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COMMUNICATION

Design of A First-in-Class, Highly Potent Irreversible Inhibitor Targeting the Menin-MLL Protein-Protein Interaction

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Abstract: We report the structure-based design of M-525 (**10**), a first-in-class, highly potent, irreversible small-molecule inhibitor of the menin-MLL protein-protein interaction. M-525 targets cellular menin protein at sub-nanomolar concentrations and achieves low nanomolar potencies in cell growth inhibition and in suppression of MLL-regulated gene expression in MLL leukemia cells. It demonstrates high cellular specificity over non-MLL leukemia cells and is >30-times more potent than the corresponding reversible inhibitors. Mass spectroscopic analysis and the co-crystal structure of M-525 in a complex with menin firmly establish its mode of action. A single administration of M-525 effectively suppresses MLL-regulated gene expression in tumor tissue. An efficient synthetic procedure was developed to obtain enantiomerically pure M-525 and its analogues. Our study demonstrates that irreversible inhibition of menin may represent a promising therapeutic strategy for MLL leukemia.

MLL leukemia, which is characterized by chromosomal translocations at 11q23 and expression of MLL fusion proteins, has a very poor prognosis and is resistant to current therapies[1]. MLL fusion proteins interact directly with menin, a protein encoded by the *MEN1* gene, to regulate the expression of *MEIS1* and *HOX* genes, which drive leukemogenesis in MLL leukemia[2]. Consequently, targeting the menin-MLL protein-protein interaction using small-molecule inhibitors (here called menin inhibitors) is a promising therapeutic strategy for MLL leukemia[3].

Several classes of peptidomimetic compounds and non-peptide small-molecule menin inhibitors have been reported in recent years[4]. To date, all published menin inhibitors are reversible in nature. Preclinical data show that extended drug exposure is required for menin inhibitors to achieve anti-leukemia activity *in vitro*[4d]. We hypothesized that since irreversible inhibitors in general can achieve target inhibition superior to that of reversible inhibitors[5], irreversible menin inhibitors may have much greater anti-leukemia activity and efficacy than reversible menin inhibitors. In this paper, we report the design and characterization of the first-in-class, highly potent and irreversible menin inhibitors.

For the design of irreversible menin inhibitors, we sought first to obtain a highly potent reversible menin inhibitor. We used MIV-6 (**1**), a previously reported, moderately potent, reversible inhibitor[4c], as the starting point for our structure-based optimization efforts, which are summarized in Figure 1.

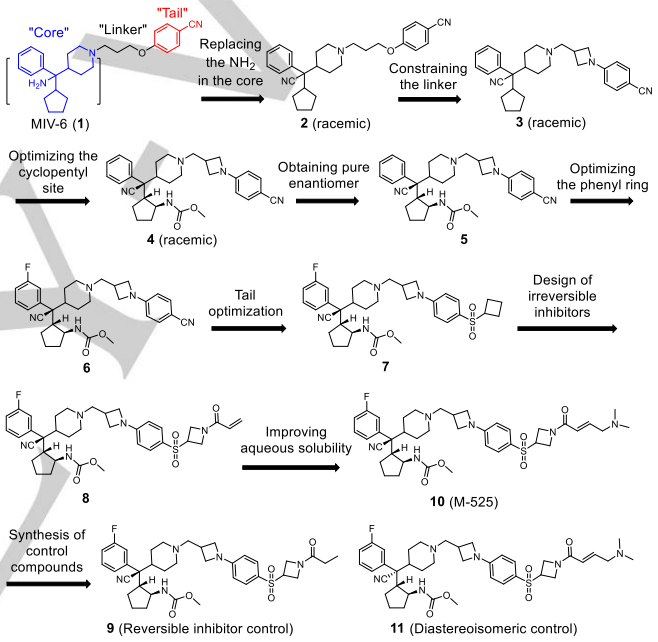

Figure 1. Structure-based design of irreversible menin inhibitors and control compounds. Compounds **5**~**11** are enantiopure.

The primary amino group in MIV-6 was shown to be a major metabolic softspot and in addition, the synthetic yield for the "core" structure bearing this primary amino group was very low[4c]. Consequently, we replaced this free amino group in MIV-6 with a metabolically stable nitrile group, producing compound **2**, which binds to menin with an IC_{50} value of 3.1 μM (Table 1). Although **2** is 17-times less potent than MIV-6 in binding to menin, its synthetic yield is high, and it represents a useful starting point for further optimization.

MIV-6 and compound **2** have a flexible linker connecting their respective "core" structure to the same "tail" group. Because restriction of rotatable bonds is an effective means of reducing conformational space and potential off-target activity of small-molecule drugs, we restricted the linker conformation using 3-methylazetidine. This led to compound **3** that binds to menin with IC_{50} = 3.0 μM and is as potent as compound **2**.

Analysis of the co-crystal structure of MIV-6 complexed with menin shows that there is a well-defined pocket around the

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cyclopentyl group of MIV-6[4c]. We reasoned that addition of an appropriate group to the cyclopentyl ring to capture additional interactions at this site could greatly enhance the binding affinity to menin. Extensive modifications at this site showed that installation of a reverse carbamate at the 2-position of the cyclopentyl ring yielded compound **4** which showed dramatically improved binding affinity to menin. Compound **4** binds to menin with an IC_{50} value of 29 nM, and is >100-times more potent than compounds **2** or **3**.

Compound **4** was initially synthesized as a racemic compound. To determine its stereospecificity for binding to menin, we developed an efficient synthetic route to compound **5**, a pure enantiomer of **4**. Compound **5** binds to menin with an IC_{50} value of 15 nM, and is twice as potent as **4**.

Introduction of a fluorine substituent into the free phenyl ring of previously examined menin inhibitors[4c, 6] had been shown to improve the binding affinity to menin and accordingly, we systematically investigated the effect of a fluorine substituent in the free phenyl group of **5**. This effort resulted in **6**, which binds to menin with an IC_{50} value of 9 nM (K_i < 1 nM).

Table 1. Binding affinity and cell growth inhibition of menin inhibitors.

ID	Binding Affinity to menin in FP Assay (IC_{50}, nM)	Cell growth inhibition assay (4 days) (IC_{50}, μM)	
		MV4;11 (MLL fusion)	HL60 (No MLL fusion)
1 (MIV-6)	185 ± 24	3.6 ± 0.2	4.9 ± 0.8
2	3100 ± 200	2.8 ± 0.32	> 10
3	3000 ± 200	> 10	> 10
4	29 ± 2	0.83 ± 0.04	> 10
5	15 ± 1	0.20 ± 0.01	> 10
6	9.1 ± 1.0	0.16 ± 0.03	> 10
7	6.9 ± 1.1	0.21 ± 0.02	> 10
8	3.0 ± 1.0	0.006 ± 0.002	3.2 ± 0.7
9	8.0 ± 1.0	0.263 ± 0.039	> 10
10 (M-525)	3.3 ± 0.4	0.0027 ± 0.0005	2.0 ± 0.4
11	> 2000	1.01 ± 0.14	1.5 ± 0.3

These high-affinity menin inhibitors (**4**-**6**) were tested for their activity and selectivity in a 4-day cell growth inhibition assay in MV4;11 (MLL-AF4) and HL-60 (no MLL fusion) leukemia cell lines and the resulting data are summarized in Table 1. Compounds **4**, **5** and **6** achieve IC_{50} values of 0.83, 0.20, and 0.16 μM, respectively, in the MV4;11 cell line. Significantly, these three compounds have IC_{50} values of >10 μM in the HL-60 cell line, demonstrating excellent cellular selectivity for MLL leukemia.

Although compounds **5** and **6** bind to menin with very high affinities (K_i < 1 nM), they achieve only submicromolar IC_{50} values in the MV4;11 cell line carrying an MLL fusion. We next explored the possibility of designing covalent, irreversible menin inhibitors based upon the potent reversible inhibitor **6**. Our goal was a dramatic improvement of the cellular potency of menin inhibitors through covalent and irreversible inhibition of menin.

Analysis of the co-crystal structure of the MIV-6/menin complex shows that the Cys329 residue in menin is in close proximity to the inhibitor molecule and may be used in the design of covalent menin inhibitors. Our modeled structure of **6** in a complex with menin shows that the terminal nitrile group of **6** is the closest to the sulfur atom of Cys329 but is still 6.7 Å away, suggesting that the nitrile group needs to be extended if it is to form a covalent bond with Cys329 (Supplementary Information - SI - Figure S1a). We thus replaced the nitrile group in **6** with a cyclobutylsulfonyl group, yielding **7**. Modeling suggests that the sulfonyl group can maintain the hydrogen bond with the indole group of Trp321 and the cyclobutyl moiety can lodge in the surface hydrophobic pocket formed by Cys329, Val367, Val371 and Trp341 (SI Figure S1b). Indeed, it was found that **7** binds to menin with IC_{50} = 7 nM (K_i < 1 nM).

We next designed and synthesized **8** as a potential covalent inhibitor by installation of an acrylamide group, a Michael acceptor, on the 4-membered ring of **7**. We also synthesized **9** as a reversible inhibitor control by reducing the ethenyl moiety in **8** to an ethyl group.

To establish the mode of action of **8** and **9**, we performed mass spectroscopic analysis (SI Table S1), which showed that **8** forms a covalent complex upon incubation with recombinant human menin protein but **9**, lacking the acrylamide, the Michael acceptor group, fails to do so. Using bio-layer interferometry (BLI), we demonstrated that while **8** binds to menin protein irreversibly, **9** binds to menin protein reversibly (SI Figure S2). These data clearly show that **8** is a covalent, irreversible menin inhibitor, while **9** is a non-covalent, reversible menin inhibitor.

In the cell growth inhibition assay, the irreversible menin inhibitor **8** achieves an IC_{50} value of 6 nM in the MV4;11 cell line, and its corresponding reversible inhibitors, **7** and **9,** have IC_{50} values of 210 and 263 nM respectively (Table 1). Hence, **8** is >30-times more potent than the corresponding reversible inhibitors **7** and **9**. In the cell growth inhibition assay, **8** has an IC_{50} value of 3.2 μM in the HL-60 cell line lacking MLL fusion, thus displaying >500-times cellular selectivity in the MV4;11 cell line harboring an MLL fusion over the HL-60 cell line lacking MLL fusion.

To improve the solubility of compound **8**, we attached a dimethylaminomethyl group to the acrylamide group, yielding compound **10**, which binds to menin with an IC_{50} value of 3 nM. In the cell growth inhibition assay, **10** achieves an IC_{50} value of 3 nM in the MV4;11 cell line and has an IC_{50} value of 2.0 μM in the HL-60 cell line, again displaying >500-times cellular selectivity for the MV4;11 cell line over the HL-60 cell line.

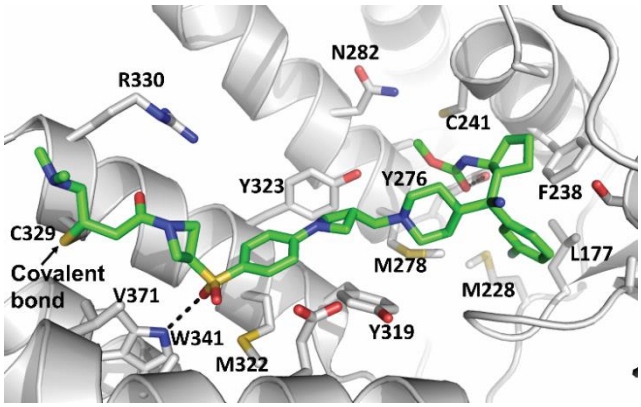

Figure 2. Co-crystal structure of compound **10** (green) complexed with menin (gray) at 2.61 Å resolution (PDB: 6B41). A covalent bond was found between Cys329 and inhibitor **10**.

10.1002/anie.201711828

To establish precisely the mode of interaction of **10** with menin, we determined the co-crystal structure of **10** complexed with human menin protein at 2.61 Å resolution (Figure 2, SI Figure S3). This co-crystal structure clearly shows that, consistent with our design, **10** through its acrylamide group forms a covalent bond with the sulfur atom of Cys329. The reverse carbamate group on the cyclopentyl ring inserts nicely into the available pocket in menin, its carbonyl group forms a strong hydrogen bond with the hydroxyl group of Tyr276, and the methyl group enjoys hydrophobic contacts with the side chains of Met278 and Cys241. The fluorine substituent on the phenyl ring inserts into a hydrophobic pocket formed by Met228, Met278 and Leu177. This co-crystal structure provides a solid structural basis for the high-affinity, covalent binding of **10** with menin. The mass spectroscopic and BLI data further confirm that **10** is a covalent and irreversible inhibitor (SI, Table S1 and Figure S2).

To investigate the binding stereospecificity of **10** with menin, we synthesized **11**, a diastereoisomer of **10**, by inverting the chiral center on the quaternary carbon (Figure 1). Compound **11** binds to menin with an IC_{50} value of >2000 nM, and is thus >500-times less potent than **10**, indicating the high binding stereospecificity of **10** to menin. Furthermore, **11** has an IC_{50} value of 1.0 µM in inhibition of cell growth of the MV4;11 cell line, and is >300-times less potent than **10**. These data are further evidence that the potent cellular activity achieved by **10** in the MV4;11 cell line is attributable to its binding to menin.

The cellular thermal shift assay (CETSA) is a powerful assay allowing assessment of the engagement of small-molecule inhibitors with their specific protein target in cells[7]. Our CETSA data (SI Figure S4) demonstrated that **10** enhances the thermal stability of cellular menin protein in both the MV4;11 and MOLM-13 cells with respect to the control treatment at concentrations as low as 0.4-1.2 nM, reaching a maximum effect at 30-100 nM. In comparison, **10** has no effect on WD repeat-containing protein 5 (WDR5)[8], which also binds to MLL. Since the MV4;11 and MOLM-13 cells were treated with **10** for only 1 h, the thermal stabilization of cellular menin protein by **10** is evidently a direct effect. Hence, the CETSA data provide clear evidence that **10** effectively targets the cellular menin protein at subnanomolar concentrations.

The activity and specificity of compound **10** were further evaluated in a panel of human acute leukemia cell lines with or without MLL fusions. Similar to inhibitors of other epigenetic regulators such as EZH2 and DOT1L[9], menin inhibitors exert their cell growth inhibitory activity more slowly than traditional cytotoxic agents and kinase inhibitors[4d]. Accordingly, we evaluated the growth inhibitory activity of **10** in a panel of leukemia cell lines with a 7-day treatment, and obtained the results summarized in Figure 3. Our data show that **10** achieves IC_{50} values of 2.3-31.3 nM in six leukemia cell lines harboring MLL fusions. In comparison, **10** has IC_{50} values of 0.9-6.5 µM in four leukemia cell lines lacking MLL fusion where it is 25-2000 times less potent than in leukemia cell lines carrying an MLL fusion. Thus, **10** achieves potent cellular activity in human leukemia cell lines carrying MLL fusions, independent of the MLL fusion partner, and demonstrates excellent cellular selectivity over leukemia cell lines lacking MLL fusion.

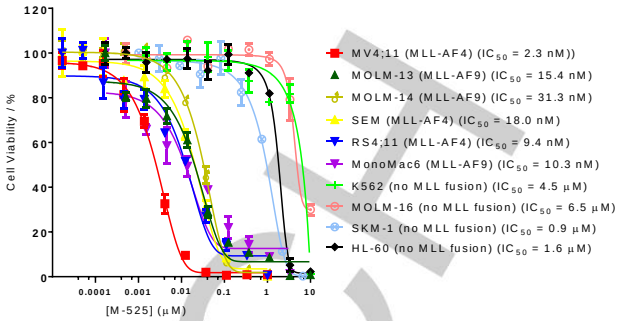

Figure 3. Cell growth inhibition in acute leukemia cell lines with or without MLL fusion. Cells were treated with compound **10** for 7 days and cell viability was determined using a WST-8 assay. Data are representative of three independent experiments.

Reversible menin inhibitors have been shown to down-regulate *MEIS1* and *HOX* genes in MV4;11 and other leukemia cell lines carrying an MLL fusion[4d]. We assessed the ability of compound **10** to regulate expression of *MEIS1* and *HOX* genes in the MV4;11 and MOLM-13 cell lines. In the MV4;11 cell line, treatment for 6 or 24 h with **10** significantly down-regulates the expression of *MEIS1*, *HOXA9* and *HOXA11* genes in a dose-dependent manner (SI, Figure S5a and S5b) and is effective at low nanomolar concentrations. Compound **10** also effectively suppresses the expression of *MEIS1* and *HOX* genes in the MOLM-13 cell line at concentrations as low as 3 nM (SI, Figure S5c). Compound **10** is therefore potent and effective in suppressing the expression of *MEIS1* and *HOX* genes in MV4;11 cells carrying the MLL-AF4 fusion and in MOLM-13 cells carrying the MLL-AF9 fusion.

We further examined in a pharmacodynamics (PD) experiment (SI, Figure 6a, b), the ability of **10** to down-regulate *MEIS1* and *HOX* genes in the MV4;11 xenograft tumor tissue in mice. In contrast to the requirement for repeated dosing with reversible menin inhibitors[4d], a single, intravenous administration of **10** effectively down-regulates *MEIS1*, *HOXA9* and *HOXA11* genes after 24 h with the effect persisting for at least 48 h. The long-lasting PD effect of a single dose of **10** on down-regulation of *MEIS1* and *HOX* genes *in vivo* suggests that infrequent administration of an irreversible menin inhibitor may be sufficient to achieve a strong anti-leukemia activity against MLL leukemia in patients, and this would represent a significant advantage over reversible menin inhibitors.

The synthesis of **10** is summarized in Scheme 1. The critical step was the construction of the key intermediate (**15a**), which contains three chiral centers and a highly hindered quaternary carbon with four distinct substituents. The synthesis of **15a** was achieved using an efficient method that generates the three chiral centers in one step. The absolute stereochemistry of **15a** was confirmed by determination of its single crystal structure. The detailed syntheses of **10** and other compounds are provided in SI.

In summary, we report the succesful design and extensive evaluation of first-in-class, irreversible inhibitors of menin, examplified by **10** (M-525). Our data clearly demonstrate that irreversible inhibitors of menin are highly potent and effective in targeting the menin-MLL interaction and should be extensively evaluated as a new class of targeted therapy for MLL leukemia.

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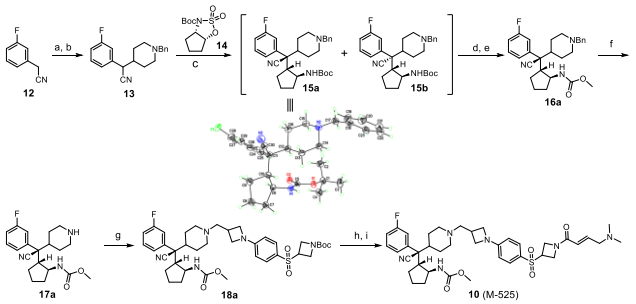

Scheme 1. Reagents and conditions: a) MeONa, MeOH, reflux; b) $NaBH_4$, MeOH, rt; c) KHMDS, 18-Crown-6, H_2SO_4, THF, 0 °C to rt; d) TFA, CH_2Cl_2, 0 °C to rt; e) Dimethyl dicarbonate, Et_3N, CH_2Cl_2, 0 °C to rt; f) 10% Pd/C, H_2, MeOH, rt; g) K_2CO_3, KI, CH_3CN, 80 °C; h) TFA, CH_2Cl_2, 0 °C to rt; i) DIPEA, CH_2Cl_2, 0 °C to rt. The absolute stereochemistry of **15a** was confirmed by x-ray crystallography (CDCC: 1581872).

Acknowledgements

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Keywords: drug design • irreversible inhibitors • menin-MLL protein-protein interaction• MLL leukemia

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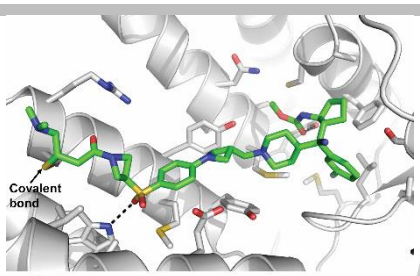
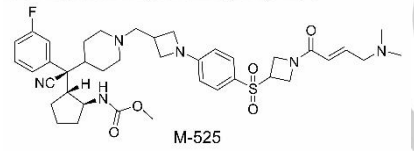
The irreversible inhibitor M-525 targets the menin-MLL protein-protein interaction. Our study demonstrates that irreversible inhibition of menin represents a promising therapeutic strategy for the treatment of MLL leukemia and may have advantages over reversible inhibition.





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Page No. – Page No.
A First-in-Class, Highly Potent Irreversible Inhibitor Targeting the Menin-MLL Protein-Protein Interaction