Structural insights into inhibition of the bivalent menin-MLL interaction by small molecules in leukemia

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Abstract

Menin functions as a critical oncogenic co-factor of Mixed Lineage Leukemia (MLL) fusion proteins in development of acute leukemias, and inhibition of the menin interaction with MLL fusion proteins represents a very promising strategy to reverse their oncogenic activity. MLL interacts with menin in a bivalent mode involving two N-terminal fragments of MLL. Here, we report the first high resolution crystal structure of human menin in complex with a small molecule inhibitor of the menin-MLL interaction, MI-2. The structure reveals that the compound binds to the MLL pocket in menin and mimics the key interactions of MLL with menin. Based on the menin-MI-2 structure, we developed MI-2-2, a compound that binds to menin with low nanomolar affinity (K_d = 22 nM) and very effectively disrupts the bivalent protein-protein interaction between menin and MLL. MI-2-2 demonstrated specific and very pronounced activity in MLL leukemia cells, including inhibition of cell proliferation, downregulation of Hoxa9 expression and differentiation. Together, our results provide the rational and essential structural basis to design next generation of inhibitors for effective targeting the menin-MLL interaction in leukemia and demonstrate a proof of concept that inhibition of complex multivalent protein-protein interactions can be achieved by a small molecule inhibitor.
Introduction

Translocations of the *MLL* gene frequently occur in aggressive human acute myeloid and lymphoid leukemias, affecting both children and adults. Fusion of *MLL* with one of over 60 different genes results in chimeric *MLL* fusion proteins which enhance proliferation and block hematopoietic differentiation, ultimately leading to acute leukemia. Patients with leukemias harboring *MLL* translocations have very unfavorable prognoses and respond poorly to currently available treatments. The relapse risk is very high using conventional chemotherapy and stem cell transplantation, leading to an overall five-year survival rate only about 35%.

All *MLL* fusion proteins preserve approximately 1,400 amino acid long N-terminal fragment of *MLL* fused in frame with the C terminus of the fusion partner. Two regions in this fragment of *MLL* have been shown to be indispensable for leukemogenic transformation: the N-terminal region, which binds to menin and to lens epithelium–derived growth factor (LEDGF), and the conserved region encompassing CXXC domain, which mediates binding to nonmethylated CpG DNA and interacts with the polymerase associated factor complex (PAFc). Targeting these interactions provides new opportunities for development of new therapeutic agents for the *MLL* leukemias.

Menin is a tumor-suppressor protein encoded by the *MEN1* (*Multiple Endocrine Neoplasia I*) gene. Mutations of *MEN1* are associated with tumors of the parathyroid glands, pancreatic islet cells and anterior pituitary gland. Menin is also a highly specific binding partner for *MLL* and *MLL* fusion proteins, and is required to regulate expression of *MLL* target genes, including *HOXA9* and *MEIS1*. Loss of the ability to bind menin abolishes the oncogenic potential of *MLL* fusion proteins *in vitro* and *in vivo*. Disruption of the interaction between...
menin and MLL fusion proteins using genetic methods blocks development of acute leukemia in mice \cite{8}, indicating that menin functions as a critical oncogenic co-factor of MLL fusion proteins and is required for their leukemogenic activity. The menin-MLL interaction represents an attractive therapeutic target for development of novel drugs for acute leukemias with MLL rearrangements.

Development of small molecule inhibitors of protein-protein interactions (PPIs) represents a challenging task \cite{18}. Menin interacts with two fragments of MLL, the high affinity motif MBM1 (menin binding motif 1, $K_d = 53$ nM) and low affinity MBM2 (menin binding motif 2, $K_d = 1.4$ µM), located within the intrinsically unstructured 43-amino acid fragment at the N-terminus of MLL \cite{19}. MBM1 and MBM2 are separated by a 7 glycine linker, and most likely bind to adjacent sites on menin. Development of small molecules effectively targeting the menin-MLL interaction would require the disruption of this bivalent interaction. Importantly, a point mutation within MBM1 is sufficient to abolish oncogenic properties of MLL fusion proteins \textit{in vivo} \cite{8}. We have recently developed small molecules which directly bind to menin and inhibit the menin-MLL interaction \textit{in vitro} and in human cells \cite{20}.

In order to understand the molecular mechanism of the menin-MLL interaction and to facilitate development of novel drugs targeting this interaction we have determined the high resolution (1.46 Å) crystal structure of human menin, as well as menin in complex with the high affinity binding motif of MLL, MBM1. We have also obtained the first crystal structure of menin in complex with \textbf{MI-2}, our recently developed small molecule inhibitor of the menin-MLL interaction \cite{20} and found that this compound binds to the MLL site on menin and mimics the key interactions of MBM1. Based on the structure of the menin-\textbf{MI-2} complex we developed a very potent second-generation inhibitor, \textbf{MI-2-2}, which binds to menin with low nanomolar affinity.
and is capable to potently inhibit the MLL-fusion protein mediated leukemogenic transformation. Our work provides an important structural insight into menin’s function as a critical co-factor of oncogenic MLL fusion proteins in leukemogenesis. The structures of menin complexes with small molecule inhibitors MI-2 and MI-2-2 shed light on inhibitor binding to the MLL binding site on menin and provide the rational and molecular basis for the development of the next generation of menin-MLL inhibitors as novel therapies in acute leukemias with MLL translocation.

Methods

Cloning, protein engineering, expression and purification of human menin

The gene encoding human menin was subcloned into pET32a vector (Novagen) and generation of deletion constructs was performed by mutagenesis according to Quick Change site-directed mutagenesis protocol to introduce different internal deletions and a stop codon. Proteins were expressed and purified using the previously described protocol. For crystallization experiments proteins were purified using size exclusion column HiLoad 16/60 Superdex 75 pg resin (GE Healthcare), and 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM TCEP as mobile phase.

Crystallization of menin and menin complexes

For crystallization experiments, 2.5 mg/mL menin was incubated with MLL MBM1 peptide (MLL residues 4-15, ordered from Genscript) in a 1-1 molar ratio. Crystals were obtained using a sitting drop technique at 10°C in 0.2 M ammonium acetate, 0.1 M HEPES pH 7.5 and 25% w/v PEG 3,350. Prior to data collection, crystals were transferred into a cryo-solution containing 20%
PEG550 MME and flash-frozen in liquid nitrogen. A similar procedure was used for crystallization of the complexes with MI-2 and MI-2-2.

Crystallographic data collection and structure determination

Diffraction data for menin and menin complexes were collected at the 21-ID-D and 21-ID-F beamlines at the Life Sciences Collaborative Access Team at the Advanced Photon Source. Data were processed with HKL-2000\(^ {22}\). Structure of the free protein was determined employing HKL-3000\(^ {23}\) and MOLREP\(^ {24}\) using menin from *Nematostella vectensis* (PDB code: 3RE2) as a search model in molecular replacement. The model was rebuilt with BUCCANNER\(^ {25}\) and RESOLVE\(^ {26}\) and the refinement was carried out using HKL-3000, REFMAC\(^ {27}\), COOT\(^ {28}\) and the CCP4 package\(^ {29}\). In the final stages, refinement was performed with addition of the TLS groups defined by the TLMSD server\(^ {30}\) or using anisotropic B-factors in the case of the high resolution structures. Validation of the structures was performed using MOLPROBITY\(^ {31}\) and ADIT\(^ {32}\). Details of data processing and refinement are summarized in Supplementary Table 1. Coordinates and structure factors have been deposited in the Protein Data Bank under accession codes 4GPQ, 4GQ6, 4GQ3 and 4GQ4.

MLL binding experiments

Dissociation constants for binding of MBM1 (MLL residues 4-15), MBM2 (residues 23-40) and MLL4-43 (MLL residues 4-43) to full-length human menin were determined by fluorescence polarization method using a previously published protocol\(^ {19}\).
Biochemical characterization of menin-MLL inhibitors

Activities of small molecules to inhibit menin-MBM1 and menin-MLL4-43 interaction were assessed using fluorescence polarization assay using a previous protocol. Details of ITC experiments for measurement of dissociation constants for menin inhibitors were described previously.

Co-immunoprecipitation experiments

HEK293 cells were transfected with 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 DMSO for 12h. Whole cell lysates were immunoprecipitated with ANTI-FLAG M-2 Magnetic beads (Sigma-Aldrich, St. Louis, MO) and were analysed by SDS-PAGE electrophoresis and Western blotting. More details were provided previously.

Viability assays

MLL-AF9 transduced mouse bone marrow cells were prepared as described previously. MV4;11, KOPN-8, ML-2, MOLM-13 cells were cultured in RPMI-1640 medium with 10% FBS, 1% penicillin/streptomycin and NEAA. MTT viability assay was carried out using recently published protocol. For growth curves, 3 x 10^5/ml 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 3 x 10^5 cells/ml and compound re-supplied. At designated time point, cell culture samples were mixed with trypan blue (GIBCO, Invitrogen, Carlsbad, CA) and counted.
Colony formation assay

The MLL-AF9 transduced murine BMC were plated in 12-well plates at the concentration of $5 \times 10^3$ cells/ml with 1 ml methylcellulose medium M3234 (StemCell Technologies) containing 20% IMDM medium, 1% penicillin/streptomycin, IL-3 and 0.25% DMSO or compounds. 6 days later colonies were stained with 100 μl iodonitrotetrazolium chloride (Sigma-Aldrich) at final concentration of 1mg/ml, incubated at 37°C for 30 min and counted.

Real-Time PCR

Total RNA was extracted from cells using RNeasy mini kit (Qiagen). 100 ng – 1000 ng of total RNA was reverse transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer’s protocol. Real-time PCR was performed using the ABI Prism 7700 sequence detection system. Taqman Gene Expression Master Mix and Taqman Gene Expression Assays for mouse Hoxa9 (Mm00439364_m1), Meis1 (Mm00487664_m1), β-Actin (4352933), human HOXA9 (Hs00365956_m1), MEIS1 (Hs00180020_m1) and 18S RNA (Hs99999901_s1) were purchased from Applied Biosystems. Relative quantification of each gene transcript was carried out using the comparative C_t method as described in the Applied Biosystems User Bullletin No. 2.

Annexin V/PI assay of inhibitor effects on apoptosis

$5 \times 10^5$ cells/ml 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 48h at 37 °C in a 5% CO₂ incubator. After incubation, $1.5 \times 10^5$ cells were harvested and resuspended in 100 μl 1× Annexin V binding buffer from the Annexin V-FITC Apoptosis kit (BD Biosciences...
Pharmingen), incubated with 4 μl of AnnexinV-FITC and 6 μl of Propidium iodide (Sigma-Aldrich) at room temperature in the dark for 10 minutes and analyzed by flow cytometry on a LSR II instrument. Data analysis was performed using WinList software.

Expression of CD11b

MV4;11 cells or MLL-AF9 transduced bone marrow cells were plated in 12-well plates at an initial concentration of 5x10^5 cells/ml and treated with compounds or 0.25% DMSO. Media were changed every 48h with viable cell concentration restored to 5x10^5 cells/ml and compounds resupplied. Six days after the experiment was set-up, the 1.5x10^5 cells were harvested and washed with FACS buffer (PBS, 1% FBS, 0.1% NaN₃). Cells were resuspended in 100 μl FACS buffer and incubated with 2 μl Pacific Blue mouse anti-human CD11b antibody (BD Biosciences) or 1 μl Pacific Blue rat anti-mouse CD11b antibody (BioLegend) at 4°C for 30 min. Cells were then washed, resuspended in 100 μl Annexin V binding buffer, and incubated with 4 μl Annexin V-FITC (BD Biosciences) and 6 μl Propidium iodide (1mg/ml, Sigma-Aldrich) at room temperature for 10 min before being analyzed by flow cytometry.

Cytospin/Wrigth-Giemsa staining

MV4;11 and mouse bone marrow cells transduced with MLL-AF9 were plated in 12-well plates (1ml/well) at an initial concentration of 5x10^5/ml cells, treated with compounds (0.25% final DMSO concentration) or 0.25% DMSO control and incubated at 37 °C in a 5% CO₂ incubator. At designated time point, 1x10^5 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).

Inhibitor effects on cell cycle of MV4;11 leukemia cells

5x10^5/ml MV4;11 cells were plated in 12-well plates (1ml/well) and treated with MI-2-2 compound (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% CO2 incubator. After incubation, 5x10^5 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 propidium iodide at 37°C for 30 minutes before being processed by flow cytometry.

Chemistry

Synthesis and characterization of the second generation of menin-MLL inhibitors (MI-2-2 and analogs) is provided in Supplementary Material.

Results

Crystal structure of human menin and menin-MLL complex

Our initial attempts to crystallize full-length human menin failed, most likely due to the presence of several internal fragments predicted to be unstructured. Based on the structure of menin homolog from Nematostella vectensis 21 we deleted three internal fragments and C-terminus in human menin, which we predicted to correspond to loops and unstructured regions.
(Figure S1A). This resulted in a construct that yielded protein crystals diffracting to a 1.46 Å resolution (Table S1). Importantly, the engineered protein retains the ability to bind MLL with a similar affinity as the wild type menin, indicating that deletion of these fragments does not alter the MLL binding site (Figure S1B). Human menin is predominantly an α-helical protein (Figure S1C), and closely resembles the structure of Nematostella menin that we recently reported 21 with only minor structural differences, localized to the peripheral fragments and loop regions. Very recently, the structure of a longer human menin construct has been described 33. Interestingly, deletion of the loops resulted in a very significant improvement in the resolution of the structure.

MLL associates with menin in a bivalent mode using two short motifs, MBM1 and MBM2, separated by a polyglycine linker (Figure 1A) 19. We previously reported that MBM1 and MBM2 peptides bind to menin with, respectively, 50nM and 1.4µM affinities. The longer fragment containing both motifs interacts with menin with 10nM affinity 19. To provide an insight into the recognition of MLL by menin we have determined the structure of menin in complex with the high affinity motif, MBM1 (Table S1). The structure revealed that MBM1 binds to the large central cavity on menin. The peptide is well ordered and its backbone adopts a U-shaped conformation, with a single β-turn comprising residues 9 to 12 (Figures 1B, S1C). The β-turn is stabilized by an intramolecular hydrogen bond between the carbonyl of Phe9\textsuperscript{MLL} and the backbone amide of Arg12\textsuperscript{MLL} (Figure 1C). The binding of MBM1 to menin is maintained by several hydrophobic contacts involving the side chains of Phe9\textsuperscript{MLL}, Pro10\textsuperscript{MLL}, Pro13\textsuperscript{MLL} (Figure 1B,C), which we demonstrated to play the most important role for MBM1 binding to menin 19. Phe9\textsuperscript{MLL} is entirely buried in the complex, and fits between the protein backbone (residues 179-181) and the side chain of Phe238 (Figure 1C). Pro10\textsuperscript{MLL} binds to the
site adjacent to Phe9\textsuperscript{MLL}, and interacts with Phe238 and Ala242, while Pro13\textsuperscript{MLL} fits into a hydrophobic pocket formed by Tyr319, Tyr323 and Met322. Mutations of Phe9\textsuperscript{MLL}, Pro10\textsuperscript{MLL} and Pro13\textsuperscript{MLL} to alanine residues result in a dramatic decrease of MBM1 binding to menin (2000-, 30- and 50-fold decrease, respectively)\textsuperscript{19}, validating the importance of these hydrophobic interactions. Additional stabilization of the MBM1-menin complex results from a salt bridge between Arg12\textsuperscript{MLL} and Glu359 and Glu363, as well as three intermolecular hydrogen bonds involving MLL backbone (residues Arg6\textsuperscript{MLL}, Trp7\textsuperscript{MLL} and Ala11\textsuperscript{MLL}) and menin side chains (Asn244, Asp136 and Tyr323) (\textbf{Figure 1B,C}). Interestingly, mutation of Arg12\textsuperscript{MLL} to alanine has a relatively modest effect on MBM1 binding (4-fold decrease)\textsuperscript{19}. Remarkably, binding of the 12 amino acid fragment of MLL does not cause any significant changes within the menin structure. Very recently, the lower resolution crystal structure of the menin-MLL complex has been reported showing a similar interaction mode\textsuperscript{33}.

We were not able to determine the crystal structure of menin with the MBM2 fragment of MLL, presumably due to the crystal contacts interfering with the binding. MBM1 and MBM2 are separated by a linker comprising seven glycine residues (\textbf{Figure 1A}), and therefore it is possible that MBM2 occupies the space in a close proximity to MBM1. The MBM2 is positively charged and using site directed mutagenesis in menin we probed whether it binds to the negatively charged site in a close proximity to the MBM1 binding site (\textbf{Figure 1D}). Introduction of two point mutations in menin, D252K and L289K, which we assumed would cause electrostatic repulsion with the MBM2 (\textbf{Figure 1D}), interferes with binding of MBM2 without affecting protein stability or the interaction with MBM1 (\textbf{Figure S2}). This indicates that the low affinity motif, MBM2, highly likely binds in the proximity of the MBM1 binding site and provides an additional anchor enhancing the interaction of MLL with menin.
**Thienopyrimidine inhibitors mimic the binding mode of MBM1**

We have recently developed small molecules with thienopyrimidine scaffold that bind to menin with sub-micromolar affinities and disrupt the menin-MLL interaction. One of these compounds, MI-2 (IC$_{50}$ = 446 nM), showed pronounced effects in MLL leukemia cells, representing a very promising lead compound for development of new anti-leukemic agents. However, lack of structural data for the MI-2 interaction with menin impeded development of more potent inhibitors of the menin-MLL interaction. To overcome this limitation, we determined the 1.56 Å resolution structure of MI-2 bound to menin (Figures 2A,B, S11, Table S1), which represents the first crystal structure of menin in complex with a small molecule inhibitor. The electron density for MI-2 inhibitor is very well defined (Figure 2B), enabling detailed analysis of its interactions with menin.

We found that MI-2 binds to the same central cavity on menin that is occupied by the MBM1 fragment of MLL. The structure of MI-2 contains an n-propyl substituted thienopyrimidine ring connected by piperazine linker to a dimethyl-thiazoline ring (Figures 2A,C). The interaction of MI-2 with menin is predominantly stabilized via hydrophobic interactions and by two hydrogen bonds between N1 and N3 nitrogen atoms of the thienopyrimidine ring and the side chains of Tyr276 and Asn282, respectively (Figures 2A,C). The n-propyl-thienopyrimidine fragment fits into a pocket formed by the main residues Ser178 to His181 and the side chains of mostly hydrophobic residues (Phe238, Leu177, Ala182, Tyr276, Met278, Cys241 and Ser155), while piperazine ring serves mostly as a linker and approaches the side chains of Met278 and Tyr319. The dimethyl-thiazoline moiety of MI-2 fits into a site
formed by two orthogonally oriented tyrosine side chains (Tyr319 and Tyr323) and the side chain of Met322 (Figure 2A).

Strikingly, MI-2 mimics the binding mode and the key interactions formed by the MBM1 with menin (Figure 2D). The n-propyl group of MI-2 fits into the pocket occupied by Phe$^{9\text{MLL}}$ while the dimethyl thiazoline ring binds to the same pocket as Pro$^{13\text{MLL}}$. The piperazine ring in MI-2 also mimics a part of the MBM1 backbone by overlapping with the main chain atoms of Arg$^{12\text{MLL}}$ (Figure 2D). Overall, MI-2 is a relatively rigid molecule, and its high binding affinity to menin is likely due to the strong shape complementarity of this compound to the binding site on menin. Despite its relatively small size ($M_w=375$ Da), MI-2 binds to menin with only three fold lower affinity than the 12-amino acid MBM1 fragment of MLL and mimics the key interactions of MBM1 with menin. This strongly emphasizes that MI-2 constitutes a very promising candidate for further development into a more potent inhibitor of the menin-MLL interaction.

**Structure-based design of a nanomolar inhibitor of the menin-MLL interaction**

We have exploited the structure of the menin-MI-2 complex to design new analogs with improved binding affinities. Inspection of the structure revealed that the n-propyl group of MI-2 does not represent an optimal substituent for interacting with the binding pocket on menin. The structure also rationalizes why substitution of the n-propyl by bulky hydrophobic groups did not improve binding affinity$^{20}$. To develop more potent compounds, we employed structural information and designed several modifications of the R1 substituent on the thienopyrimidine ring (Figure 3A). First, we introduced oxygen, which could form a hydrogen bond with the side chain hydroxyl of Ser155. However, addition of the methoxy-propyl group at R1 (MI-2-1)
resulted in three-fold decreased activity (Figure 3A). As an alternative strategy, we synthesized several analogs by addition of fluorine atoms to R1 to achieve optimal shape complementarity with the binding site on menin (Figure 3A). Indeed, the replacement of n-propyl by trifluoroethyl group (MI-2-2) substantially improved the activity (Figure 3A,B). The MI-2-2, which has been designed based on the structure of the menin-MI-2 complex, represents a second generation of menin inhibitors, and binds to menin with \( K_d = 22 \text{nM} \) as assessed by ITC (Isothermal Titration Calorimetry) (Figure 3C). The MI-2-2 inhibits the menin-MBM1 interaction with IC\(_{50} = 46 \text{nM} \) (Figure 3B), representing approximately 7 to 9 fold improvement as compared to MI-2 (IC\(_{50} = 446 \text{nM} \), \( K_d = 158 \text{nM} \)). Remarkably, MI-2-2 binds to menin with more than two-fold higher affinity than the MBM1 fragment of MLL (Figure 3B).

In order to understand the molecular basis of a significant effect caused by introduction of the trifluoroethyl group we have determined the high resolution structure (1.27 Å, Table S1) of MI-2-2-menin complex (Figure 3D, S11). We found that binding mode of MI-2-2 to menin is the same as observed for MI-2. Interestingly, one of the fluorine atoms resides in a very close proximity (3.0 to 3.1 Å) to backbone atoms of His181 (Figure 3E). Such an orthogonal orientation of the fluorine relative to protein backbone results in a favorable C-F\( \cdots \)C=O dipolar interaction that has previously been identified to significantly enhance protein-ligand interactions \(^{34}\). Introducing fluorine atoms has become common in medicinal chemistry to enhance the binding affinity of protein ligands as well as to improve their drug-like properties \(^{35}\). Our high resolution crystal structure of MI-2-2 bound to menin allows for detailed analysis of the protein interactions with fluorine and it may represent a valuable model system to better understand such interactions and improve the design of potent small molecule inhibitors of other protein-protein interactions.
Small molecules are capable to inhibit bivalent menin-MLL interaction

The intrinsically unstructured N-terminus of MLL interacts with menin via a complex mechanism, with two short MLL fragments (MBM1 and MBM2) involved in binding \(^{19}\). Deletion of a high affinity motif MBM1 is sufficient to abolish transformation by MLL-ENL \(^{8}\), which rationalizes that this interaction constitutes a hotspot for small molecule development. Because MBM2 also contributes to the binding to menin it was necessary to establish whether small molecules targeting the MBM1 site on menin are sufficient to efficiently inhibit the bivalent interaction of menin with MLL. We found that both, **MI-2** and **MI-2-2** can inhibit the interaction of menin with MLL\(_{4-43}\), which comprises the intact menin binding fragment \(^{19,36}\). As expected, the second generation inhibitor, **MI-2-2**, is about 7-fold more potent in disrupting the menin-MLL\(_{4-43}\) interaction, with \(IC_{50}=520\text{nM}\) (**Figure 4A**). To assess whether these inhibitors can dissociate menin interaction with the full length MLL-AF9 fusion protein in cells we performed the co-immunoprecipitation (co-IP) experiments. We found that both **MI-2** and **MI-2-2** inhibit the interaction of menin with MLL-AF9 in HEK293 cells in a dose dependent manner at low micromolar concentrations (**Figure 4B**), with **MI-2-2** representing approximately 4-fold improvement over **MI-2** (**Figure 4B**). These experiments provide an important evidence that small molecules that bind to menin with high affinity at the MBM1 site are capable of fully dissociating the entire menin-MLL fusion protein complex (**Figure 4C**).

**MI-2-2 has significantly improved cellular activity compared to MI-2**

Our second generation compound, **MI-2-2**, demonstrates substantially improved inhibition of the menin-MLL interaction over **MI-2**, and therefore we compared the effects of these two...
compounds in mouse bone marrow cells (BMC) transformed with MLL-AF9. Both compounds caused very significant growth inhibition at low micromolar doses (Figure 5A). Interestingly, treatment longer than 6 days revealed that only the more potent compound, MI-2-2, stably suppressed growth of MLL-AF9 transformed BMC (Figure 5A). We have also assessed the capability of MI-2 and MI-2-2 to downregulate the expression of MLL fusion protein target genes. While treatment with 6 µM MI-2 had a small effect, the same dose of MI-2-2 caused greater than 80 % downregulation of Hoxa9 and Meis1 expression (Figure 5B,C). In colony formation assay, treatment with MI-2-2 resulted in a more pronounced effect on colony number when compared to MI-2, with essentially no colonies formed at 25µM of MI-2-2 (Figures 5D,E). The colonies were also smaller and much more diffused upon treatment with MI-2-2 reflecting loss of transforming properties by MLL-AF9 (Figures 5E, S3). Furthermore, treatment with MI-2-2 resulted in a more pronounced hematopoietic differentiation than observed for MI-2, as reflected by a substantial increase in the level of CD11b, a differentiation marker of myeloid cells (Figure 5F), and very pronounced change in cell morphology. Treatment for 7 days with 12µM of MI-2-2 was sufficient to cause terminal monocytic differentiation of MLL-AF9 transformed BMCs, while MI-2 did not cause such a pronounced effect (Figures 5G, S4). In summary, the structure-based designed inhibitor MI-2-2 represents a significant improvement over MI-2 as reflected by its substantially more potent cellular activity in MLL-AF9 transduced BMCs.

**MI-2-2 exhibits potent activities in human MLL leukemia cells**

We have also tested the activity of MI-2-2 in the MV4;11 human MLL leukemia cell line, which harbors MLL-AF4 translocation. Similar to the effects observed in MLL-AF9 transduced BMC,
treatment of MV4;11 cells with **MI-2-2** caused growth inhibition of these cells (GI$_{50}$=3μM) (Figure 6A), while weak or no activity was shown in non-MLL leukemia cells (Figure S5). The MI-2-2 compound also resulted in a substantial and dose-dependent increase in the number of cells undergoing apoptosis (Figure 6B) and in G0/G1 cell cycle arrest (Figures 6C,D). The MI-2-2 also exhibited strong downregulation of *HOXA9* and *MEIS1* expression in MV4;11 cells (Figure 6E) and induced differentiation in these cells as manifested by increase in the expression of CD11b and formation of multi-lobed nuclei and highly vacuolated cytoplasm (Figures 6F,G, S6). Remarkably, the effect of MI-2-2 on growth inhibition (Figure 6H) and differentiation of MV4;11 cells was significantly more pronounced as compared to the first generation compound MI-2 20. We have also observed very similar activities of MI-2-2 on growth inhibition and differentiation in three other cell lines harboring various MLL translocations: ML-2, MOLM-13 and KOPN8 (Supplementary Figures S8, S9, S10). Overall, these results demonstrate that the second-generation inhibitor MI-2-2 exhibits very pronounced effects in human leukemia cells carrying MLL translocation, which correlates well with the improved inhibition of the menin-MLL interaction.

**Discussion**

Development of low-molecular weight compounds targeting protein-protein interactions (PPIs) is generally considered a challenging task 18. Recently, we have provided a proof of principle that targeting the PPI between menin and MLL by small molecules is feasible 20. In this work, we carried out structural studies to characterize human menin and its interaction with MLL, which provides essential molecular basis to understand the function of menin as an oncogenic co-factor of MLL fusion proteins in acute leukemias. Importantly, we have also
determined the crystal structure of menin in complex with a small molecule inhibitor MI-2, and found that this compound binds to menin in a mode that mimics the key interactions of MBM1 with menin. The structure of the menin-MI-2 complex was crucial to develop a second generation inhibitor, MI-2-2, with 8-fold improved binding affinity towards menin. The MI-2-2 compound binds to menin with $K_d=22\text{nM}$ and is capable of inhibiting both the interaction of menin with MBM1 ($IC_{50}=46\text{nM}$) and with the bivalent fragment of MLL that comprises both MBM1 and MBM2 ($IC_{50}=520 \text{nM}$). The MI-2-2 exhibits very pronounced activities at low micromolar concentrations in BMC transformed with MLL-AF9 and in MV4;11 human leukemia cell line harboring MLL-AF4 translocation. This also indicates that this compound has high cellular permeability and might be used as a valuable chemical probe to study function of the menin-MLL and menin-MLL fusion protein interactions.

The difficulty in developing potent small molecule inhibitors of the menin-MLL arises from the relatively high binding affinity ($K_d = 10 \text{nM}$) due to the bivalent interaction mode, where two MLL fragments (MBM1 and MBM2) are involved in binding to menin $^{19}$. Our previous studies demonstrated that the entire N-terminal fragment of MLL binds to menin with approximately 5-fold stronger affinity than MBM1 alone, due to the presence of a second low affinity binding motif $^{19}$. Mutational analysis indicates that deletion of MBM1 is sufficient to abolish development of leukemia in vivo $^{8}$, and therefore the menin-MBM1 interaction constitutes a primary target for inhibitor development. The small molecule compound we developed, MI-2-2, binds to menin with the affinity which is comparable with the 40-amino acid long MLL fragment encompassing both MBM1 and MBM2 $^{19}$. More importantly, MI-2-2 can efficiently disrupt not only the binding of MBM1 but also the entire MLL fusion protein to menin (Figure 4).
Multisite binding modes have also been described for the interactions of other intrinsically unstructured proteins \(^{37}\). Therefore, development of small molecules that would efficiently target such interactions represents an additional difficulty for PPI inhibitors. Our work provides an important proof of concept that the inhibition of a complex with multivalent interactions can be achieved by a low molecular weight compound, providing that the ligand binds with high affinity to the PPI hotspot.

Structural analysis of protein-ligand complexes revealed that inhibitors of PPIs tend to be large molecules forming extensive hydrophobic contacts with relatively few hydrogen bonds \(^ {38}\). The MI-2-2 has relatively low molecular weight (415 Da) and lipophilicity (cLogP=3.9), and is fully compliant with Lipinski’s rule of five for orally bioavailable drugs \(^ {39}\). Furthermore, MI-2-2 has a very favorable ligand efficiency index (LE=0.39) \(^ {40}\), which is much better than the average value of 0.24 reported for PPI inhibitors \(^ {38}\). In summary, MI-2-2 has several advantageous qualities as a protein-protein inhibitor, including nanomolar affinity and favorable drug-like properties. The availability of the high resolution (1.27 Å) crystal structure of the MI-2-2 in complex with menin provides an excellent foundation to develop novel drugs for effective targeting of menin-MLL interaction in acute leukemias with MLL rearrangements.
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Authorship

A.S. synthesized compounds, M.J.M., T.H. and G.R. crystallized menin and performed biochemical experiments; M.C. and G.L. determined the crystal structures, S.H. and T.P. performed cellular assays, and J.G. and T.C. planned the experiments and wrote the manuscript with an input from all authors.

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References


**Figure legends**

**Figure 1. Structure of menin-MLL complex.** (A) Sequence of the N-terminal fragment of MLL with MBM1 and MBM2 motifs. (B) Details of the menin-MBM1 interaction. Structure of the MBM1 is shown in stick representation (green carbon atoms) and MLL residues are labeled in blue. Menin is presented as a gray ribbon and selected side chains involved in contacts with MBM1 are shown as sticks (cyan carbon atoms); hydrogen bonds are shown as dashed lines. (C) Most significant contacts between MBM1 (green carbons) and selected menin side chains (cyan carbons). (D) Probing the MBM2 binding site on menin. MBM1 (shown in sticks) occupies negatively charged central cavity on menin. The positions of D252 and L289 that were mutated to lysines are labeled. The electrostatic potential was calculated using APBS and mapped onto menin structure 41.
Figure 2. Crystal structure of menin-MI-2 complex. (A). Details of MI-2 interaction with menin. Selected menin side chains are shown in sticks (cyan carbons) and hydrogen bonds are shown as dashed lines (B) Menin-MI-2 complex determined at 1.56 Å resolution with corresponding 2Fo-Fc electron density map contoured at 1σ level. (C) Diagram depicting VdW contacts and hydrogen bonds (dashed lines) between MI-2 and menin. (D) Superposition of MI-2 (green carbons) with fragment of MBM1 motif (MLL residues 9-13, gray carbons) in menin bound conformation.

Figure 3. Development of second-generation menin-MLL inhibitors. (A) Structures and activities of new compounds designed based on the structure of the menin-MI-2 complex. IC50 values for the inhibition of the menin-MBM1 complex are provided in parenthesis. (B) FP experiments comparing activities of MI-2, MI-2-2 and MBM1 for disruption of the menin-MBM1 interaction demonstrating that MI-2-2 is a more potent inhibitor than MLL derived peptide. (C) ITC showing the binding of MI-2-2 to menin. N represents a stoichiometry of binding. (D) Crystal structure of the menin-MI-2-2 complex determined at 1.27 Å resolution with corresponding 2Fo-Fc electron density map contoured at 1σ level. Water molecules were omitted for clarity. (E) Orthogonal dipolar interactions between MI-2-2 fluorine and backbone atoms of His181. The distances are shown in Å.

Figure 4. Small molecules targeting the MBM1 site efficiently disrupt bivalent menin-MLL interaction. (A) Fluorescence polarization experiments demonstrating displacement of MLL4-43 from menin by MI-2 and MI-2-2. Data represent mean values from two experiments ± s.d. (B) Co-IP experiment in HEK293 cells transfected with Flag-MLL-AF9, demonstrating that MI-2
and MI-2-2 disrupt the interaction of menin with MLL-AF9 in human cells. Input shows the levels of menin and MLL. Amount of menin bound to Flag-MLL-AF9 was detected by co-immunoprecipitation using anti-Flag antibody followed by immunoblotting using menin antibody. (C) Model of the disruption of bivalent MLL-menin interaction by MI-2-2 via binding to MBM1 site in menin.

Figure 5. Second generation inhibitor MI-2-2 exhibits strongly enhanced cellular activities compared to MI-2. (A) Growth curves for MLL-AF9 transduced BMC grown in liquid culture treated with DMSO, MI-2 and MI-2-2. Experiment was performed two times. (B,C) Quantitative real-time PCR showing the expression of Hoxa9 (B) and Meis1 (C) in MLL-AF9 transduced BMC upon 6 days of treatment with MI-2 and MI-2-2. Expression of Hoxa9 and Meis1 has been normalized to β-actin and is referenced to the DMSO treated cells. Data represents the mean values for duplicates ± s.d. Experiment was performed three times. (D) Colony counts for methylcellulose colony assay performed with MLL-AF9 transduced BMC treated for 7 days with MI-2-2 and MI-2-2. Error bars indicate SD from duplicate experiments; experiments were performed two times. (E) Representative colonies shown for DMSO, MI-2, and MI-2-2 treated MLL-AF9 transduced BMC plated on methylcellulose. Black lines represent the scale bars (500 μm). (F) Quantification of CD11b expression in MLL-AF9 transduced BMC treated for 7 days with the menin-MLL inhibitors as detected by flow cytometry. Data represents the mean values for duplicates ± s.d. Experiment was performed two times. (G) Wright-Giemsa stained cytospins for MLL-AF9 transformed BMC after 7 days of treatment with DMSO, MI-2 (12 μM) and MI-2-2 (12 μM). Black lines represent the scale bars (50 μm). Statistical analysis and calculation of p-values was performed using Two-way ANOVA method.
Figure 6. MI-2-2 exhibits pronounced activity in MV4;11 human leukemia cells with MLL-AF4 translocation. (A) Inhibition of cell proliferation in MV4;11 cells induced by MI-2-2 after 72h treatment, as detected by the MTT cell viability assay. Data represent mean values for four samples ± s.d. Experiment was performed three times. (B) Apoptosis and cell death induced by MI-2-2 in MV4;11 cells as detected by flow cytometry using AnnexinV/propidium iodide (PI) staining. Data represent mean values for duplicates ± s.d. (C). Histograms from cell cycle analysis performed by FACS after PI staining in MV4;11 cells treated with DMSO and MI-2-2. (D) Dose dependent effect of MI-2-2 on cell cycle progression measured by FACS in MV4;11 cells after PI staining. Data represents the mean values for four experiments ± s.d. (E) Expression of the HOXA9 and MEIS1 genes normalized to 18S rRNA determined by qRT-PCR in MV4;11 cells treated for 4 days with MI-2-2 referenced to DMSO treated cells; Data represents the mean values for duplicates ± s.d. (F) Quantification of CD11b expression in MV4;11 cells treated for 10 days with the MI-2-2 as detected by flow cytometry. Data represents the mean values for duplicates ± s.d. Experiment was performed two times. (G) Wright-Giemsa stained cytospins demonstrating differentiation of MV4;11 cells upon 10 days of treatment with MI-2-2 and compared to DMSO; scale is 25μm. (H). Comparison of growth curves for MV 4;11 cells treated with DMSO, MI-2 and MI-2-2.
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Structural insights into inhibition of the bivalent menin-MLL interaction by small molecules in leukemia

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