d, J=6Hz), 7.33 (1H, d, J=6Hz), 8.49 (1H, s); ^{13}C NMR (150 MHz, $CDCl_3$): δ_C 46.15, 48.28, 116.65, 120.56, 121.98, 153.12, 158.91, 169.50; *m/z* (ES^+) 221.1 $(M+H)^+$.

MI-8 (6)

4-(piperazin-1-yl)-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidine (MI-8) was purchased from Asinex (cat # ASN17326948): ^{1}H NMR (600 MHz, $CDCl_3$): δ_H 1.83(2H, m), 1.95 (2H, m), 2.89 (4H, m), 3.41 (4H, t, J=5Hz), 3.79 (4H, t, J=5Hz), 8.55 (1H, s), 10.01 (2H, broad s); ^{13}C NMR (150 MHz, $CDCl_3$): δ_C 22.66, 22.80, 25.82, 26.32, 42.65, 47.32, 121.63, 126.35, 137.04, 151.45, 160.93, 168.94; *m/z* (ES^+) 275.2 $(M+H)^+$.

MI-9 (7)

4-(4-(pyrazin-2-yl)piperazin-1-yl)-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidine (MI-9) was purchased from Enamine (cat # T5813607): ^{1}H NMR (600 MHz, $CDCl_3$): δ_H 1.84 (2H, m), 1.96 (2H, m), 2.91 (2H, t, J=6.5Hz), 2.98 (2H, t, J=5Hz), 3.54 (4H, t, J=5Hz), 3.79 (4H, t, J=5Hz), 7.91 (1H, d, J=2.6Hz), 8.11 (1H, d, J=2.6Hz), 8.22 (1H, s), 8.56 (1H, s); ^{13}C NMR (150 MHz, $CDCl_3$): δ_C 22.79, 23.07, 25.87, 26.86, 44.48, 50.42, 121.67, 126.94, 131.33, 133.62, 135.72, 141.79, 151.62, 155.23, 162.25, 168.67; *m/z* (ES^+) 353.2 $(M+H)^+$.

MI-10 (8)

6-ethyl-4-(4-(pyridin-2-yl)piperazin-1-yl)thieno[2,3-d]pyrimidine (MI-10) was purchased from Chembridge (cat # 5251813): ^{1}H NMR (600 MHz, $CDCl_3$): δ_H 1.41 (3H, t, J=7.5Hz), 3.00 (2H, q, J=7.5Hz), 4.24(4H, t, J=5Hz), 4.41 (4H, t, J=5Hz), 7.00 (1H, t, J=6.6Hz), 7.07 (1H, d, J=9Hz), 7.22 (1H, s), 7.99 (1H, t, J=7.7Hz), 8.25 (1H, d, J=6.6Hz), 8.55 (1H, s); ^{13}C NMR (150

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MHz, 10% DMSO-d_6 in $CDCl_3$): δ_C 15.46, 24.36, 40.64, 45.85, 111.21, 113.29, 116.24, 117.10, 138.36, 144.40, 147.27, 151.94, 156.47; *m/z* (ES^+) 326.2 $(M+H)^+$.

MI-11 (9)

6-ethyl-4-(4-(pyrimidin-2-yl)piperazin-1-yl)thieno[2,3-d]pyrimidine (MI-11) was purchased from Interbioscreen (cat # IS-24791): ^1H NMR (600 MHz, $CDCl_3$): δ_H 1.41(3H, t, J=8Hz), 3.00 (2H, q, J=8Hz), 4.08 (2H, t, J=6.5Hz), 4.22 (2H, t, J=5Hz), 4.30 (2H, t, J=6.5Hz), 4.34 (2H, t, J=5Hz), 6.78 (1H, t, J=5Hz), 7.25 (1H, s), 8.31 (1H, s), 8.48 (1H, d, J=5Hz), 8.57 (1H, d, J=5Hz); ^{13}C NMR (150 MHz, 10% DMSO-d_6 in $CDCl_3$): δ_C 15.43, 24.12, 43.01, 43.44, 110.62, 116.50, 119.39, 144.85, 145.20, 147.46, 156.04, 157.49, 158.31, 159.23; *m/z* (ES^+) 327.2 $(M+H)^+$.

MI-4 (10)

4-(4-benzylpiperazin-1-yl)-6-ethylthieno[2,3-d]pyrimidine (10) was purchased from Chemical Diversity Inc. (cat # 0866-0033): ^1H NMR (600 MHz, DMSO-d_6): δ_H 1.31 (3H, t, J=7.5Hz), 2.92 (2H, q, J=7.5Hz), 3.20 (2H, s), 3.41 (2H, m), 3.64 (2H, m), 4.39 (2H, m), 4.61 (2H, m), 7.39 (1H, s), 7.48 (3H, m), 7.64 (2H, m), 8.46 (1H, s), 11.38 (1H, broad s); ^{13}C NMR (600 MHz, DMSO-d_6): δ_c 15.38, 23.74, 43.30, 50.21, 58.71, 116.89, 128.80, 129.53, 131.44, 143.71, 151.79, 156.94, 167.96; *m/z* (ES^+) 339.5 $(M+H)^+$.

MI-12 (11)

4-(4-(4-chlorobenzyl)piperazin-1-yl)-6-ethylthieno[2,3-d]pyrimidine (MI-12) was purchased from Princeton Biomolecular Research Inc. (cat # OSSK_851527): ^1H NMR (600 MHz, $CDCl_3$): δ_H 1.39 (3H, t, J=7.5Hz), 2.95 (2H, q, J=7.5Hz), 3.20 (2H, m), 3.48 (2H, m), 4.28 (4H, m), 4.76

(2H, s), 7.06 (1H, s), 7.42 (2H, d, J=8.5Hz), 7.71 (2H, d, J=8.5Hz), 8.52 (1H, s); ^{13}C NMR (150 MHz, 10% DMSO-d_6 in $CDCl_3$): δ_C 15.45, 24.42, 44.10, 50.58, 59.72, 115.59, 117.36, 120.98, 126.53, 129.49, 131.63, 133.19, 136.44, 148.49, 156.78; *m/z* (ES^+) 373.2 $(M+H)^+$.

MI-13 (12)

4-(4-((1H-benzo[d]imidazol-2-yl)methyl)piperazin-1-yl)-6-ethylthieno[2,3-d]pyrimidine (MI-13) was purchased from Princeton Biomolecular Research Inc. (cat # OSSK_851537): ^{1}H NMR (600 MHz, 10% DMSO-d_6 in $CDCl_3$): δ_H 1.38 (3H, t, J=7.5Hz), 2.95 (2H, q, J=7.5Hz), 3.22 (2H, s), 4.36 (4H, m), 4.68 (4H, m), 7.15 (1H, s), 7.52 (2H, m), 7.86 (2H, m), 8.55 (1H, s); ^{13}C NMR (150 MHz, DMSO-d_6): δ_C 15.36, 23.53, 40.02, 44.81, 51.73, 114.74, 116.55, 117.16, 124.45, 125.62, 134.24, 143.68, 150.78, 156.79, 162.55, 165.65; *m/z* (ES^+) 379.2 $(M+H)^+$.

MI-14 (13)

4-(4-(2,4-dichlorobenzyl)piperazin-1-yl)-6-ethylthieno[2,3-d]pyrimidine (MI-14) was purchased from Princeton Biomolecular Research Inc. (cat # OSSK_851528): ^{1}H NMR (600 MHz, 10% DMSO-d_6 in $CDCl_3$): δ_H 1.39 (3H, t, J=7.5Hz), 2.96 (2H, q, J=7.5Hz), 3.22 (2H, s), 4.34 (2H, m), 4.47 (4H, s), 4.80 (2H, m), 7.11 (1H, s), 7.44 (1H, d, J=8.7Hz), 7.50 (1H, s), 8.24 (1H, d, J=8.7Hz), 8.55 (1H, s); ^{13}C NMR (150 MHz, 10% DMSO-d_6 in $CDCl_3$): δ_C 15.34, 24.19, 43.99, 50.73, 55.93, 115.68, 117.22, 124.75, 128.61, 129.98, 135.63, 136.14, 137.25, 146.56, 147.19, 147.70, 156.52; *m/z* (ES^+) 407.1 $(M+H)^+$.

MI-15 (14)

4-(4-(benzo[d][1,3]dioxol-5-ylmethyl)piperazin-1-yl)-6-ethylthieno[2,3-d]pyrimidine (MI-15) was purchased from Enamine (cat # T5473121): ^{1}H NMR (600 MHz, 10% DMSO-d_6 in

$CDCl_3$): δ_H 1.39 (3H, t, J=7.5Hz), 2.94 (2H, q, J=7.5Hz), 3.03 (2H, m), 3.47 (2H, m), 4.16 (4H, m), 4.63 (2H, m), 6.00 (2H, s), 6.84 (1H, m), 6.97 (1H, s), 7.09 (1H, m), 7.25 (1H, s), 8.47 (1H, s); ^{13}C NMR (150 MHz, 10% DMSO-d_6 in $CDCl_3$): δ_C 15.19, 24.29, 43.75, 50.47, 60.60, 101.62, 108.60, 111.51, 115.13, 117.43, 120.97, 125.74, 146.15, 148.18, 149.24, 150.66, 157.02; *m/z* (ES^+) 383.2 $(M+H)^+$.

MI-16 (15)

6-ethyl-4-(4-(thiazol-2-yl)piperazin-1-yl)thieno[2,3-d]pyrimidine (MI-16) was synthesized according to the Supplementary Scheme 1 (Note: 2-(piperazin-1-yl)thiazole was used instead of 30 for coupling with 29 to obtain MI-16): ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.41 (s, 1H), 7.39 (s, 1H), 7.22 (d, 1H), 6.90 (d, 1H), 4.01 (t, 4H), 3.60 (t, 4H), 2.93 (q, 2H), 1.31 (t, 3H); ^{13}C NMR (150 MHz, $CDCl_3$): δ_C 14.44, 23.44, 45.21, 47.68, 106.71, 114.46, 116.56, 136.74, 144.18, 150.66, 156.85, 170.19, 179.25; MS (ES^+): *m/z* 332 $(M + H)^+$.

MI-17 (16)

4-(4-(5,5-dimethyl-4,5-dihydrothiazol-2-yl)piperazin-1-yl)-2-methyl-6-propylthieno[2,3-d]pyrimidine (MI-17) was synthesized according to the Supplementary Scheme 1: ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 0.99 (3H, t, J=7Hz), 1.53 (6H, s), 1.72 (2H, m), 2.53 (3H, s), 2.82 (2H, t, J=7.4Hz), 3.56 (4H, t, J=5Hz), 3.74 (2H, s), 3.88 (4H, t, J=5Hz), 6.89 (1H, s); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 13.76, 24.50, 25.86, 28.95, 33.18, 46.64, 47.94, 60.24, 73.40, 114.97, 116.45, 141.27, 157.99, 161.58, 164.00, 169.71; 390.1 $(M+H)^+$.

MI-19 (18)

4-(4-(5,5-dimethyl-4,5-dihydrothiazol-2-yl)piperazin-1-yl)-6-ethylthieno[2,3-d]pyrimidine (MI-19) was synthesized according to the Supplementary Scheme 1: ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.45 (s, 1H), 7.38 (s, 1H), 4.08 (m, 4H) 3.9-3.5 (m, 6H), 2.93 (q, 2H), 1.62 (s, 6H), 1.31 (t, 3H); ^{13}C NMR (150 MHz, $CDCl_3$): 15.66, 24.50, 28.25, 45.41, 47.51, 49.23, 51.75, 58.15, 62.49, 115.77, 117.68, 130.53, 147.25, 149.52, 156.85, 170.60; δ_C MS (ES^+): *m/z* 362 $(M+H)^+$.

MI-20 (19)

4-(4-(5,5-dimethyl-4,5-dihydrothiazol-2-yl)piperazin-1-yl)-6-butylthieno[2,3-d]pyrimidine (MI-20) was synthesized according to the Supplementary Scheme 1: ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 0.96 (3H, t, J=7Hz), 1.38-1.45 (2H, m), 1.54 (6H, s), 1.59-1.75(2H, m), 2.88 (2H, J=8Hz), 3.58 (4H, t, J=5Hz), 3.75 (2H, s), 3.89 (4H, t, J=5Hz), 6.94 (1H, s), 8.46 (1H, s);^{13}C NMR (100 MHz, $CDCl_3$): δ_C 13.95, 22.35, 28.98, 30.97, 33.35, 46.78, 47.92, 60.37, 73.48, 116.44, 117.64, 143.07, 152.30, 158.12, 163.94, 168.92; *m/z* (ES^+) 390.1 $(M+H)^+$.

MI-21 (20)

6-(sec-butyl)-4-(4-(5,5-dimethyl-4,5-dihydrothiazol-2-yl)piperazin-1-yl)thieno[2,3-d]pyrimidine (MI-21) was synthesized according to the Supplementary Scheme 1: ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 0.92 (3H, t, J=7.4Hz), 1.35 (3H, d, J=7Hz), 1.54 (6H, s), 1.70 (2H, m), 2.98 (1H, m), 3.60 (4H, t, J=5Hz), 3.75 (2H, s), 3.90 (4H, t, J=5Hz), 6.95 (1H, s), 8.46 (1H, s); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 12.08, 22.40, 28.99, 31.77, 38.39, 46.80, 47.95, 60.38, 73.47, 115.19, 117.41, 149.09, 152.31, 158.20, 163.98, 168.63; *m/z* (ES^+) 390.1 $(M+H)^+$.

MI-22 (21)

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4-(4-(5,5-dimethyl-4,5-dihydrothiazol-2-yl)piperazin-1-yl)-6-phenylthieno[2,3-d]pyrimidine (MI-22) was synthesized according to the Supplementary Scheme 1: ^{1}H NMR (600 MHz, $CDCl_3$): δ_H 1.69 (6H, s), 3.75 (4H, t, J=5Hz), 3.93 (2H, s), 4.15 (4H, t, J=5Hz), 7.40 (1H, s), 7.44-7.50 (3H, m), 7.68(2H, m), 8.54 (1H, s); ^{13}C NMR (150 MHz, $CDCl_3$): δ_C 27.73, 44.82, 48.0158.32, 62.46, 114.52, 118.59, 126.73, 129.27, 132.95, 141.98, 152.03, 157.86, 161.39, 168.23, 171.13; *m/z* (ES^+) 410.2 $(M+H)^+$.

MI-23 (22)

6-benzyl-4-(4-(5,5-dimethyl-4,5-dihydrothiazol-2-yl)piperazin-1-yl)thieno[2,3-d]pyrimidine (MI-23) was synthesized according to the Supplementary Scheme 1: ^{1}H NMR (600 MHz, $CDCl_3$): δ_H 1.68 (6H, s), 2.06 (2H, s), 3.67(4H, t, J=5Hz), 3.93 (2H, s), 4.02 (4H, t, J=5Hz), 7.04 (1H, s), 7.26-7.35 (5H, m), 8.47 (1H, s); ^{13}C NMR (150 MHz, 10% DMSO-d_6 in $CDCl_3$): δ_C 27.94, 36.84, 45.88, 48.30, 50.78, 58.18, 62.11, 116.97, 117.70, 127.29, 128.58, 128.95, 138.17, 144.43, 147.66, 156.79, 170.70; *m/z* (ES^+) 423.4 $(M+H)^+$.

MI-24 (23)

4-(4-(5,5-dimethyl-4,5-dihydrothiazol-2-yl)piperazin-1-yl)-6-phenethylthieno[2,3-d]pyrimidine (MI-24) was synthesized according to the Supplementary Scheme 1: ^{1}H NMR (600 MHz, $CDCl_3$): δ_H 1.54 (6H, s), 3.02 (2H, t, J=7.5Hz), 3.19 (2H, t, J=7.5Hz), 3.55(4H, t, J=5Hz), 3.75 (2H, s), 3.82 (4H, t, J=5Hz), 6.78 (1H, s), 7.18 (2H, d, J=7.5Hz), 7.21 (1H, t, J=8.2Hz), 7.28 (2H, dd, J=7.5, 8.2Hz), 8.45 (1H, s); ^{13}C NMR (150 MHz, $CDCl_3$): δ_C 28.78, 32.83, 37.29, 46.57, 47.87, 60.16, 73.14, 117.13, 117.36, 126.39, 128.50, 128.54, 140.30, 141.01, 152.25, 158.04, 164.04, 168.80; *m/z* (ES^+) 438.2 $(M+H)^+$.

MI-25 (24)

4-(4-(5,5-dimethyl-4,5-dihydrothiazol-2-yl)piperazin-1-yl)-6-(3-phenylpropyl)thieno[2,3-d]pyrimidine (MI-25) was synthesized according to the Supplementary Scheme 1: ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 1.52 (6H, s), 2.08 (2H, m), 2.63 (2H, t, J=7Hz), 2.90 (2H, t, J=7Hz), 3.51 (4H, t, J=5Hz), 3.74 (2H, s), 3.89 (4H, t, J=5Hz), 6.89 (1H, s), 7.11-7.20 (3H, m), 7.23-7.37 (2H, m), 8.44 (1H, s); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 29.00, 30.70, 32.74, 35.31, 46.79, 48.02, 60.36, 73.21, 116.83, 117.63, 126.27, 128.67, 141.53, 142.43, 152.43, 156.70, 158.15, 164.16, 169.05; *m/z* (ES^+) 452.2 $(M+H)^+$.

MI-26 (25)

4-(4-(5,5-dimethyl-4,5-dihydrothiazol-2-yl)piperazin-1-yl)-6-(4-phenylbutyl)thieno[2,3-d]pyrimidine (MI-26) was synthesized according to the Supplementary Scheme 1: ^{1}H NMR (600 MHz, $CDCl_3$): δ_H 1.55 (6H, s), 1.77 (4H, m), 2.67 (2H, m), 2.90 (2H, m), 3.64 (4H, t, J=5Hz), 3.79 (2H, s), 3.91 (4H, t, J=5Hz), 6.92 (1H, s), 7.17-7.20 (2H, m), 7.26-7.30 (3H, m), 8.47 (1H, s); ^{13}C NMR (150 MHz, $CDCl_3$): δ_C 28.74, 30.27, 30.88, 35.51, 35.60, 46.53, 48.81, 59.81, 73.12, 116.13, 117.23, 126.03, 128.59, 142.16, 142.87, 149.34, 152.20, 157.97, 168.61, 169.19; *m/z* (ES^+) 466.2 $(M+H)^+$.

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