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

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Design of the 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 as the first-in-class, highly potent, irreversible small-molecule inhibitor of the menin-MLL 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. M-525 demonstrates high cellular specificity over non-MLL leukemia cells and is >30-times more potent than its corresponding reversible inhibitors. Mass spectroscopic analysis and co-crystal structure of M-525 in 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 procedure was developed to synthesize M-525. 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 directly interact 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 (hereafter called menin inhibitors) represent a promising therapeutic strategy for MLL leukemia[3].

Several classes of peptidomimetics and non-peptide small-molecule menin inhibitors were reported in recent years[4]. To date, all reported menin inhibitors are reversible in nature. Preclinical data showed that extended drug exposure is required for menin inhibitors to achieve anti-leukemia activity *in vitro*[4d]. Therefore, we hypothesize that irreversible menin inhibitors may achieve much greater anti-leukemia activity than reversible menin inhibitors in MLL leukemia. Herein, we report the design and characterization of the first-in-class, highly potent and irreversible menin inhibitors.

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For the design of irreversible menin inhibitors, we sought to first 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 is summarized in Figure 1.

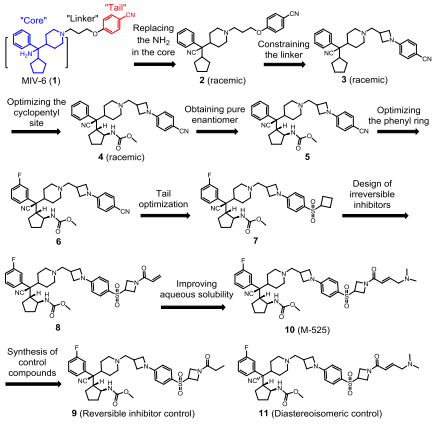


Figure 1. Structure-guided design of irreversible menin inhibitors and control compounds. Compounds **5**~**11** are enantiopure.

The primary amine group in MIV-6 was shown to be a major metabolic softspot and the synthetic yield for the "core" structure bearing this primary amine group was very low[4c]. We thus replaced this free amine in MIV-6 with a metabolically stable nitrile group, which led to compound **2** that binds to menin with an IC_{50} value of 3.1 μM (Table 1). Although compound **2** is 17-times less potent than MIV-6 in binding to menin, its synthetic yield is high, representing a reasonable 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 improving target selectivity for small-molecule drugs, we have identified 3-methylazetidine as a suitable, conformationally constrained linker, which led to compound **3**. Compound **3** 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 cyclopentyl group of MIV-6[4c]. We reasoned that installation of an appropriate group onto the cyclopentyl ring to optimize the interactions at this site could greatly enhance the binding affinity to menin. Our extensive modifications at this site showed that installation of a reverse carbamate at the 2-position of the cyclopentyl ring dramatically improves the binding affinity to menin, which yielded compound **4**. Compound **4** binds to menin with an IC_{50} value of 29 nM, and is thus > 100-times more potent than compounds **2** and **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 obtain 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 phenyl ring group was shown to modestly improve the binding affinity of

previous menin inhibitors[4c, 5] and accordingly, we systematically investigated the effect of a fluorine substituent in the 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	0.16 ± 0.03	> 10
7	7 ±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 potent menin inhibitors 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) 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 the MLL fusion. Since irreversible inhibitors can achieve enhanced target inhibition over reversible inhibitors[6], we next explored the possibility of designing covalent, irreversible menin inhibitors based upon the potent reversible inhibitor **6**.

Analysis of the co-crystal structure of the MIV-6/menin complex shows that the Cys329 residue in menin is close to the inhibitor and may be used for 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 in order 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 lodges in the surface hydrophobic pocket formed by Cys329, Val367, Val371 and Trp341 (SI Figure S1b). Indeed, **7** binds to menin with an IC_{50} value of 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, in 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 analyses (SI Table S1), which showed that **8** forms a covalent complex upon incubation with recombinant human menin protein, but **9**, lacking the Michael acceptor group fails to do so. Using bio-layer interferometry (BLI) by Octet Red, we demonstrated that while **8** binds to menin protein irreversibly, **9** binds to menin protein reversibly (SI Figure S2). Taken together, these data clearly show that **8** is a covalent and 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 same 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 installed a dimethylaminomethyl group onto the acrylamide group, yielding **10**. Compound **10** 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.

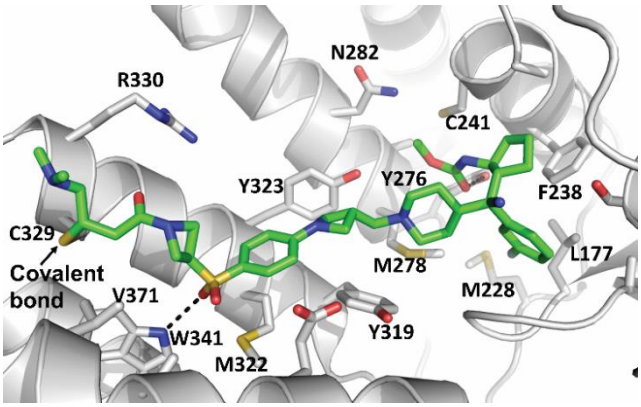


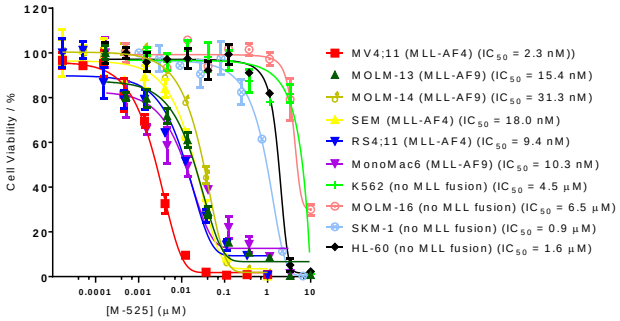
Figure 2.Co-crystal structure of compound **10** (green) complexed with menin (gray) at 2.61 Å resolution (PDB: 6B41). A covalent bond between Cys329 and inhibitor **10** was detected.

To precisely establish the mode of interactions of **10** with menin, we determined its co-crystal structure 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, with its carbonyl group forming 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. Additionally, our mass spectroscopic and BLI data further confirmed 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 a 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 provide further evidence that the potent cellular activity achieved by **10** in the MV4;11 cell line is attributed to its binding to menin.

The cellular thermal shift assay (CETSA) is a recently developed, powerful assay to assess 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 at concentrations as low as 0.4-1.2 nM over the control treatment and reaches 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.

Compound **10** was further evaluated for its activity and specificity 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, with 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 and 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.

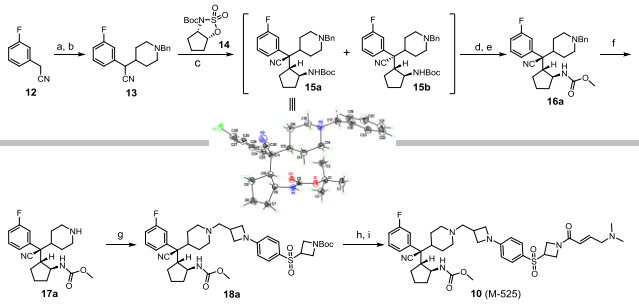


Reversible menin inhibitors have been shown to down-regulate *MEIS1* and *HsOX* genes in MV4;11 and other leukemia cell lines carrying a MLL fusion[4d]. We tested **10** for its ability to regulate expression of *MEIS1* and *HOX* genes in MV4;11 and MOLM-13 cell lines. In the MV4;11 cell line, treatment 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 MLL-AF4 fusion and in MOLM-13 cells carrying MLL-AF9 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.

We further examined the ability of **10** to down-regulate *MEIS1* and *HOX* genes in the MV4;11 xenograft tumor tissue in mice in a pharmacodynamic (PD) experiment (SI Figure 6a, b). 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 at 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 agaisnt 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 consists of three chrial 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 synthesis of **10** and other compounds are provided in SI.



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

In summary, we report the design and extensive evaluation of the 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.

Acknowledgements

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

[1] a) P. Ernst, J. Wang, S. J. Korsmeyer, *Curr. Opin. Hematol.* **2002**, *9*, 282-287; b) R. Marschalek, *Br. J. Haematol.* **2011**, *152*, 141-154; c) R. Popovic, N. J. Zeleznik-Le, *J. Cell. Biochem.* **2005**, *95*, 234-242; d) R. K. Slany, *Hematol. Oncol.* **2005**, *23*, 1-9; e) D. Tomizawa, K. Koh, T. Sato, N. Kinukawa, A. Morimoto, K. Isoyama, Y. Kosaka, T. Oda, M. Oda, Y. Hayashi, M. Eguchi, K. Horibe, T. Nakahata, S. Mizutani, E. Ishii, *Leukemia* **2007**, *21*, 2258-2263.

[2] a) C. Caslini, Z. Yang, M. El-Osta, T. A. Milne, R. K. Slany, J. L. Hess, *Cancer Res.* **2007**, *67*, 7275-7283; b) Y.-X. Chen, J. Yan, K. Keeshan, A. T. Tubbs, H. Wang, A. Silva, E. J. Brown, J. L. Hess, W. S. Pear, X. Hua, *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 1018-1023; c) A. Yokoyama, T. C. Somervaille, K. S. Smith, O. Rozenblatt-Rosen, M. Meyerson, M. L. Cleary, *Cell* **2005**, *123*, 207-218; d) R. K. Slany, *Haematologica* **2009**, *94*, 984-993; e) Y. Zhang, A. Chen, X. M. Yan, G. Huang, *Int. J. Hematol.* **2012**, *96*, 428-437.

[3] a) A. Yokoyama, T. C. P. Somervaille, K. S. Smith, O. Rozenblatt-Rosen, M. Meyerson, M. L. Cleary, *Cell* **2005**, *123*, 207-218; b) A. Yokoyama, M. L. Cleary, *Cancer Cell* **2008**, *14*, 36-46.

[4] a) J. Grembecka, S. He, A. Shi, T. Purohit, A. G. Muntean, R. J. Sorenson, *Nat. Chem. Biol.* **2012**, *8*, 277-284; b) A. Shi, M. J. Murai, S. He, G. Lund, T. Hartley, T. Purohit, *Blood* **2012**, *120*, 4461-4469; c) S. He, T. J. Senter, J. Pollock, C. Han, S. K. Upadhyay, T. Purohit, R. D. Gogliotti, C. W. Lindsley, T. Cierpicki, S. R. Stauffer, J. Grembecka, *J. Med. Chem.* **2014**, *57*, 1543-1556; d) D. Borkin, S. He, H. Miao, K. Kempinska, J. Pollock, J. Chase, T. Purohit, B. Malik, T. Zhao, J. Wang, B. Wen, H. Zong, M. Jones, G. Danet-Desnoyers, Monica L. Guzman, M. Talpaz, Dale L. Bixby, D. Sun, Jay L. Hess, Andrew G. Muntean, I. Maillard, T. Cierpicki, J. Grembecka, *Cancer Cell* **2015**, *27*, 589-602.

[5] H. Zhou, L. Liu, J. Huang, D. Bernard, H. Karatas, A. Navarro, M. Lei, S. Wang, *J. Med. Chem.* **2013**, *56*, 1113-1123.

[6] J. Singh, R. C. Petter, T. A. Baillie, A. Whitty, *Nat. Rev. Drug Discov.* **2011**, *10*, 307-317.

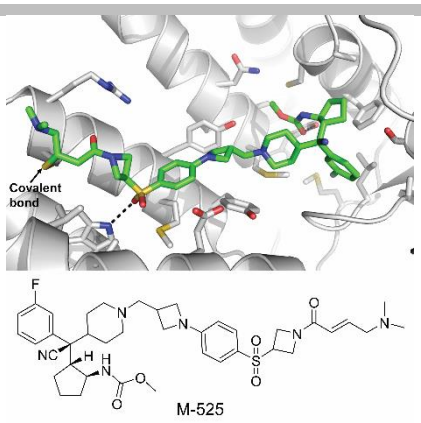
[7] a) D. M. Molina, R. Jafari, M. Ignatushchenko, T. Seki, E. A. Larsson, C. Dan, L. Sreekumar, Y. Cao, P. Nordlund, *Science* **2013**, *341*, 84-87; b) R. Jafari, H. Almqvist, H. Axelsson, M. Ignatushchenko, T. Lundbäck, P. Nordlund, D. M. Molina, *Nat. Protoc.* **2014**, *9*, 2100-2122.

[8] J.-J. Song, R. E. Kingston, *J. Biol. Chem.* **2008**, *283*, 35258-35264.

[9] a) S. K. Knutson, N. M. Warholic, T. J. Wigle, C. R. Klaus, C. J. Allain, A. Raimondi, M. Porter Scott, R. Chesworth, M. P. Moyer, R. A. Copeland, V. M. Richon, R. M. Pollock, K. W. Kuntz, H. Keilhack, *Proc. Natl. Acad. Sci. U.S.A.* **2013**, *110*, 7922-7927; b) S. R. Daigle, E. J. Olhava, C. A. Therkelsen, A. Basavapathruni, L. Jin, P. A. Boriack-Sjodin, C. J. Allain, C. R. Klaus, A. Raimondi, M. P. Scott, N. J. Waters, R. Chesworth, M. P. Moyer, R. A. Copeland, V. M. Richon, R. M. Pollock, *Blood* **2013**, *122*, 1017-1025.

COMMUNICATION

Irreversible inhibitor M-525 targets the menin-MLL 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 inhibitors.



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