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Progress towards small molecule menin-mixed lineage leukemia (MLL) interaction inhibitors with in vivo utility

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ABSTRACT

A series of substituted hydroxymethyl piperidine small molecule inhibitors of the protein–protein interaction between menin and mixed lineage leukemia 1 (MLL1) are described. Initial members of the series showed good inhibitory disruption of the menin-MLL1 interaction but demonstrated poor physicochemical and DMPK properties. Utilizing a structure-guided and iterative optimization approach key substituents were optimized leading to inhibitors with cell-based activity, improved in vitro DMPK parameters, and improved half-lives in rodent PK studies leading to MLPCN probe ML399. Ancillary off-target activity remains a parameter for further optimization.

Misregulation or mutation of histone-modifying enzymes plays a major role in the development of a wide-range of human cancers.[1,2] Mixed lineage leukemia 1 (MLL1) is one of six known members of the SET1-family of human histone methyltransferases (HMT) and participates in catalyzing the mono-, di-, and trimethylation of histone H3 lysine 4 (H3K4).[3] Genetic rearrangements of MLL1 are associated with acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) and can be found in 5–10% of adult AML patients[4] and up to 80% of infants with ALL.[5] Patients with acute leukemias harboring MLL translocations suffer a poor prognosis with existing therapies providing only a ~35% overall 5-yr survival rate.[6,7] Thus, novel targeted therapies are urgently needed to treat these leukemias and enhance patient long-term outcome. The most common MLL1 rearrangements are balanced MLL1 translocations, in which one MLL1 allele is truncated and fused in frame with one of over 70 partners to produce oncogenic fusion proteins. MLL fusion partners preserve the N-terminal fragment of approximately 1400 amino acids and participate in a key protein-protein interaction (PPI) with the oncogenic co-factor menin. Menin is a ubiquitously expressed nuclear protein capable of binding directly to both wild type MLL1, MLL2, and MLL1 fusion protein complexes and is thought to function by targeting their recruitment to the target genes which includes the homeotic genes *Hoxa9*, *Hoxc6*, and *Hoxc8*.[8,9] Menin contains a large and well defined central cavity (human menin ~5000 Å^3) that represents the MLL binding site. In addition, its architecture remains relatively rigid upon binding of MLL, thus making menin a druggable and yet challenging PPI interface for the development of small molecule inhibitors. Current inhibitors of the menin-MLL interaction[9] include peptidomimetics such as MCP-1 (**1**)[10] and two classes of small molecule inhibitors including the thienopyrimidines MI-2 (**2**) and MI-2-2 (**3**)[11,12] and hydroxymethylpiperidines ML227 (**4**)[13,14] (Fig. 1); the later class of which was discovered from an NIH Molecular Libraries Production Center Network (MLPCN) sponsored screen of the Molecular Libraries Small Molecule Repository (MLSMR) of ~280 K compounds. To date, systemically available menin-MLL inhibitor tool compounds for studying menin biology and proof-of-mechanism using MLL driven xenograft leukemogenic animal models have not been described.[15] The development of potent small molecule inhibitors of the menin-MLL interaction with good selectivity, metabolic stability, and acceptable oral bioavailability will permit chronic systemic administration in animal models of MLL leukemia. These studies are critical in order to understand the anticipated efficacy, PK-PD relationship, and therapeutic index for the mechanism.

Recent menin-inhibitor co-crystal structures developed in these laboratories for the individual enantiomers of **4** reveal key

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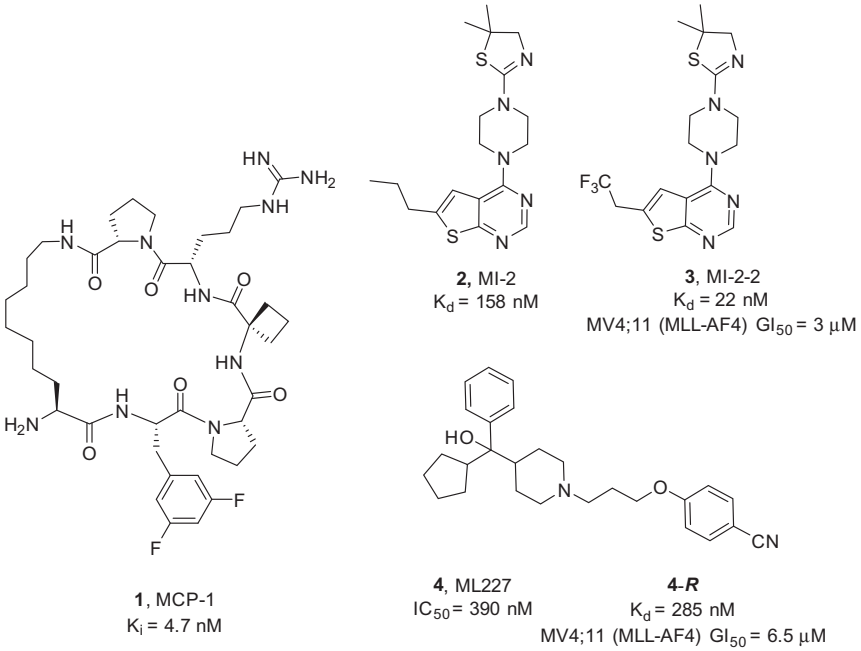


Figure 1. Small-molecule menin-MLL PPI inhibitors **1**–**5**.

interactions that closely mimic the menin-MLL protein-protein interaction.[14] In cell based assays the slightly more potent *R* enantiomer of **4** (**4*R***, K_d = 285 nM, Fig. 1) was found to disrupt menin-MLL fusion protein interactions in co-immunoprecipitation studies, dose-dependently reduce *Hoxa9* expression levels by qRT-PCR, and selectivity inhibit cell proliferation in MLL human leukemia cell lines with GI_{50s} comparable to **3**, between 6.5 and 7.4 μM (MLL-AF4, MLL-AF9 containing).[13,14] Although ML227 is a useful in vitro tool compound, it possesses several key limitations precluding its use in animals (Fig. 2). These include poor metabolic stability and off-target activity against multiple monoaminergic and ion channel targets. Other challenges noted within the profile of ML227 include moderate inhibition of human cytochrome P450 (CYP) enzymes 2D6 and 3A4 and high plasma protein binding (Fig. 2). In an effort to develop a useful in vivo probe compound within the series we initiated a Molecular Libraries extended probe optimization campaign using an integrated approach focused on menin binding affinity, tier one DMPK properties (e.g., predicted hepatic clearance, plasma protein binding, and major CYP450 inhibition), and ancillary screening for selected candidates. Herein our progress towards reaching this goal is described.

As shown in Figure 2, the lead structure ML227 possessed four structural pharmacophore elements: (1) a lipophilic head group containing a stereogenic tertiary carbinol, (2) a piperidine core, (3) an propoxy linker, and (4) a benzonitrile tail group. Within the binding pocket of menin, the lipophilic head group of ML227 mimics key interactions normally occupied by the F9 and P10 residues of MLL. The removal of either the phenyl or cyclopentyl ring within ML227 results in >500-fold loss of binding to menin. Similarly, removal of the tail group cyano moiety, which participates in a unique hydrogen bond accepting interaction with W341 results in loss of inhibitory activity. Thus, these particular pharmacophore elements are considered most critical to maintain high binding affinity to menin.

Based on the high calculated Log*P* for ML227 (*c*Log*P* >5, Fig. 2) structure-based design strategies predicted to attenuate lipophilicity and maintain a favorable binding interaction with menin were prioritized; specifically within the lipophilic carbinol and eastern tail group region. In addition to IC_{50} measurements as determined from our fluorescence polarization assay using a displaceable MLL tagged substrate peptide,[11,16] efficiency metrics, including ligand efficiency (LE) and lipophilicity-dependent ligand efficiency (LELP)[17] were utilized to assess physicochemical impact from structural modifications and to identify preferred enthalpy-driven SAR.

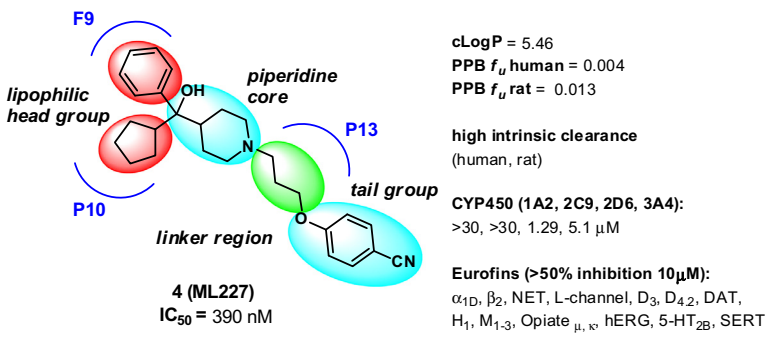
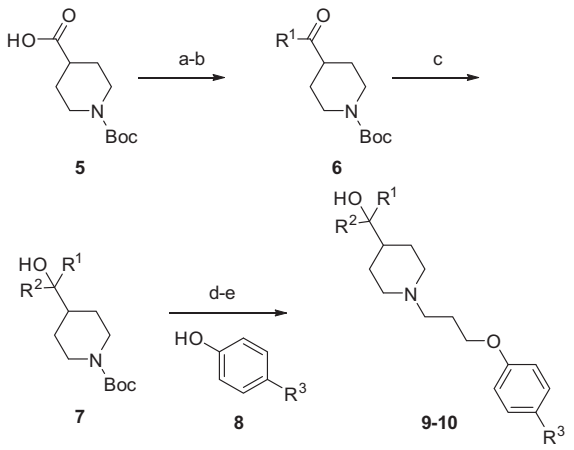


Figure 2. In vitro profile summary of MLPCN probe ML227 (**4**).



Scheme 1. Reagents and conditions: (a) *N,O*-dimethyl hydroxylamine-HCl, CDI, Et_3N, DCM, 75–90%; (b) R_1-MgBr, THF, 0 °C to rt, 60–85%; (c) R_2-MgBr, THF, 0 °C to rt, 30–75%; (d) 4.0 M HCl/dioxanes, MeOH, rt, 2 h, 93–100%; (e) (i) K_2CO_3, DMF, 1-bromo-3-chloropropane, rt, 3 h, (ii) **8**, K_2CO_3, 40 °C, 2 h, 42–78%.

Similar to our prior synthetic strategies, analogs within the hydroxymethylpiperidine series **9** were synthesized according to Scheme 1.[14] Initial Grignard addition to the Weinreb amide of 1-(*t*-butoxycarbonyl)piperidine-4-carboxylic acid (**5**) proceeded in high yield. A second Grignard addition to the resulting ketone **6** provided the tertiary carbinols **7**. Deprotection, followed by sequential alkylation of the piperidine and desired phenol provided the final products in good overall yields (42–78%), while allowing for a high degree of control over three regions of interest for analog synthesis.

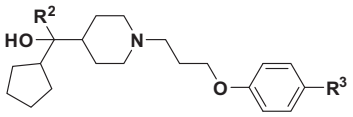
Inhibitory potency and calculated properties for cyclopentyl analogs **9a–h** and ML227 (**4**) for comparison are shown in Table 1. As previously reported,[14] introduction of the correct heterocycle moiety (e.g., **9a–9c**) within the carbinol head group leads to a slight increase in potency while reducing *c*Log*P* an order of magnitude. Thus, with the retained potency, improvements in calculated LELP were observed. For example, the 2-pyridyl and

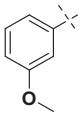
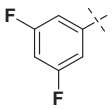
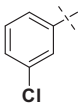
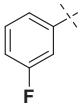
Table 2
In vitro tier one DMPK and rat IV pharmacokinetics of cyclopentyl hydroxymethylpiperidines **4** (ML227), **9a**, **9c**[18]

Entry[a]	CL_{int} (h, r)[b]	CL_{hep} (h, r)[b]	PPB f_u (h, m)	CL_p[c]	$T_{1/2}$ (h)	V_{ss}[d]
4	124, 919	18, 65	0.004, 0.013	40	1.6	2.9
9a	77.4, 558	16, 62	0.117, 0.192	NT	NT	NT
9c	16.2, 271	9.1, 57	0.291, 0.342	293	0.2	4.3

NT = not tested.
[a] Human (h), rat (r), mouse (m).
[b] mL/min/kg, see Ref. 18.
[c] mL/min/kg, rat IV dose 0.2 mg/kg.
[d] V_{ss} = volume distribution, L/kg.

Table 1
Potency and physical properties of cyclopentyl hydroxymethyl piperidine menin-MLL inhibitors **4**, **9a–h**[13,14]



Entry	R^2	R^3	IC_{50} (nM)	*c*Log*P*	LE/LELP
4 (ML227)			390	5.46	0.29/17.9
9a			242	3.80	0.30/11.2
9b			302	3.90	0.29/13.8
9c			114	2.78	0.28/9.9
9d			1100	5.37	0.25/21.74
9e			437	5.74	0.26/21.77
9f			226	6.16	0.28/21.69
9g			197	5.59	0.29/19.53
9h			28,000	4.60	0.2./22.90

thiazolyl congeners **9a** and **9b**, demonstrated an LELP reduction from ~18 for ML227 to less than 14. In general LELP proved to be a responsive indicator in lead optimization for this program and informed prioritization of compounds for in vitro DMPK studies. As previously noted, analysis of **4** in complex with menin revealed a hydrogen bond interaction between the eastern nitrile of **4** with tryptophan residue 341, and this information was used to investigate additional polar substituents that contained hydrogen bond acceptor moieties. Sulfonamide **9c** (Table 1) proved particularly successful, with an IC_{50} of 114 nM as a racemic mixture.[13,14] Relative to **9b** this modification led to a further reduction in lipophilicity (*c*Log*P* = 2.78) and provided an inhibitor with an attractive calculated LELP of ~10.

Table 2 describes results from in vitro DMPK studies for the cyclopentyl congeners **9a** and **9c** relative to lead **4**. In addition, parameters from IV pharmacokinetic studies in rat are summarized for **4** versus **9c** (IV 0.2 mg/kg: CL_p, $T_{1/2}$, V_{ss}). Inhibitor **4** was found in rat and human microsomal intrinsic clearance assays to demonstrate high in vitro metabolism with predicted hepatic clearance near the rate of blood flow in both human and rat,[18] while the fraction unbound from plasma proteins was ~1% or lower (PPB f_u = 0.004, 0.013, human and rat, respectively). Due in part to the high protein binding and weak base properties of **4**, rat IV pharmacokinetics afforded a compound with moderate plasma clearance and volume of distribution (Table 2). Replacement of the phenyl to afford thiazole **9a** (Table 2) led to a notable decrease in intrinsic clearance whereas substitution of phenyl with a 2-pyridyl, and replacement of the nitrile with a sulfonamide to afford **9c**, further attenuated intrinsic and predicted hepatic clearance. Unfortunately **9c** suffered from a short half-life in vivo (IV rat, $T_{1/2}$ <0.2 h, Table 2) probably owing to the greater than ten-fold improvement in fraction unbound that accompanied these structural changes (PPB f_u 29–34%). Hence, further effort was spent on reducing metabolic clearance. Alcohol or primary sulfonamide conjugation were suspected to contribute to the high clearance of **9c**; however, soft-spot metabolite analysis in microsomal incubations implicated the cyclopentyl ring was a retained liability for extensive oxidative metabolism. Although mimicry of the MLL proline

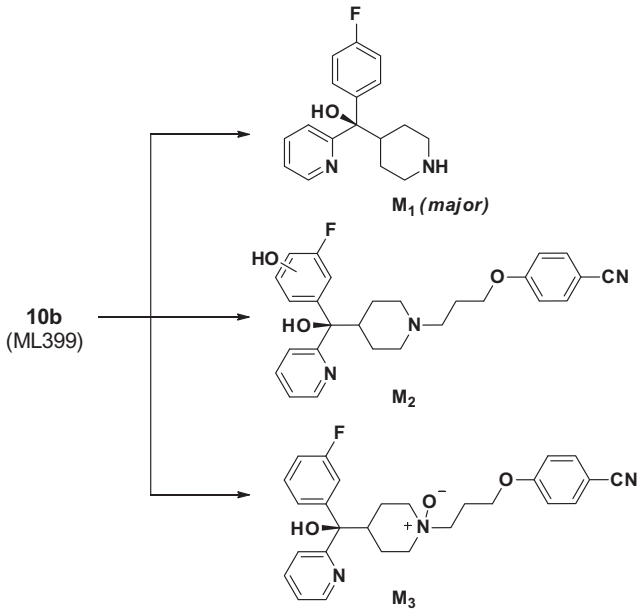
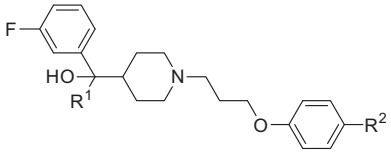


Figure 3. Identification of major metabolites of **10b** in rat S9.

10 residue by the cyclopentyl moiety was evident, X-ray structures of the individual ML227 enantiomers bound to menin demonstrated that aromatic replacements within the P10 pocket were feasible. With respect to prior diaryl carbinol SAR this type of head group appeared challenging for potency, as simple phenyl within the P10 pocket[13] was shown to lead to a >30-fold loss in IC_{50} relative to **4**.[14] By targeting a conserved water molecule found within the P10 pocket the introduction of either a 2-pyridine or 2-thiazolyl to engage in a solvent mediated hydrogen bond with Y276 proved successful as a near equipotent replacement for the cyclopentyl P10 mimic. Heteroaryl P10 cyclopentyl replacements, in conjunction with a 3-fluorophenyl within the F9 pocket[14] were predicted to be favorable for potency while also mitigating sites

Table 3
In vitro, calculated, and in vivo characterization of advanced aryl/heteroaryl hydroxymethyl piperidine menin-MLL inhibitors



Entry[a]	R^2	R^3	IC_{50}	*c*Log*P*	LE/LELP	CL_{int} (h, r)[b]	CL_{hep} (h, r)[b]	PPB f_u (h, r, m)	CL_p[c]	$T_{1/2}$ (h)	V_{ss}[d]
10a			460	3.62	0.27/13.4	69, 273	16, 56	0.04, 0.108, NT	32	2.8	7.2
10b (ML399) 1st *peak SFC* (*R*)			90	3.57	0.30/12.2	20, 87	10, 39	0.062, 0.074, 0.079	12	10.1	7.4
10c			354	2.36	0.29/11.6	50, 206	15, 52	0.170, 0.322, 0.166	26	1.4	4.4
10d			246	3.00	0.28/9.1	17, 21	14, 11	0.267, NT, 0.178	32	2.5	4.6

NT = not tested.
[a] Human (h), rat (r), mouse (m).
[b] mL/min/kg, see Ref. 18.
[c] mL/min/kg, rat IV dose 0.2 mg/kg.
[d] V_{ss} = volume distribution, L/kg.

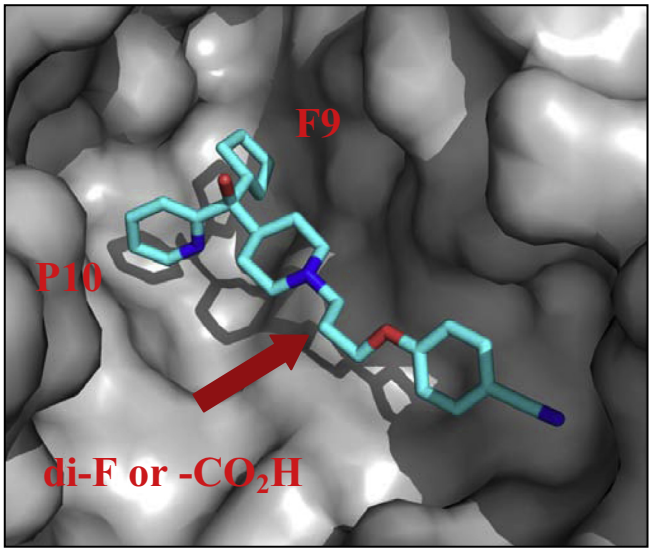


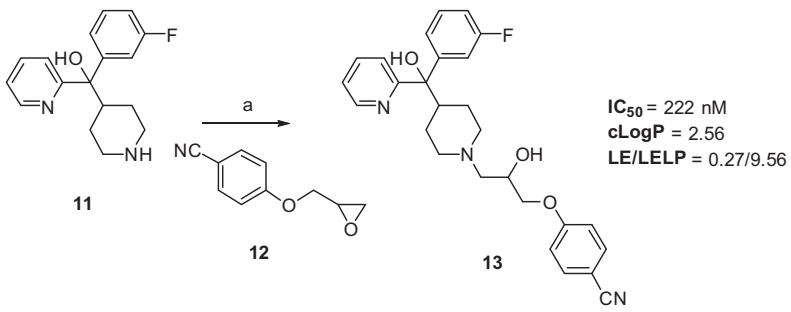
Figure 4. X-ray crystal structure of **9b** in complex with menin (PDB: 4OGS) and proposed modification to central propyl linker carbon.

for oxidative metabolism. Thus, hybrid compounds **10a**–**10d** containing a heteroaryl and aryl moiety within the carbinol head group, in addition to either a 4-cyano or 4-sulfonamide substituent within the tail group were prepared according to methods in Scheme 1. As shown in Table 3 these compounds consistently lowered *c*Log*P* from ~5 to <3.6, while improving LELP to as low as 9.1 (e.g., **10d**), affording inhibitors with comparable or improved potency. In the case of single enantiomer **10b**, an IC_{50} less 100 nM was measured (IC_{50} = 90). Efforts to resolve sulfonamide **10d** using chiral SFC were unsuccessful. Overall improvements in these metrics were reflected well in in vitro DMPK studies, providing menin-MLL inhibitors with predicted hepatic clearance less than half of liver blood flow based on human and rat microsomal incubation experiments. Analogs **10a**–**10d** were subsequently advanced to an in vivo IV cassette study in rat (Table 3).

Importantly, replacement of the cyclopentyl group, an identified liability in soft-spot metabolite studies, led to compounds with improved half-lives in vivo ($T_{1/2}$ from 1.4–10 h). Of particular interest was **10b**, which demonstrated a 10 h half-life in rat and 5.5 h half-life in mouse (mouse data not presented), with a somewhat high volume of distribution in rat (V_{ss} = 7.4 L/kg). Metabolite identification experiments using **10b** in rat hepatic S9 fractions revealed N-dealkylation at the piperidine core as the major metabolite, followed by oxidation of the 3-fluorophenyl substituent (Fig. 3). Menin-MLL inhibitor **10b** was subsequently tested in a cell viability assay for its impact on proliferation using murine bone marrow cells (MBC) transformed with MLL-AF9, a prevalent fusion protein observed in both AML and ALL patients.[19] Using this leukemic cell line with a 72 h incubation protocol[14] significant growth inhibition was observed for **10b** with a GI_{50} of 4 μM. These results demonstrate an association between in vitro inhibition of the menin-MLL interaction and inhibition of cell growth in MLL leukemia cells; however, a broader assessment of anti-proliferative activity in additional leukemia and non-MLL containing cell lines is needed.

Based on the overall in vitro and in vivo profile obtained, menin-MLL inhibitor **10b** was declared second generation probe ML399. In a Eurofins radioligand binding panel of over 60 G protein-coupled receptors, ion channels, and transporters at a concentration of 10 μM several significant activities at ancillary targets were found for ML399, similar to ML227 (Fig. 2). These included binding to the hERG potassium ion channel, a known off-target activity for Terfenadine-like scaffolds.[20,21] Although diminished, notable hERG binding was also observed for the more polar sulfonamide **10d**. Efforts to address hERG binding and related off-target activity continue. Strategies include attenuation of basicity of the piperidine moiety and introduction of alternative polar groups, including carboxylate to engender zwitterionic character.[21] Structural studies of **9b** in complex with menin[14] revealed that the propyl linker region occupied a relatively unhindered region of the binding pocket (Fig. 4), and might readily tolerate further derivatization. Thus, hydroxyl-substituted analog **13** was synthesized according to Scheme 2. Piperidine starting material **11** was accessed as before according to Scheme 1. Subsequent opening of epoxide **12** using **11** was achieved in good yield leading to **13**. When tested as a mixture of four diastereomers piperidine **13** was found to inhibit the menin-MLL interaction with an IC_{50} of 222 nM, approximately two-fold less potent than ML399 (**10b**), demonstrating that polar functionality within this region of the linker is well tolerated. In addition, secondary alcohol **13** may provide a useful synthetic handle for introduction of a β-difluoro or carboxylic acid modification as proposed (see Fig. 4). Efforts to capitalize on this strategy and its potential impact continue to be investigated.

Ongoing efforts are required in order to develop potent, selective, and orally available menin inhibitors to test in animal models of mixed lineage leukemia.[22] In summary, starting from ML227 an iterative strategy incorporating analysis of physical properties and metrics, as well as rational design informed by metabolite identification studies were used to develop inhibitors of the menin-MLL interaction with improved potency and stability in vivo, leading to ML399.[23] Efforts to enhance selectivity within the series, as well as oral dosing studies using ML399 in naïve animals to assess bioavailability and tolerability are in progress and will be reported in due course.



Scheme 2. Reagents and conditions: (a) acetonitrile, **12**, K_2CO_3, 30 °C, 4 h, 67%.

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22. During the review of this manuscript a study demonstrating suppressed development of acute leukemia in a mouse model via menin-MLL inhibition with a small molecule was reported, see: Borkin, D.; He, S.; Miao, H.; Kempinska, K.; Pollock, J.; Chase, J.; Purohit, T.; Malik, B.; Zhao, T.; Wang, J.; Wen, B.; Zong, H.; Jones, M.; Danet-Desnoyers, G.; Guzman, M. L.; Talpaz, M.; Bixby, D. L.; Sun, D.; Hess, J. L.; Muntean, A. G.; Maillard, I.; Cierpicki, T.; Grembecka, J. *Cancer Cell* **2015**. http://dx.doi.org/10.1016/j.ccell.2015.02.016. in press.
23. For information on the MLPCN and information on how to request probe compounds, such as ML399, see: http://mli.nih.gov/mli/mlpcn/.