Clinical-Stage Menin Inhibitor KO-539 is Synergistically Active with Multiple Classes of Targeted Agents in KMT2A/MLL-r and NPM1-Mutant AML Models.

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Introduction

MLL1 (KMT2A) is a large (3696 amino acids) transcriptional regulator and a histone-lysine-N-methyltransferase. N-terminal 1400 amino acids of MLL1 act as a transcription factor containing Menin binding domains (MBDs), and the C-terminal SET domain acts as a histone methyltransferase that mediates histone H3 lysine 4 trimethylation. Menin is a scaffold protein that binds to MBD. Encoded by MEN1 gene, Menin is a 610 amino acid, scaffold protein. Menin binds to MBD within the N-terminal 1-40 residues of MLL1 and forms a Menin-MLL1-LEDGF ternary complex, which tethers MLL1 to chromatin. Menin-KMT2A complex has a critical role in regulating HOX genes cluster, including the leukemogenic homeobox A9 (HOXA9) and its co-factor MEIS1 in myeloid stem progenitor cells, which are involved in embryonic development and hematopoiesis. HOXA9 functions as a pioneer factor and along with its co-factor MEIS1 recruits CEBPα and MLL3/MLL4 to reprogram the enhancer landscape to promote leukemogenesis. MLL1 knockout (KO) is embryonic lethal, but conditional KO undermines self-renewal of hematopoietic stem cells (HSCs). In MLL1-r AML, N-terminus of the MLL1 gene is fused to the C-terminus of any of over 80 fusion partners, including AF4, AF9, ENL and ELL. These MLL fusion partners are components of (and recruit) the super elongation complex (SEC) and DOT1L to induce H3K4Me3 and H3K79Me2 marks on active chromatin, driving aberrant expression of HOXA9, MEIS1, PBX3, MEF2C and CDK6. Conditional KO of MEN1 prevents MLL-r AML. In AML with mtNPM1 (NPM1c), the wild type menin-KMT2A complex is the main oncogenic regulator of HOXA9, MEIS1 and FLT3, promoting self-renewal of myeloid progenitor cells. Treatment with clinical, investigational, orally bioavailable, drug candidate Menin inhibitor (MI) KO-539 (Kura Pharmaceuticals) disrupts the Menin-KMT2A protein-protein interaction. Preclinical data show that KO-539 and close analogs induce differentiation and loss of viability of AML cells. Monotherapy with KO-539 exerted profound anti-leukemic activity in multiple PDX models harboring MLL-FP or NPM1c when dosed continuously QD for 3-6 weeks. In an ongoing phase 1/2A KOMET-001 clinical trial (NCT04067336) that is evaluating KO-539 in adult patients with relapsed and/or refractory AML, significant biological activity as well as complete remission lacking minimal residual disease have been observed.

The present studies were focused on further elucidating biologic effects and on determining synergistic activity of KO-539 with other novel agents, including those targeting BCL2, BET proteins and CDK6. Treatment with KO-539 dose-dependently inhibited in vitro growth, as well as induced differentiation (by both morphology and increased CD11b expression) and loss of viability of MOLM13 (MLL-AF9 and FLT3-ITD) and OCI-AML3 (NPM1c and homozygous NRAS mutation) cells and patient-derived (PD) MLL-r or NPM1c AML cells. This was associated with repression of protein levels of Menin, MEIS1, FLT3, CDK6 and BCL2, but upregulated MCL1 and CD11b proteins in MOLM13 and OCI-AML3 cells. KO-539 treatment also reduced protein levels of Menin, MEIS1, MEF2C, PBX3, FLT3, CDK6 and BCL2 in phenotypically characterized AML stem cells (with high expression of CLEC12A, CD123, CD244, CD99, but low expression of CD11b). KO-539-mediated Menin depletion was associated with polyubiquitylation and proteasomal degradation of Menin protein. Notably, co-treatment in vitro with KO-539 in combination with venetoclax, OTX015 (pan-BET inhibitor) or abemaciclib (CDK6 inhibitor) for 72-96 h induced synergistic loss of viability in cultured cell lines and PD AML cells from both MLL-r and NPM1c AML but not normal CD34+ progenitor cells or AML cells lacking MLL-FP or NPM1c. As compared to treatment with KO-539 or venetoclax or vehicle control, co-treatment with KO-539 and venetoclax exerted superior in vivo anti-AML efficacy without any host toxicity in PDX (patient derived xenograft) model of MLL-r AML. These preclinical findings highlight the molecular correlates of anti-AML efficacy of KO-539 and demonstrate potential superior KO-539-based combinations with inhibitors of BCL2, BET proteins and CDK6 against MLL-r or NPM1 mutant AML.

Conclusions

1. Treatment with KO-539 dose-dependently induces growth inhibition, differentiation and loss of viability of AML cells with MLL1-r or NPM1c. This is associated with attenuation of levels of MEIS1, FLT3, CDK6, Bcl-xL and BCL2, but upregulation of MCL1 and CD11b.
2. Treatment with KO-539 reduces mRNA expression of MLL1 target genes, as well as reduces their protein expressions in immune-phenotypically-characterized AML stem-progenitor cells.
3. KO-539 treatment destabilizes and reduces protein levels of Menin, which are restored by co-treatment with proteasome inhibitor carfilzomib.
4. KO-539 treatment exerts lethal activity against MOLM13 cells, MOLM13-TP53-R175H and MOLM13-TP53KO cells. Compared to control MOLM13 or MOLM13-TP53KO cells, MOLM13-TP53-R248Q cells are relatively resistant to KO-539.
5. Co-treatment with KO-539 and venetoclax, BET inhibitor or CDK6 inhibitor is synergistically lethal against AML cells with MLL-r or mutant NPM1.
6. Compared to treatment with KO-539 or venetoclax or vehicle control, co-treatment with KO-539 and venetoclax exerted superior in vivo anti-AML efficacy without any host toxicity in a PDX model of MLL-r AML.
7. These preclinical findings highlight the molecular correlates of anti-AML efficacy of KO-539 and demonstrate potential superior KO-539-based combinations with inhibitors of BCL2, BET proteins and CDK6 against MLL-r or NPM1 mutant AML.

Results

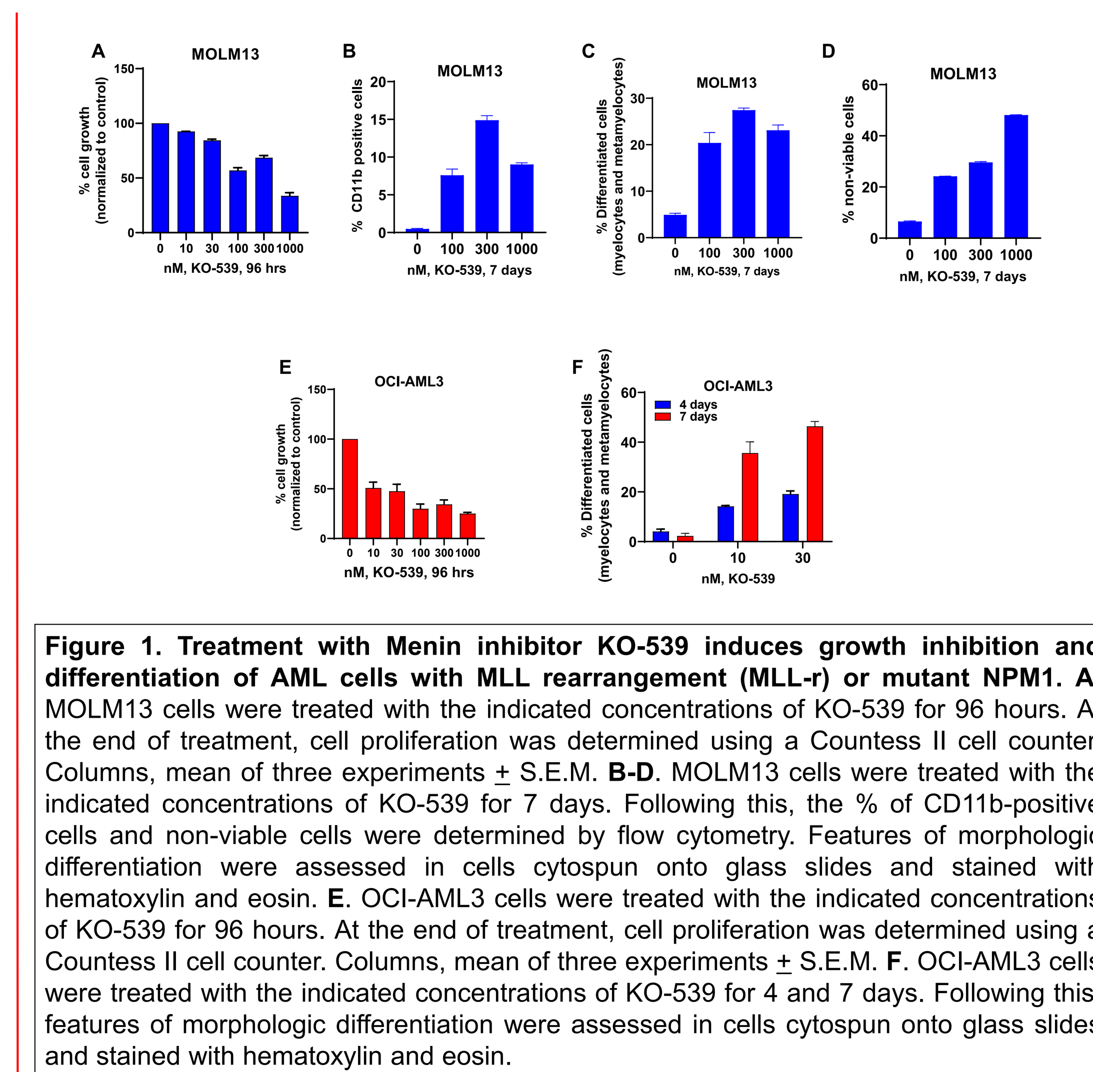
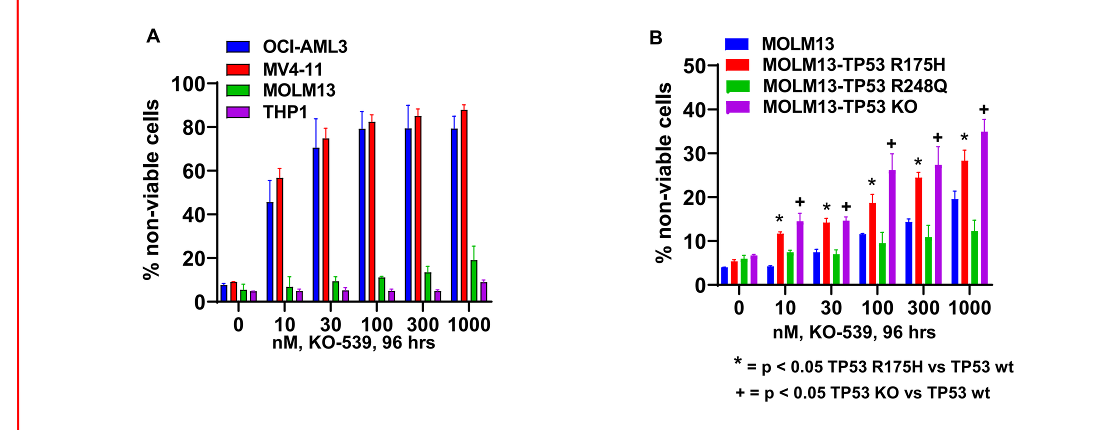


Figure 1. Treatment with Menin inhibitor KO-539 induces growth inhibition and differentiation of AML cells with MLL rearrangement (MLL-r) or mutant NPM1. A. MOLM13 cells were treated with the indicated concentrations of KO-539 for 96 hours. At the end of treatment, cell proliferation was determined using a Countess II cell counter. Columns, mean of three experiments ± S.E.M. **B-D**. MOLM13 cells were treated with the indicated concentrations of KO-539 for 7 days. Following this, the % of CD11b-positive cells and non-viable cells were determined by flow cytometry. Features of morphologic differentiation were assessed in cells cytospun onto glass slides and stained with hematoxylin and eosin. **E**. OCI-AML3 cells were treated with the indicated concentrations of KO-539 for 96 hours. At the end of treatment, cell proliferation was determined using a Countess II cell counter. Columns, mean of three experiments ± S.E.M. **F**. OCI-AML3 cells were treated with the indicated concentrations of KO-539 for 4 and 7 days. Following this, features of morphologic differentiation were assessed in cells cytospun onto glass slides and stained with hematoxylin and eosin.



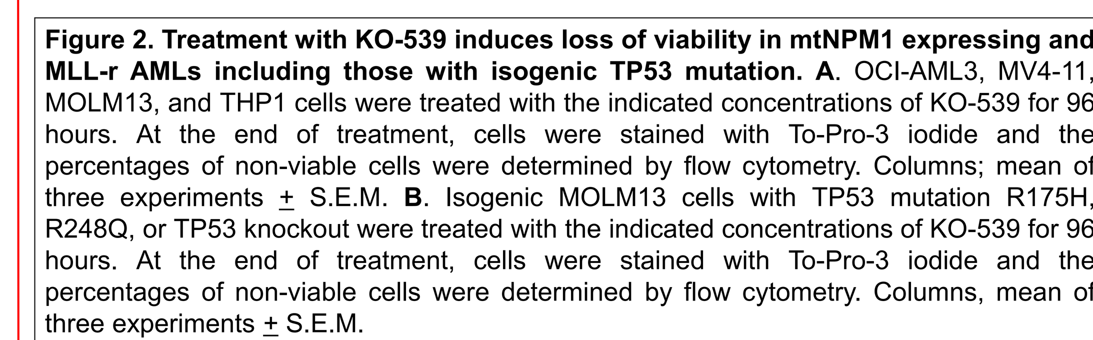
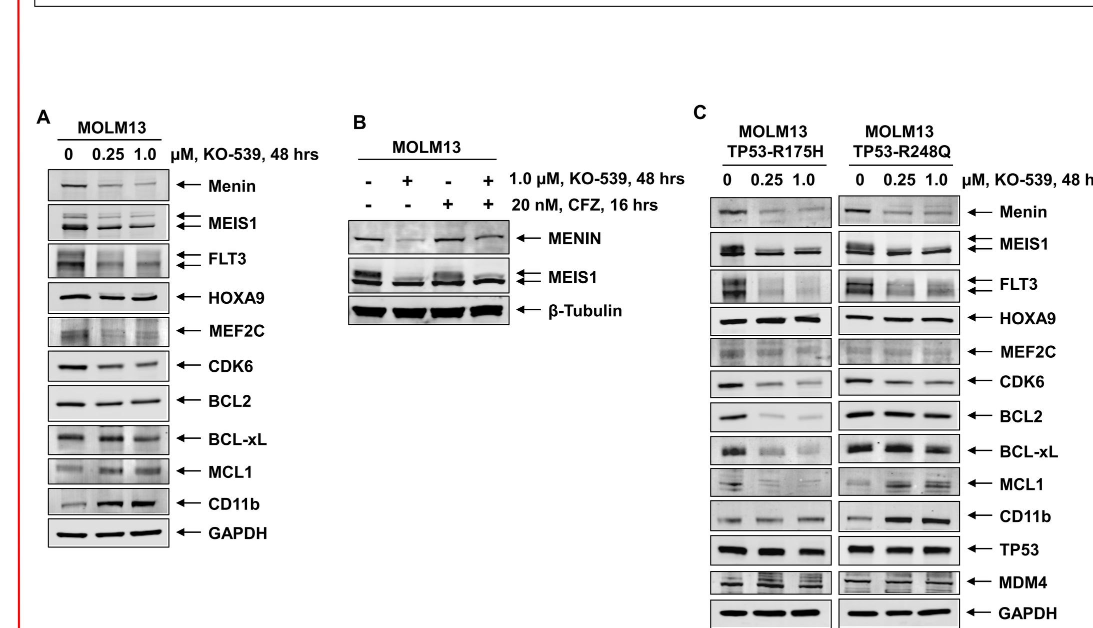


Figure 2. Treatment with KO-539 induces loss of viability in mtNPM1 expressing and MLL-r AMLs including those with isogenic TP53 mutation. A. OCI-AML3, MV4-11, MOLM13, and THP1 cells were treated with the indicated concentrations of KO-539 for 96 hours. At the end of treatment, cells were stained with To-Pro-3 iodide and the percentages of non-viable cells were determined by flow cytometry. Columns; mean of three experiments ± S.E.M. **B**. Isogenic MOLM13 cells with TP53 mutation R175H, R248Q, or TP53 knockout were treated with the indicated concentrations of KO-539 for 96 hours. At the end of treatment, cells were stained with To-Pro-3 iodide and the percentages of non-viable cells were determined by flow cytometry. Columns, mean of three experiments ± S.E.M.



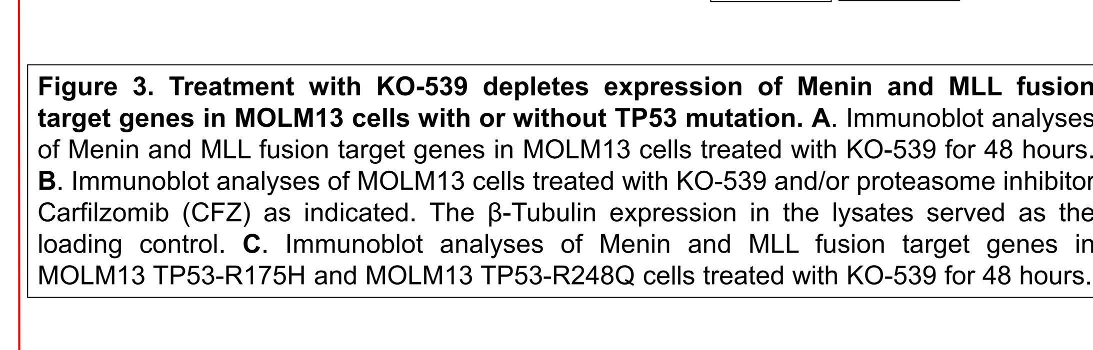


Figure 3. Treatment with KO-539 depletes expression of Menin and MLL fusion target genes in MOLM13 cells with or without TP53 mutation. A. Immunoblot analyses of Menin and MLL fusion target genes in MOLM13 cells treated with KO-539 for 48 hours. **B**. Immunoblot analyses of MOLM13 cells treated with KO-539 and/or proteasome inhibitor Carfilzomib (CFZ) as indicated. The β-Tubulin expression in the lysates served as the loading control. **C**. Immunoblot analyses of Menin and MLL fusion target genes in MOLM13 TP53-R175H and MOLM13 TP53-R248Q cells treated with KO-539 for 48 hours.

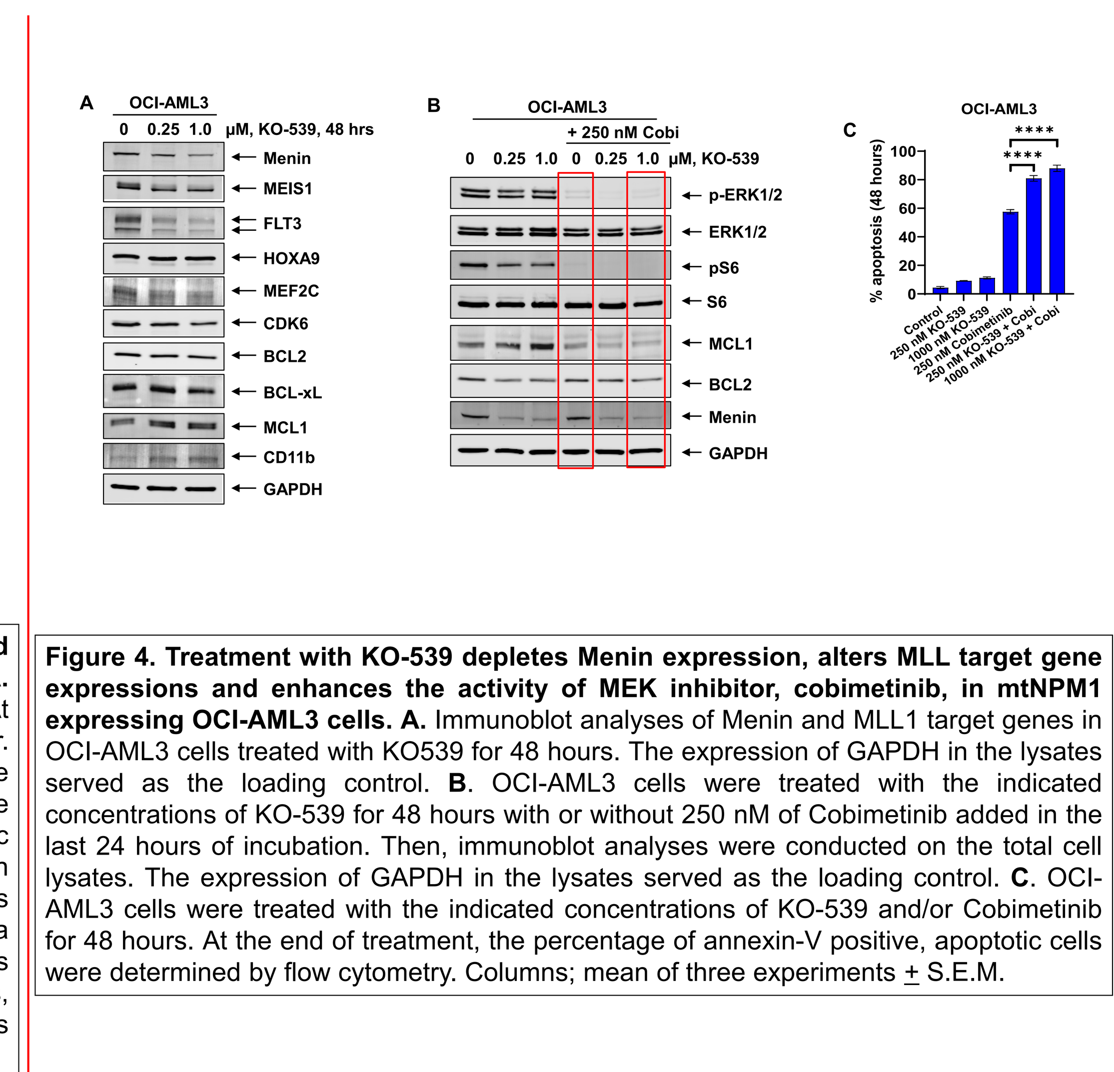
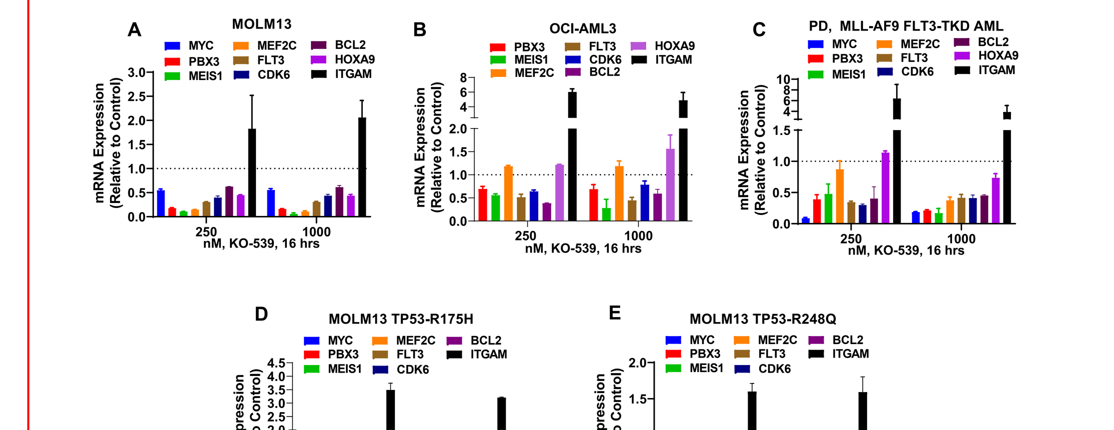


Figure 4. Treatment with KO-539 depletes Menin expression, alters MLL target gene expressions and enhances the activity of MEK inhibitor, cobimetinib, in mtNPM1 expressing OCI-AML3 cells. A. Immunoblot analyses of Menin and MLL1 target genes in OCI-AML3 cells treated with KO539 for 48 hours. The expression of GAPDH in the lysates served as the loading control. **B**. OCI-AML3 cells were treated with the indicated concentrations of KO-539 for 48 hours with or without 250 nM of Cobimetinib added in the last 24 hours of incubation. Then, immunoblot analyses were conducted on the total cell lysates. The expression of GAPDH in the lysates served as the loading control. **C**. OCI-AML3 cells were treated with the indicated concentrations of KO-539 and/or Cobimetinib for 48 hours. At the end of treatment, the percentage of annexin-V positive, apoptotic cells were determined by flow cytometry. Columns; mean of three experiments ± S.E.M.



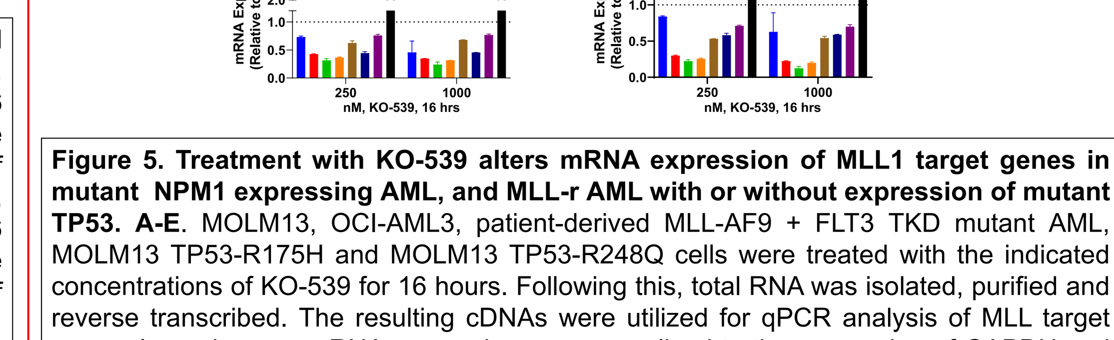
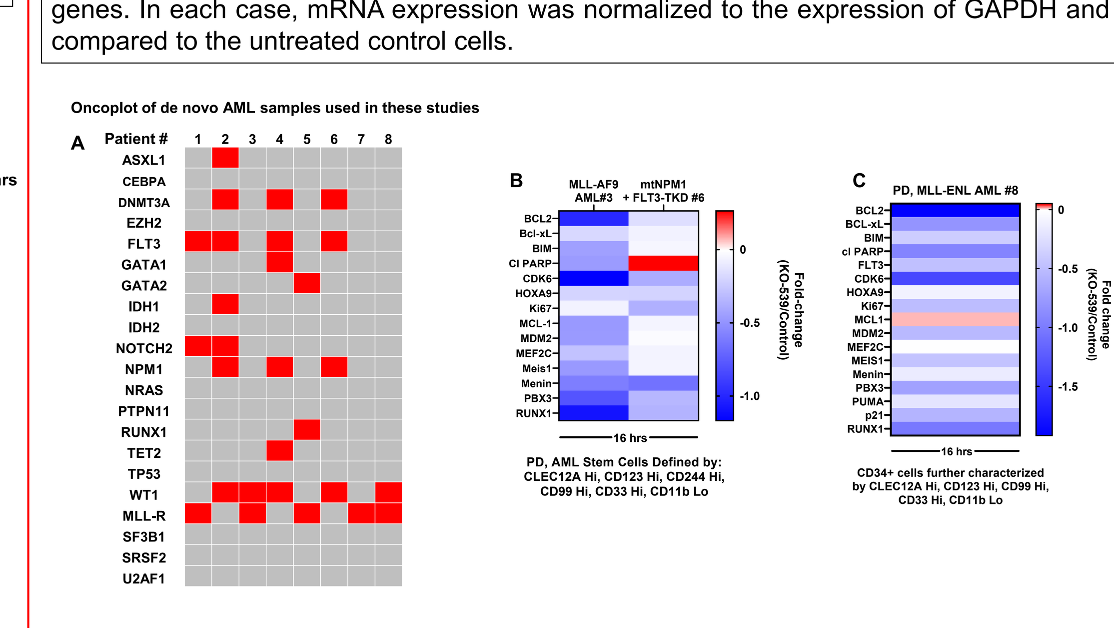


Figure 5. Treatment with KO-539 alters mRNA expression of MLL1 target genes in mutant NPM1 expressing AML, and MLL-r AML with or without expression of mutant TP53. A-E. MOLM13, OCI-AML3, patient-derived MLL-AF9 + FLT3 TKD mutant AML, MOLM13 TP53-R175H and MOLM13 TP53-R248Q cells were treated with the indicated concentrations of KO-539 for 16 hours. Following this, total RNA was isolated, purified and reverse transcribed. The resulting cDNAs were utilized for qPCR analysis of MLL target genes. In each case, mRNA expression was normalized to the expression of GAPDH and compared to the untreated control cells.



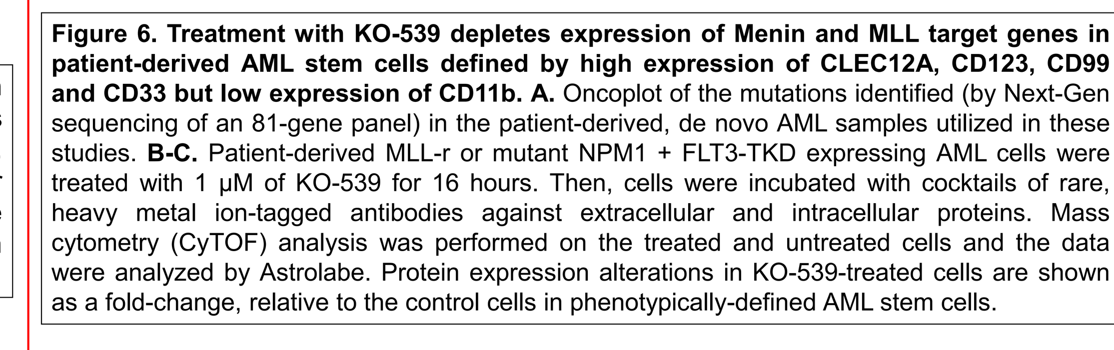
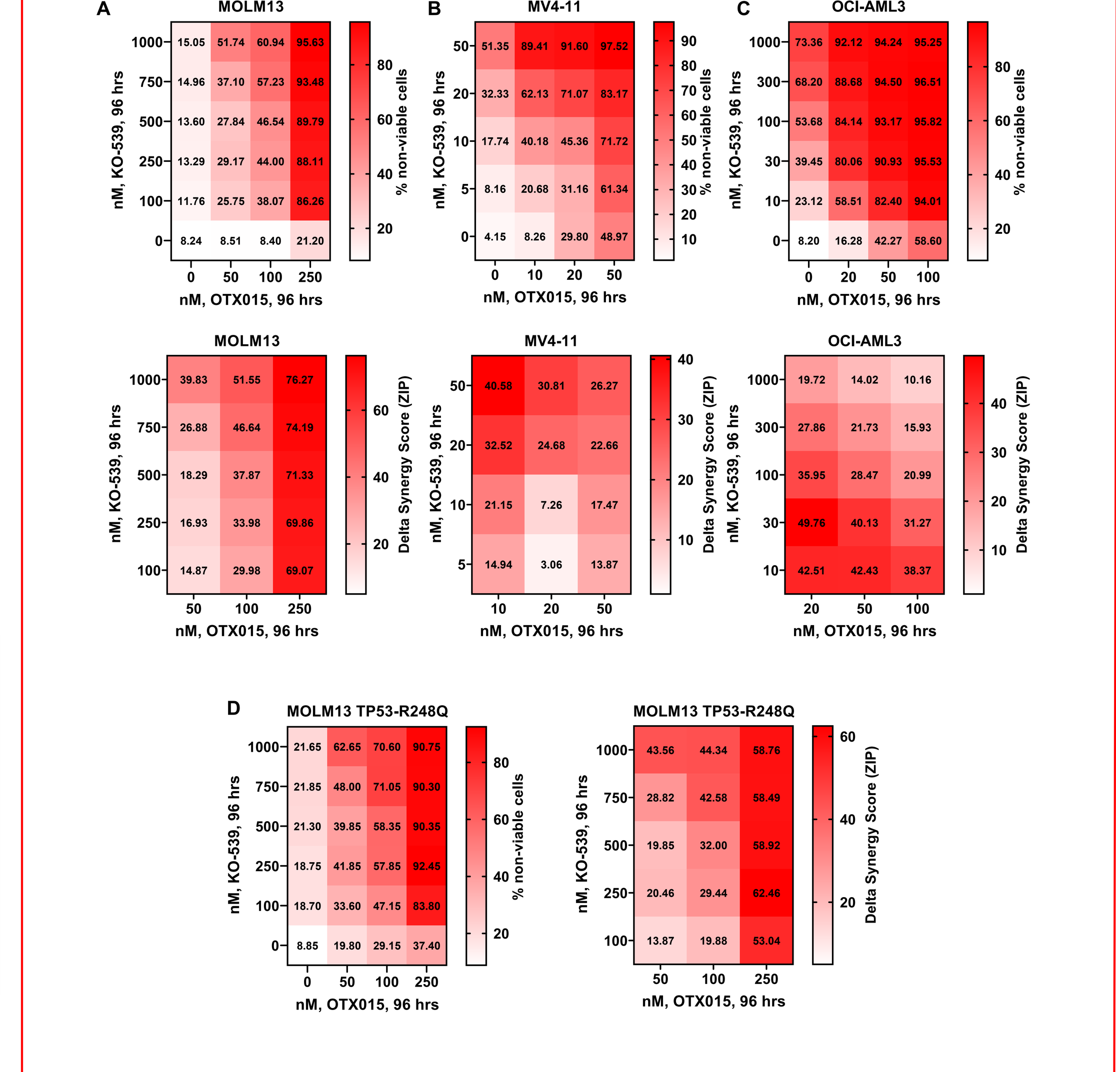


Figure 6. Treatment with KO-539 depletes expression of Menin and MLL target genes in patient-derived AML stem cells defined by high expression of CLEC12A, CD123, CD99 and CD33 but low expression of CD11b. A. Oncoplot of the mutations identified (by Next-Gen sequencing of an 81-gene panel) in the patient-derived, de novo AML samples utilized in these studies. **B-C.** Patient-derived MLL-r or mutant NPM1 + FLT3-TKD expressing AML cells were treated with 1 μM of KO-539 for 16 hours. Then, cells were incubated with cocktails of rare, heavy metal ion-tagged antibodies against extracellular and intracellular proteins. Mass cytometry (CyTOF) analysis was performed on the treated and untreated cells and the data were analyzed by Astrolabe. Protein expression alterations in KO-539-treated cells are shown as a fold-change, relative to the control cells in phenotypically-defined AML stem cells.



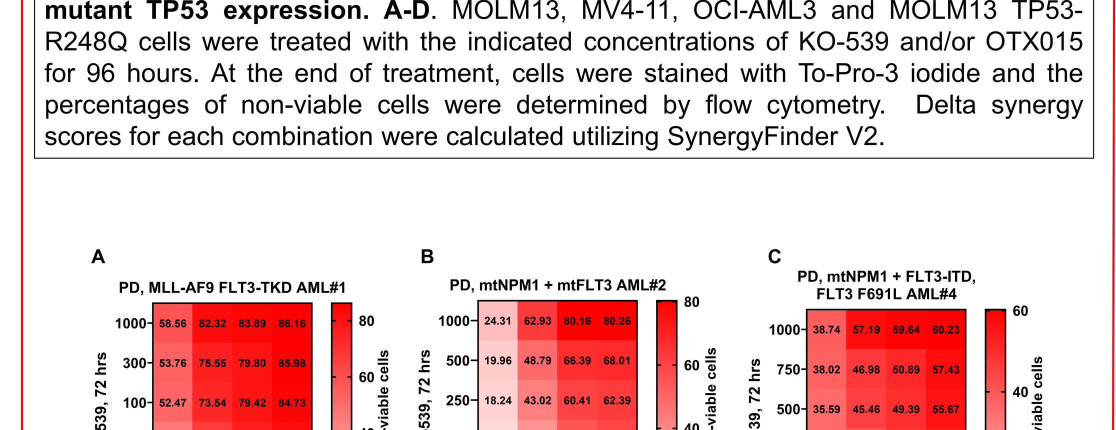
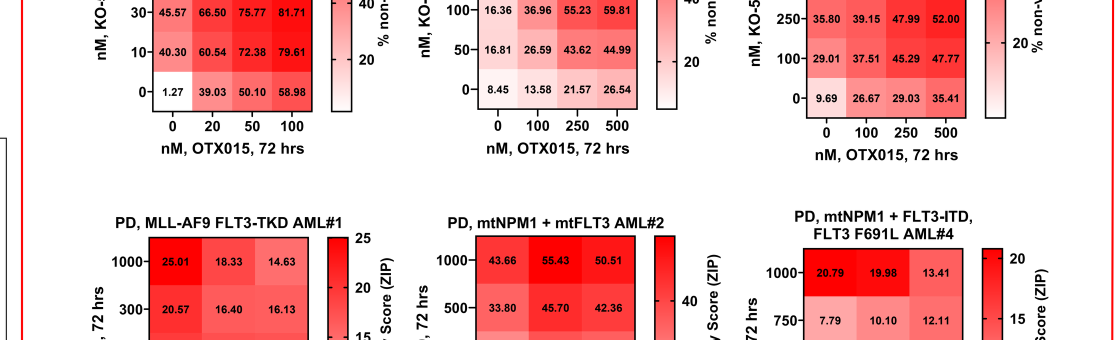


Figure 7. Co-treatment with KO-539 and BET inhibitor, OTX015, induces synergistic lethality in AML cells expressing mtNPM1 or MLL rearrangement with or without mutant TP53 expression. A-D. MOLM13, MV4-11, OCI-AML3 and MOLM13 TP53-R248Q cells were treated with the indicated concentrations of KO-539 and/or OTX015 for 96 hours. At the end of treatment, cells were stained with To-Pro-3 iodide and the percentages of non-viable cells were determined by flow cytometry. Delta synergy scores for each combination were calculated utilizing SynergyFinder V2.



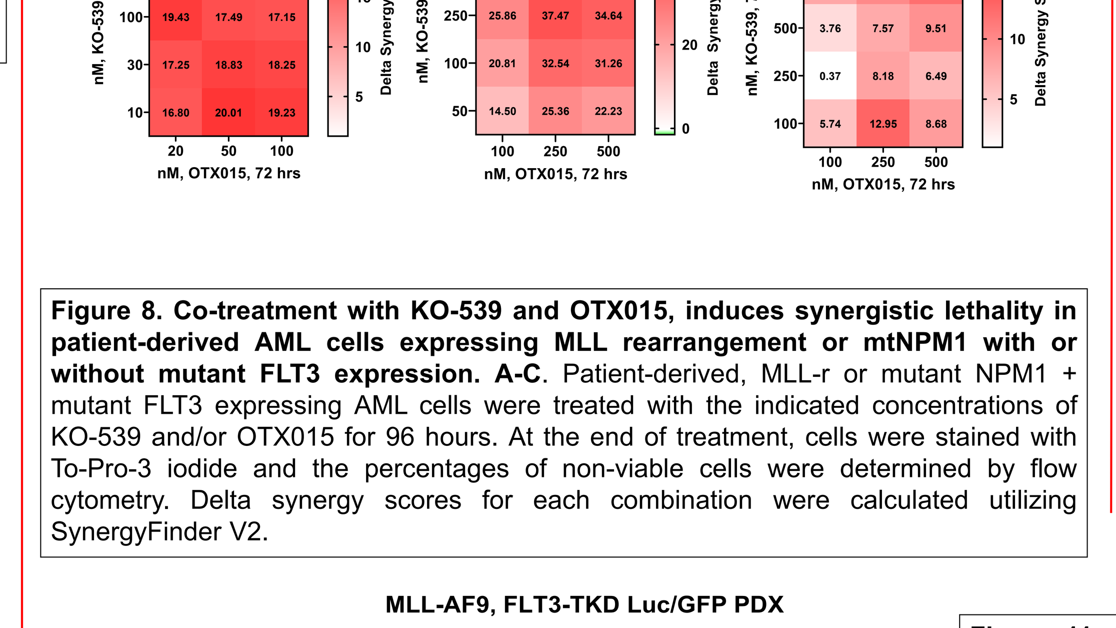


Figure 8. Co-treatment with KO-539 and OTX015, induces synergistic lethality in patient-derived AML cells expressing MLL rearrangement or mtNPM1 with or without mutant FLT3 expression. A-C. Patient-derived, MLL-r or mutant NPM1 + mutant FLT3 expressing AML cells were treated with the indicated concentrations of KO-539 and/or OTX015 for 96 hours. At the end of treatment, cells were stained with To-Pro-3 iodide and the percentages of non-viable cells were determined by flow cytometry. Delta synergy scores for each combination were calculated utilizing SynergyFinder V2.

Figure 9. Co-treatment with KO-539 and BCL2 inhibitor, venetoclax, induces synergistic lethality in AML cells expressing MLL rearrangement or mtNPM1. A-C. MOLM13, MV4-11, and OCI-AML3 cells were treated with the indicated concentrations of KO-539 and/or venetoclax for 96 hours. At the end of treatment, cells were stained with To-Pro-3 iodide and the percentages of non-viable cells were determined by flow cytometry. Delta synergy scores for each combination were calculated utilizing SynergyFinder V2. **D**. MOLM13 cells were treated with the indicated concentrations of KO-539 and/or abemaciclib for 96 hours. At the end of treatment, cells were stained with To-Pro-3 iodide and the percentage of non-viable cells was determined by flow cytometry. Delta synergy scores for each combination were calculated utilizing SynergyFinder V2.

Figure 10. Co-treatment with KO-539 and venetoclax induces synergistic lethality in patient-derived AML cells expressing MLL rearrangement or mtNPM1 with or without mutant FLT3 expression. A-C. Patient-derived MLL-r or mtNPM1 + mutant FLT3 expressing AML cells were treated with the indicated concentrations of KO-539 and/or venetoclax for 96 hours. At the end of treatment, cells were stained with To-Pro-3 iodide and the percentages of non-viable cells were determined by flow cytometry. Delta synergy scores for each combination were calculated utilizing SynergyFinder V2.

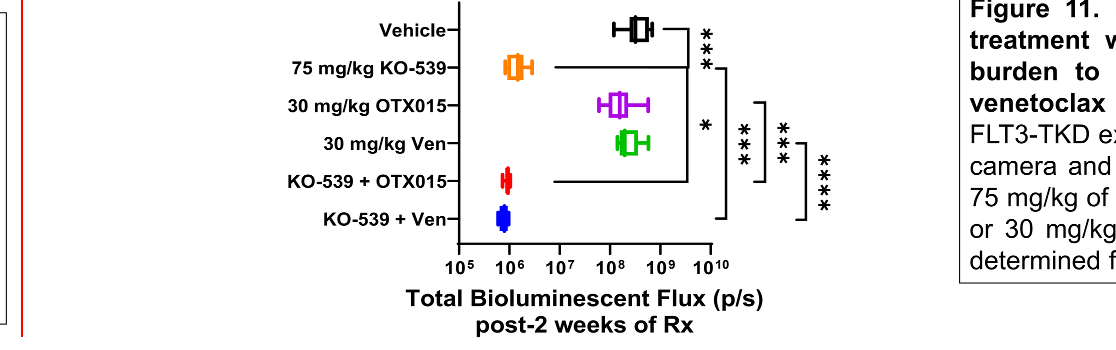


Figure 11. In NSG mice bearing an MLL-AF9 + FLT3-TKD-Luc/GFP AML PDX, co-treatment with KO-539 and OTX015 or KO-539 and venetoclax reduced leukemia burden to a significantly greater extent than treatment with KO-539, OTX015 or venetoclax alone. NSG mice were engrafted with luciferized, patient-derived MLL-AF9 + FLT3-TKD expressing AML cells and monitored for 5-7 days. Mice were imaged by Xenogen camera and randomized to equivalent bioluminescent flux. Mice were treated with vehicle, 75 mg/kg of KO-539 (P.O., daily x 5 days) and/or 30 mg/kg of OTX015 (P.O., daily x 5 days), or 30 mg/kg venetoclax (P.O., daily x 5 days) for 2 weeks. Total bioluminescent flux was determined for each cohort by Xenogen camera. * = p< 0.05, *** = p< 0.005, ****= p< 0.001.