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Research paper

High-affinity small molecular blockers of mixed lineage leukemia 1 (MLL1)-WDR5 interaction inhibit MLL1 complex H3K4 methyltransferase activity

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ABSTRACT

MLL1-WDR5 protein-protein interaction is essential for MLL1 H3K4 methyltransferase activity. Targeting MLL1 enzymatic activity to regulate expression level of MLL-dependent genes represents a therapeutic strategy for acute leukemia harboring MLL fusion proteins. Herein we reported a series of biphenyl compounds disturbed MLL1-WDR5 interaction. These compounds effectively inhibited MLL1 histone methyltransferase (HMT) activity in *vitro* and in MV4-11 cell line. The representative compound **30** (**DDO-2084**) inhibited proliferation and induced apoptosis of MV4-11 cells through deregulating expression level of *Hoxa9* and *Meis-1* genes, which emphasized our compounds were on-target. Optimization of compound **30** led to high-affinity inhibitors. Especially, compound **42** (**DDO-2117**, IC_{50} = 7.6 nM) bearing an amino and a 4-aminobutanamido group was the most potent inhibitor reported to-date, and showed the most potent inhibitory activity (IC_{50} = 0.19 μM) in HMT assay.

1. Introduction

Epigenetic regulation plays a vital role in gene expression as the changes of DNA sequences and transcription factors. DNA methylation and post-translational modification of histone are two main categories of epigenetic modification [1]. Two copies of each histone (H2A, H2B, H3 and H4) wrapped with DNA double helix constitute the basic units of nucleosome [2]. Modifications of histones include methylation, acetylation, phosphorylation, ubiquitination, sumoylation and other types of marks [3]. Catalyzed by protein methyltransferases, methyl groups were transferred from *S*-adenosyl-methionine (SAM) to the side chain nitrogen atoms of lysine and arginine residues of histones [4]. Lysine residues can be mono-, di-, and trimethylated by histone methyltransferases (HMTs) [5].

MLL1 was one of the HMTs family, specially catalyzing mono-, di-, and trimethylation of histone 3 lysine 4 (H3K4) through its evolutionarily conserved SET domain [6]. H3K4 methylation marks were recognized by different common structural features such as the royal superfamily and the PHD-finger superfamily (including the PHD fingers of BPTF and ING proteins) [7], which was essential for definitive hematopoiesis by regulating transcription activation of *Hox* genes and associated cofactors [8].

Dysregulation of MLL1 catalytic function increased the expression of *Hox* genes, which was associated with acute lymphoid leukemia (ALL) and acute myeloid leukemia (AML) [9]. Chromosomal rearrangements associated with MLL1 have been shown to cause MLL fusion proteins that were observed in kinds of leukemia [10]. MLL fusion proteins, lacking the SET domain and thus the H3K4 HMT enzymatic activity, cooperated with wild type MLL1 complex to active MLL1 target genes and lead to leukemogenesis [11].

MLL1 protein alone had weak histone methyltransferase

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activity, but the enzymatic activity massively enhanced upon complex formation of MLL1 with WDR5, ASH2L, and RBBP5 [12]. Disturbing MLL1-WDR5 protein-protein interaction (PPI) with peptides or small molecules dramatically inhibited the MLL1 H3K4 HMT activity and thus the expression of *Hox* and *Meis-1* genes [11–15]. Hence, blocking the interaction of MLL1-WDR5 with inhibitors may have a potential for the treatment of leukemia harboring MLL fusion proteins.

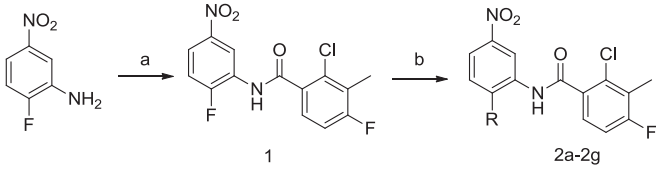
The first inhibitor of MLL1-WDR5 interaction was commenced by the report of **Ac-ARA-NH_2**, truncated from WIN motif of MLL1 as the minimum peptide, bound to WDR5 with high affinity [13]. Based on **Ac-ARA-NH_2**, linear peptidomimetic inhibitors of MLL1-WDR5 PPI were defined by modification of the Ala residue [11]. Then cyclic peptidomimetic **MM-401** was designed by constraining conformation of the linear peptide **MM-102** (Fig. 1a) [15]. Three small molecular inhibitors (**WDR5-0101-0103**) were disclosed by screening diverse compounds libraries with fluorescence polarization (FP) assay [14], then **WDR5-47** (**Fig. 1b**) was obtained from the optimization of **WDR5-0102** on the basis of the co-crystal structure of **WDR5-0102** and WDR5 protein [16]. A more potent antagonist **OICR-9429** (Fig. 1b) was reported to explore the mechanism of p30-dependent transformation and establish the essential p30 cofactor WDR5 as a therapeutic target in *CEBPA*-mutant AML [17,18].

However, even the most potent reported small molecular inhibitor, **OICR-9429** bound to WDR5 protein with only a moderate activity (K_d = 93 nM). Here, based on our reported inhibitor **W-26** (Fig. 1c) and SAR information [16,18,19], we designed and synthetized a series of biphenyl inhibitors to block MLL1-WDR5 PPI. Among those compounds, **30** effectively inhibited MLL1 HMT activity in *vitro* and in MV4-11 cell line. Down-regulating MLL-target genes expression level and inducing apoptosis of MV4-11 cells of compound **30** emphasized that our compounds were on-target. Optimization of compound **30** led to high-affinity inhibitors. Especially, up to today, compound **42** was the most potent small molecular inhibitor of the MLL1-WDR5 interaction (IC_{50} = 7.6 nM, K_d = 13.6 nM), and showed the most potent inhibitory activity (IC_{50} = 0.19 μM) in HMT assay.

2. Results and discussion

2.1. Synthesis of MLL1-WDR5 interaction inhibitors (see supplying information)

Compounds **2a-g** with *N*-methylpiperazine moiety modification were acquired following the synthetic route of **WDR5-47** (Scheme 1). 2-Fluoro-5-nitroaniline reacted with 2-chloro-4-fluoro-3-methylbenzoyl chloride to access intermediate **1**. Subsequent displacement of the fluoro with various amines, giving rise to the



Scheme 1. Synthesis of compounds 2a-g. Reaction: a. 2-chloro-4-fluoro-3-methylbenzoyl chloride, pyridine, DCM, r.t., 3 h; b. K_2CO_3, CH_3CN, reflux, 6h (**2a**) or amines, DIPEA, DMF, 80 °C, 4 h (**2b-2g**).

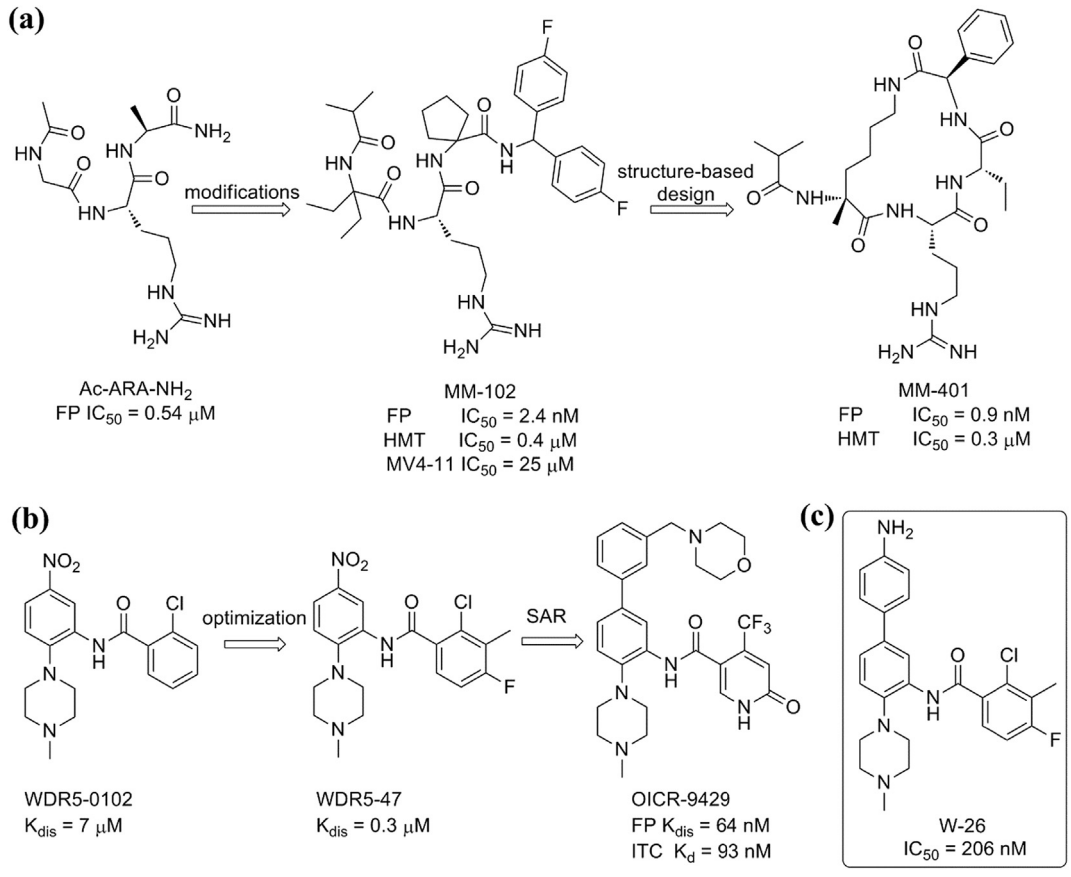


Fig. 1. Peptidomimetic and small molecular Inhibitors of MLL1-WDR5 interaction.

substituted benzamides **2a-g** in high yields.

All *N*-(4-(4-methylpiperazin-1-yl)-[1,1′-biphenyl]-3-yl) benzamide compounds (Fig. 2) in this article were synthetized from 4-bromo-1-fluoro-2-nitrobenzene. In Scheme 2, **6a-c** were prepared through Suzuki coupling reaction with different boronic acids. The fluoro of **6a-c** was substituted with *N*-methylpiperazine generating **7a-c**, followed by reducing **7a-c** with Tin (II) dichloride dehydrate to product **8a-c**. With nitration of 2-chloro-4-fluoro-3-methylbenzoic acid **3**, 2-chloro-4-fluoro-3-methyl-5-nitrobenzoic acid **4** was acquired. **8a-c** reacted with 2-chloro-4-fluoro-3-methyl-5-nitrobenzoyl chloride **5** or 4-fluoro-3-nitrobenzoyl chloride to get intermediate benzamides **W-27**, **16**–**17** or **26**. Subsequently, nitro compounds **W-27**, **16**–**17** and **26** were reduced to yield anilines **18**–**20** and **27**.

Compounds **15**, **21** and **28** were synthetized from 2-chloro-4-fluoro-3-methyl-5-nitrobenzoic acid **4** in Scheme 3. Compound **4** was reduced and acetylated to provide **13**. Treatment **11** or **8b** with 5-acetamido-2-chloro-4-fluoro-3-methylbenzoyl chloride **14** generated compounds **15** or **28**.**15** was reduced to yield target compound **21**.

The synthetic route of compounds **22**–**25** and **29**–**44** was listed in Scheme 4. 4-Bromo-1-fluoro-2-nitrobenzene was used as starting material through the alkylation with methyl piperazine to form **9** and compound **9** was reduced to produce **10**. Compound **10** reacted with 4-nitrophenylboronic acid through Suzuki coupling reaction catalyzed by Dichlorobis(triphenylphosphine)palladium(II) to give compound **11**. Compounds **22**, **23** and **29** were prepared from compound **11** and respective substituted nitrobenzoyl chlorides. Compounds **24**, **25** and **30** were obtained by reduction of compounds **22**, **23** and **29** in the following step. Compound **30** was acylated with different acids catalyzed by Reagent Castros to afford compounds **31**–**36**, **37B**–**42B** and **43**–**44**. Then the *t*-butyloxycarboryl group was removed from compounds **37B**–**42B** with TFA to provide target compounds **37**–**42**.

2.2. Identification of amino compounds as MLL1-WDR5 PPI potent inhibitors

2.2.1. Binding affinity evaluated with FP assay and isothermal titration calorimetry (ITC)

In previous report, the protonated *N*-methylpiperazine moiety played a critical role in binding to WDR5 protein through a key water-mediated hydrogen bond with Cys261 [16]. The Arg3765 of MLL *win* peptide and MLL peptidomimetic was sandwiched between two aromatic rings from Phe133 and Phe263 [11]. To explore different substitutes in this part and involve in another π-π stacking interaction with Phe263, a series of acyclic chains (**2a-c**) were append to the core phenyl ring and the methyl of *N*-methylpiperazine was substituted with aromatic rings (**2d-g**). Unfortunately,

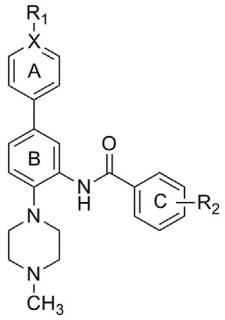


Fig. 2. Structure of *N*-(4-(4-methylpiperazin-1-yl)-[1,1′-biphenyl]-3-yl)benzamide compounds.

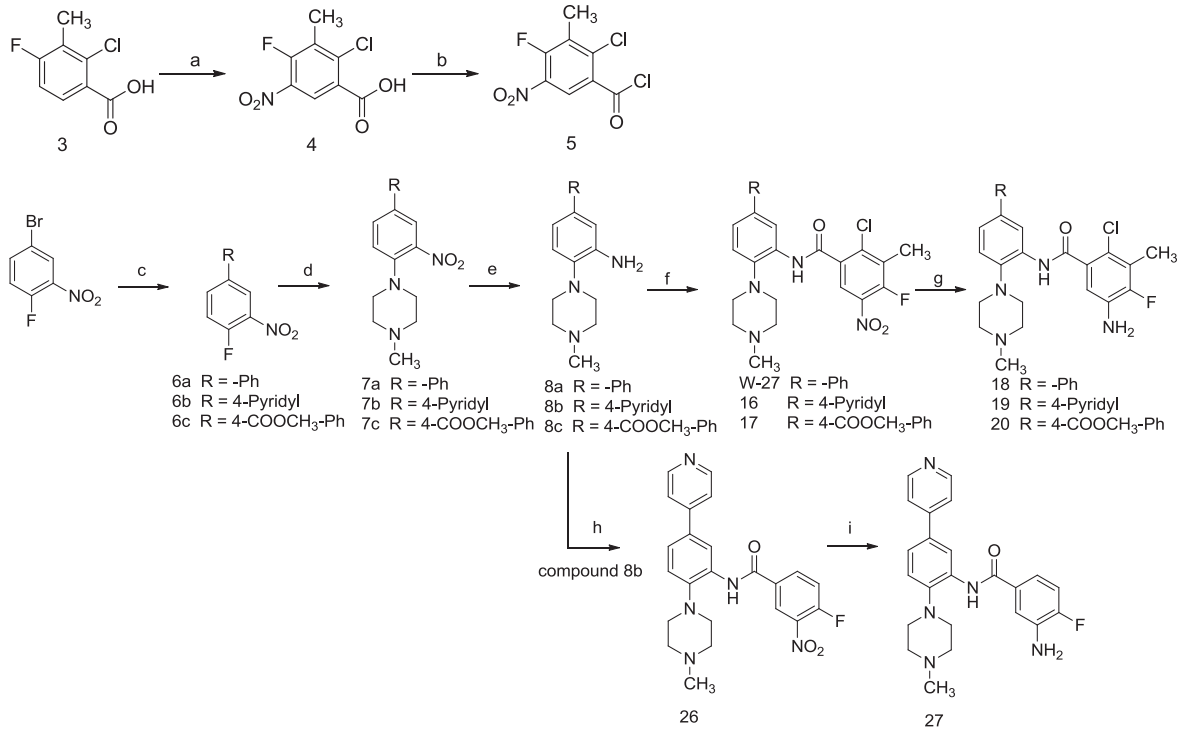
almost all of these modifications led to inactive compounds (**Table 1**). That proved the criticality of the *N*-methylpiperazine moiety. Hence, the *N*-methylpiperazine moiety was retained for further optimization.

We had previously reported that the introduction of aromatic ring A (Fig. 2) into 5-position of the structure of *N*-(2-(4-methylpiperazin-1-yl) phenyl) benzamide was accepted. The aromatic ring occupied the hydrophobic groove of WDR5 surrounded by side chains of Phe133, Phe149 and Tyr191 [19]. Given this position was located near to the solvent area and the π-π stacking interaction with an electron-rich aromatic ring Tyr191, a hydrophilic or withdrawing substituent at the 4-position of aromatic ring A was more suitable. That modifications of amide resulted in complete loss of activity showed the necessity of the amide for this structure binding to WDR5 [18,19]. Based on SAR information of this structure, more potent inhibitors were designed and synthetized.

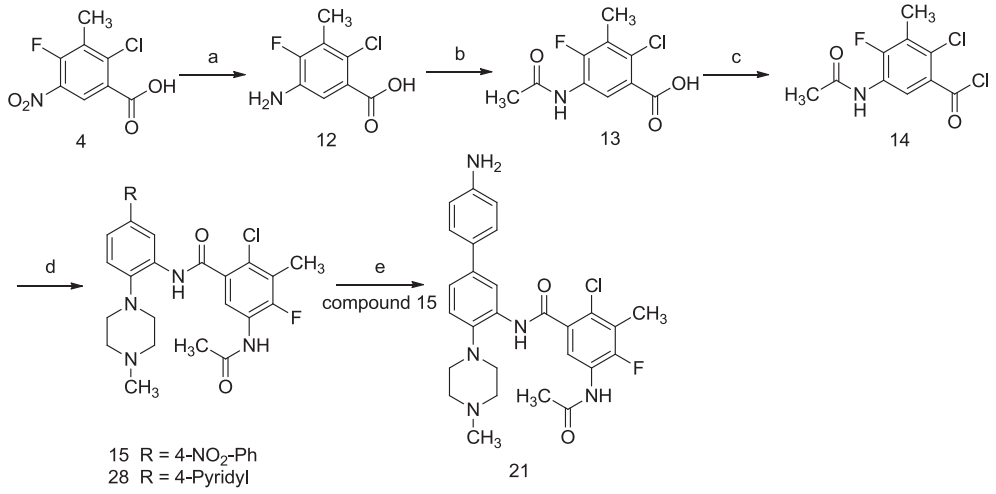
The benzamide moiety (ring C) of *N*-(4-(4-methylpiperazin-1-yl)-[1,1′-biphenyl]-3-yl) benzamide was optimized firstly. As reported, the introduction of an electron withdrawing group at the *o*-position of fluoro led to an appreciable potency gain (Table 2, compounds **W-27** and **16** versus compound **W-21** and **W-23** in reference 19, respectively), which was consistent with that an electron withdrawing group at the *o*-position of fluoro of the benzamide moiety decreased the electron density, thus strengthened the amide to Ser91 hydrogen bond interaction. This data indicated that an electron withdrawing group at the benzamide moiety was favorable in increasing activity of this structure.

Asp107 of WDR5 played a significant role in driving the binding of MLL1 to WDR5. Mutating Asp107 to Ala greatly impaired this binding [20]. Hence, an amino was introduced into the benzamide (ring C) to explore the interaction with side chain of Asp107. As anticipated, all compounds (Table 2, compounds **18, 19,** and **30**, IC_{50} = 47.9, 18.2, and 88.7 nM, respectively) achieved great gain in potency compared with those without amino (**W-21**, **W-23** and **W-26**, IC_{50} = 465.7, 103.9, and 206.4 nM, respectively). As the interaction of the pyridone of compound **OICR-9429** to Asp107, this series of compounds with a key amino were seen a direct and a water mediated hydrogen bonds to Asp107 in the docking model [17] (Fig. 3), which may account for their great increase in potency. To further verify the critical hydrogen bond interaction of the amino to Asp107, the amino was occluded with an acetyl (Table 2, compounds **15**, **21** and **28**). As expected, the inhibition activity of all compounds dramatically decreased. The additional acetyl may impede the hydrogen bond interaction with Asp107. Moreover, considering the groove occupied by benzamide was narrow, the increasing steric hindrance of acetyl may result in the potency loss. That hinted an exposed amino was suitable for the interaction with Asp107 and the narrow pocket. These results revealed that the amino of the benzamide was essential for compounds to enhance activity through forming hydrogen bonds with Asp107.

The hydrophobic substituents of benzamide were critical for potency improvement [16]. When the methyl, chloro and (or) fluoro groups (Table 3) were removed from corresponding compounds only keeping nitro, their activities in blocking MLL1-WDR5 interaction were decreased (**22**, **23** and **26**, IC_{50} = 1.1, 0.3 and 0.2 μM, respectively), and compounds with a single amino (**24**, **25** and **27**) even showed loss in potency. That directly proved the criticality of hydrophobic groups in benzamide and may indirectly indicate that an electron withdrawing group strengthened the hydrogen bond interaction between amide and Ser91 (**22** versus **24**, **23** versus **25**, and **26** versus **27**). But in compounds **19**, **20** and **30**, the methyl and chloro substituents of benzamide maintained interaction with the surrounding hydrophobic side chain of Ala47, Ala65 and Leu321 (Fig. 3) and kept activity. That further proved the



Scheme 2. Synthesis of compounds 16–20 and 26–27. Reaction: a. HNO_3, H_2SO_4, r.t., 4 h; b. $SOCl_2$, reflux, 6 h; c. boronic acids, $Pd(PPh_3)_2Cl_2$, Cs_2CO_3, dioxane, reflux, 20 h; d. *N*-methyl piperazine, DIPEA, DMF, 80 °C, 2 h; e. $SnCl_2.2H_2O$, ethyl acetate, reflux, 4 h; f. **5**, pyridine, DCM, r.t., 4 h; g. $SnCl_2.2H_2O$, ethyl acetate, reflux, 5 h; h. 4-fluoro-3-nitrobenzoyl chloride, pyridine, DCM, r.t., 4 h; i. $SnCl_2.2H_2O$, ethyl acetate, reflux, 6 h.

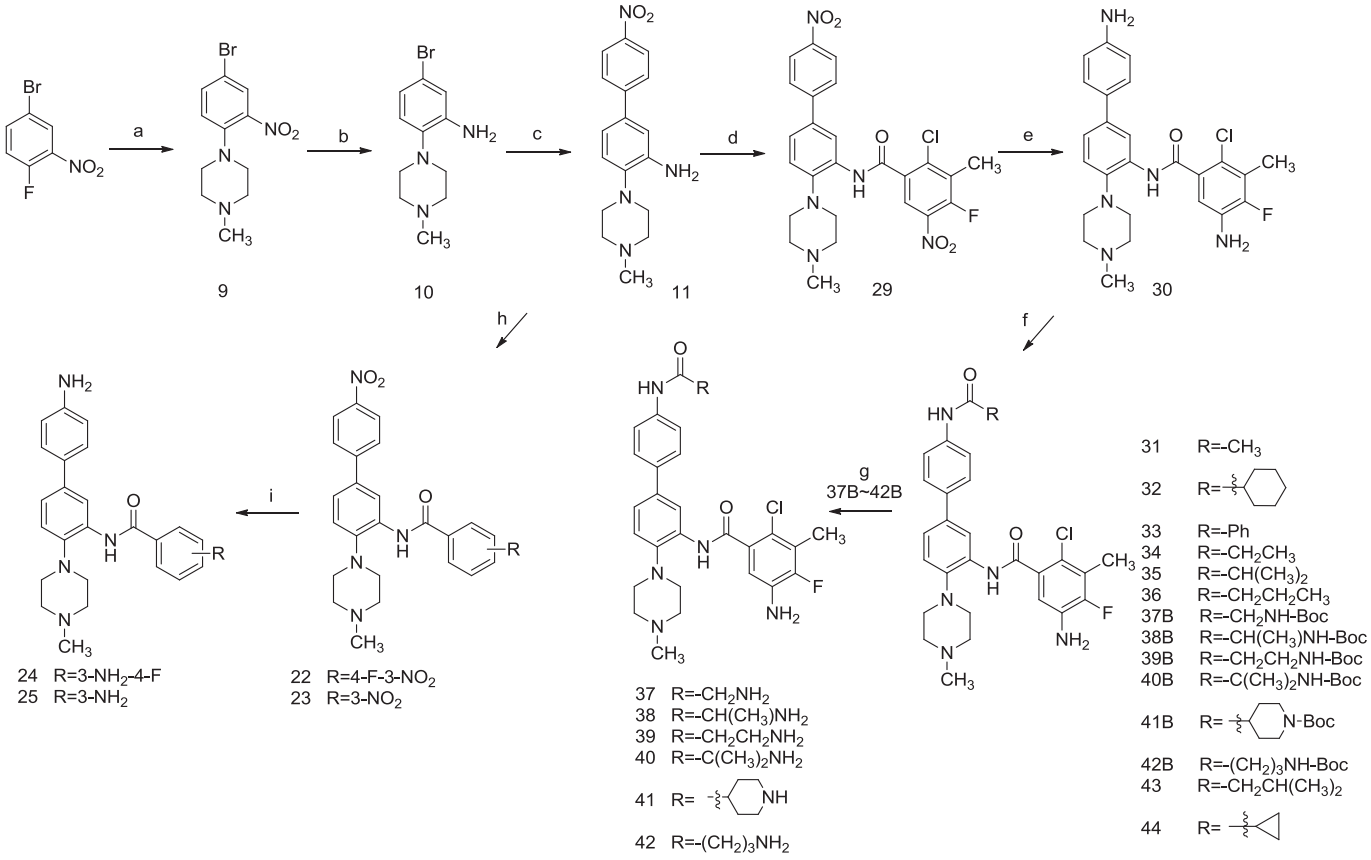


Scheme 3. Synthesis of compounds 15, 21 and 28. Reaction: a. Pd/C, H_2, CH_3OH, r.t., 10 h; b. CH_3COCl, DMF, pyridine, r.t., 4 h; c. $SOCl_2$, reflux, 6 h; d. **11** or **8b**, DCM, pyridine, r.t., 2 h; e. $SnCl_2.2H_2O$, ethyl acetate, reflux, 6 h.

criticality of hydrophobic groups in benzamide, and proper hydrophobic substitutes should be explored in future study.

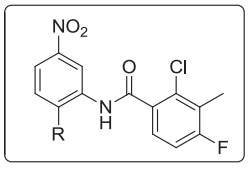
Docking study was applied to explore the binding model of compound **30** (Fig. 3a). Compound **30** recapitulated the interaction of **WDR5-47** to WDR5 protein including the hydrogen bonds between amide and the side chains of Cys261 and Ser91, the water mediated hydrogen bond between protonate *N*-methylpiperazine and Cys261, the π-π stacking interaction with Phe133, and the hydrophobic interaction with the pocket formed by Ala65, Ala47, and Leu321. But the additional amino formed a direct and an indirect hydrogen bonds interaction with Asp107 of WDR5 protein. Aromatic ring A occupied the hydrophobic groove of WDR5, surrounded by side chains of Phe133, Phe149 and Tyr191 and formed another π-π stacking interaction with Tyr191.

Overall, the additional direct and indirect hydrogen bonds with Asp107, along with the occupation of the hydrophobic pocket by the methyl, chloro and fluoro groups led to greater binding affinity of compounds with amino (**18**–**20**, and **30**). Direct binding of compound **30** to WDR5 protein was assessed by ITC experiments. With low K_d value of **30** (K_d = 202.0 nM), this series of compounds were confirmed to directly bind to WDR5 protein (Fig. 4). Considering the physicochemical property of these compounds, compound **30** (IC_{50} = 88.7 nM) was selected to verify the on-target biological activities in MV4-11 cell line and for further optimization.



Scheme 4. Synthesis of compounds 22–25 and 29–44. Reaction: a. *N*-methyl piperazine, DIPEA, DMF, 80 °C, 4 h; b. $SnCl_2.H_2O$, ethyl acetate, reflux, 6 h; c. 4-Nitrophenylboronic acid, $Pd(PPh_3)_2Cl_2$, Cs_2CO_3, dioxane, reflux, 20 h; d. **5**, pyridine, DCM, r.t., 4 h; e. $SnCl_2.H_2O$, ethyl acetate, reflux, 6 h; f. acids, BOP, TEA, DMF, r.t., 12 h. g. **37B–42B**, TFA, DCM, r.t., 2 h; h. 4-fluoro-3-nitrobenzoyl chloride or 3-nitrobenzoyl chloride, pyridine, DCM, r.t., 4 h; i. $SnCl_2.H_2O$, ethyl acetate, reflux, 6 h.

Table 1
Activity of compounds with *N*-methylpiperazine modification disturbing the interaction of MLL1 probe-WDR5.



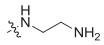
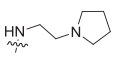
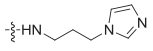
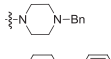
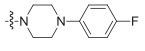
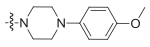
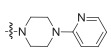
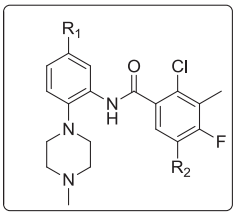
Cpd.	R	IC_{50}/μM (FP)
2a		>20
2b		>20
2c		>20
2d		>20
2e		>20
2f		>20
2g		>20
WDR5-47		338.2 ± 31.7 (nM)
MM-102	–	1.7 ± 0.4 (nM) (2.4 ± 1.7 nM reported)

Table 2
Activity of compounds with R_1 and R_2 modification disturbing the interaction of MLL1 probe-WDR5.



Cpd.	R_1	R_2	IC_{50}/nM (FP)
W-21	–Ph	–H	465.7 ± 24.6
W-23	4-Pyridyl	–H	103.9 ± 11.9
W-26	4–NH_2–Ph	–H	206.1 ± 26.4
W-27	–Ph	–NO_2	207.2 ± 34.7
15	4–NO_2–Ph	–$NHCOCH_3$	>20 (μM)
16	4-Pyridyl	–NO_2	77.1 ± 6.1
17	4–$COOCH_3$–Ph	–NO_2	92.5 ± 7.7
18	–Ph	–NH_2	47.9 ± 3.8
19	4–Pyridyl	–NH_2	18.2 ± 0.3
20	4–$COOCH_3$–Ph	–NH_2	70.0 ± 5.5
21	4–NH_2–Ph	–$NHCOCH_3$	3186 ± 101
28	4-Pyridyl	–$NHCOCH_3$	1363 ± 226
29	4–NO_2–Ph	–NO_2	285.3 ± 8.8
30	4–NH_2–Ph	–NH_2	88.7 ± 4.9
MM-102	–	–	1.7 ± 0.4 (2.4 ± 1.7 reported)

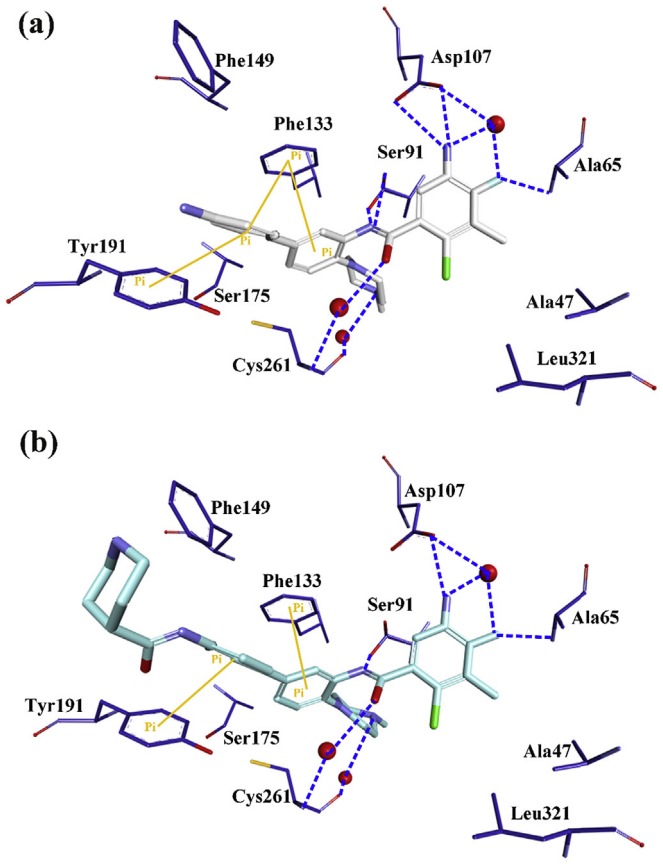
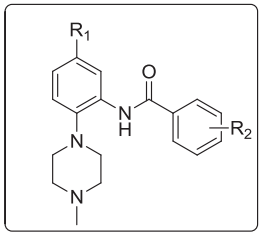


Fig. 3. Docking study of compounds **30** (a) and **41** (b) to WDR5 protein (PDB code: 4IA9). The carbon atoms of compounds **30**, **41** and WDR5 residues were colored white, light green, and purple, respectively. Hydrogen bonds were represented as blue dashed lines and π-π stacking interaction were plotted in orange lines. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Activity of compounds removing the chloro, methyl and (or) fluoro disturbing the interaction of MLL1 probe-WDR5.



Cpd.	R_1	R_2	IC_{50}/nM (FP)
22	4–NO_2–Ph	4–F–3–NO_2	1093 ± 50
23	4–NO_2–Ph	3–NO_2	285.1 ± 40
24	4–NH_2–Ph	4–F–3–NH_2	13.6 ± 1.0 (μM)
25	4–NH_2–Ph	3–NH_2	>20 (μM)
26	4–Pyridyl	4–F–3–NO_2	197.6 ± 24.5
27	4–Pyridyl	4–F–3–NH_2	5.8 ± 1.3 (μM)

2.2.2. Compound **30** inhibited MLL complex HTM activity in vitro and in MV4-11 cells

MLL1-WDR5 interaction was critical for the integrity of MLL1 complex, and thus the H3K4 methyltransferase activity [20–22]. Our compounds, which were designed to disturb this interaction, should inhibit the catalytic activity of MLL complex in *vitro* and in leukemia cells carrying MLL fusion proteins.

To explore the inhibition activity for MLL complex methyltransferase in *vitro*, compound **30** was evaluated in a recombinant MLL complex (MLL1, WDR5, RbBP5, and ASH2L proteins) Alpha Screen assays in *vitro*. The reported peptidomimetic **MM-102** (87% inhibition at 30 μM, IC_{50} = 0.75 μM) was selected as a positive control. With an amino at the benzamide (compared with **W-26,** 20% inhibition at 30 μM), compound **30** improved inhibition activity of MLL1-WDR5 interaction and MLL1 HMT activity (Fig. 5b, 75% inhibition at 30 μM, IC_{50} = 1.65 μM).

After characterizing the inhibition of MLL1 HMT activity in *vitro*, MV4-11 cells harboring MLL-AF4 fusion protein were applied to evaluate the inhibition activity of HMT. As well as in *vitro* study, the reported peptidomimetic **MM-102** was selected as a positive control. Compound **30** was able to reduce MLL1-dependent H3K4me1 at 5 μM, while no obvious differences in H3K4me1 was observed with 10 μM of **MM-102**. Consistent with the inhibition in *vitro*, H3K4me2 was reduced concentration-dependently when treated with a range of concentrations of **30** for 7 days, and 5 μM of **30** was at the same level with 10 μM of **MM-102** in inhibiting H3K4me2 in western blotting (Fig. 6).

Taken together, compound **30** was originally designed to block the interaction of MLL1-WDR5, effectively and potently inhibiting the MLL1 H3K4 methyltransferase activity in recombinant MLL complex Alpha Screen assays and in MV4-11 cell line.

2.2.3. Compound **30** reduced the expression level of Hoxa9 and Meis-1 genes

H3K4me markers were essential for the activation of MLL1 target *Hox* genes and cofactor *Mesi-1* gene [23,24], which were correlated with the methyltransferase activity of MLL complex [25]. MLL1-WDR5 interaction was required for the integrity of MLL1 complex, and therefore, its HMT activity. To assess whether compounds disturbing MLL1-WDR5 interaction affect the expression level of *Hoxa9* and *Meis-1* genes, RT-PCR experiments were performed in MV4-11 cell line harboring MLL-AF4 fusion protein.

Treating MV4-11 cells with compound **30** resulted in a strong and concentration dependent reduction in the expression level of *Hoxa9* and *Meis-1* genes as compared to the DMSO control (Fig. 7). Approximately 50% and 80% reduction in both genes expression were observed with 2.5 and 10 μM of compound **30**, respectively. These results suggested that inhibiting MLL1-WDR5 interaction with our compounds was an efficient strategy to regulate expression levels of MLL-fusion protein dependent genes, emphasizing on-target effects for these compounds and validating their specific mechanism of action.

2.2.4. Compounds **30** selectively inhibited proliferation of leukemia cells harboring MLL fusion proteins by inducing apoptosis

Down-regulation or suppression the expression level of *Hoxa9* and *Meis*-1 genes selectively inhibited growth of acute leukemia cell lines carrying MLL fusion protein [26]. Compound **30** was evaluated the inhibition of cells growth harboring with or without MLL fusion protein, and **MM-102** (IC_{50} = 20.7 μM) was selected as positive control. Compound **30** (IC_{50} = 17.7 μM) strongly inhibited cell proliferation in MV4-11 cells. When treating K562 and THP-1 cell lines carrying no MLL fusion protein with compounds **30**, no inhibition was observed (89.1%, 84.2% activity, respectively) at the concentration of 35 μM.

To further evaluate the anti-proliferation activity of our compounds, apoptosis induction experiment was carried out in MV4-11 leukemia cell line. As illustrated in Fig. 8, compound **30** effectively induced apoptosis of MV4-11 cells in a concentration-dependent manner and about 65% cells underwent death with treatment of

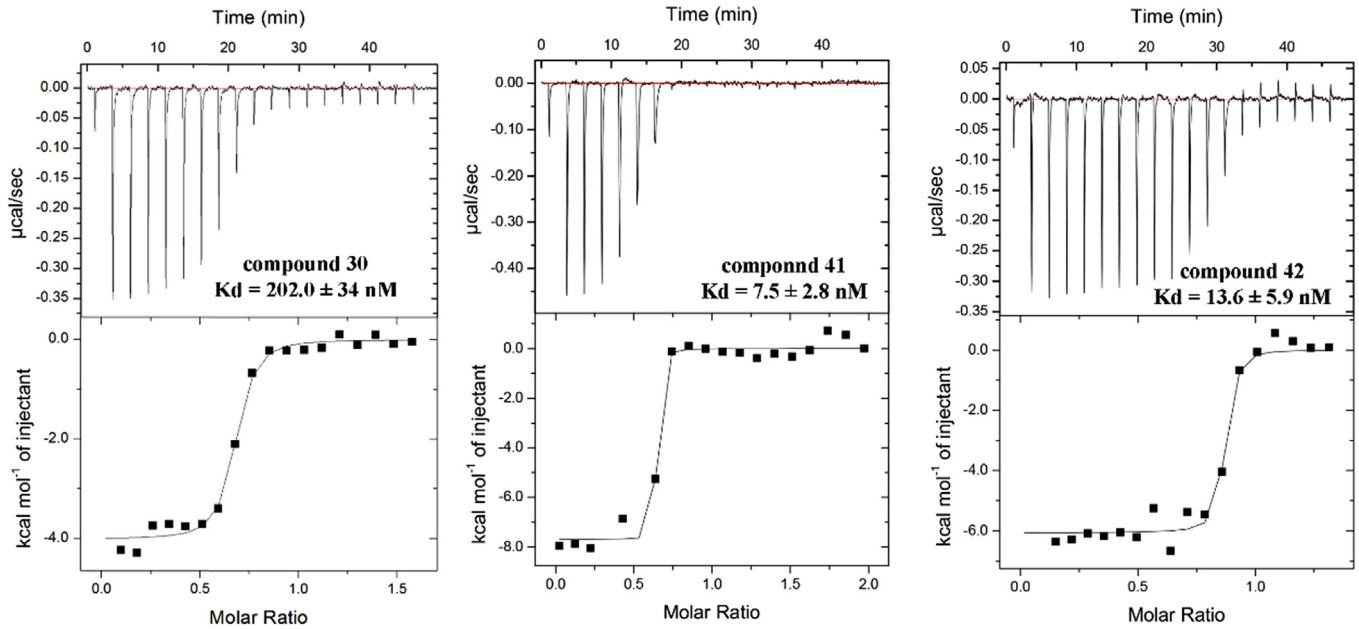


Fig. 4. Binding affinity of compounds **30**, **41** and **42** evaluated with ITC. Upper panels show heat of binding plotted versus time. Lower panels show fit to a single-site binding model to the binding isotherms. Kd (S.D.) derived from the fit is indicated.

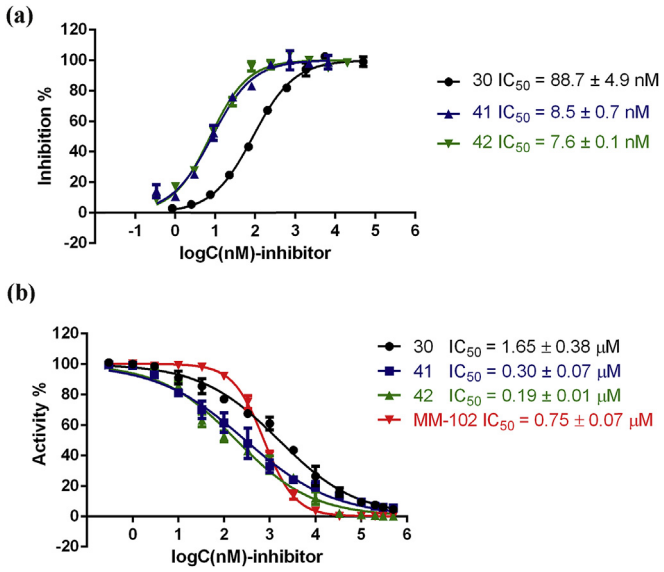


Fig. 5. (a) Competitive binding curves of compounds **30**, **41** and **42** determined using FP assay. 10mer-Thr-FAM was chosen as the fluorescent probe. (b) Inhibition of HMT activity of reconstituted MLL1 core complex as measured with Alpha Screen assays, and **MM-102** was selected as positive control. Values are shown as mean ± SD (n = 3).

30 at 10 µM for 72h. These data proved our compounds effectively and specially inhibit proliferation of leukemia cells carrying MLL fusion protein through inducing cells apoptosis.

The on-target effects of compound **30** were verified, but **30** bound to WDR5 protein with moderate activity (IC_{50} = 88.7 nM). Thus, **30** was selected as a candidate compound for further optimization.

2.3. Further modification of compound *30*

2.3.1. Binding affinity evaluation of compounds *31*–*44*

Retaining the 5-amino-2-chloro-4-fluoro-3-methylbenzamide

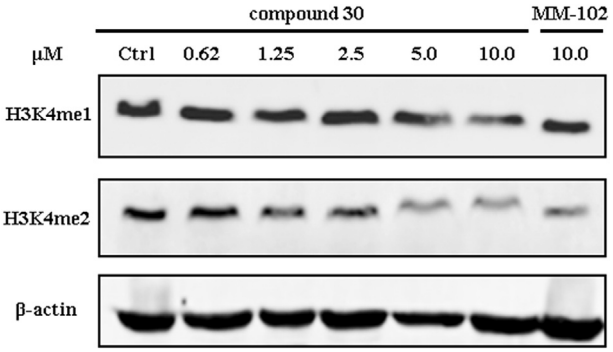


Fig. 6. Western blot analyses for the inhibition of HMT activity after treatment of MV4-11 cells with DMSO and 0.625 µM, 1.25 µM, 2.5 µM, 5.0 µM, and 10 µM compound **30**. **MM-102** was selected as positive control. H3K4me1 and H3K4me2 were determined using β-actin as loading control.

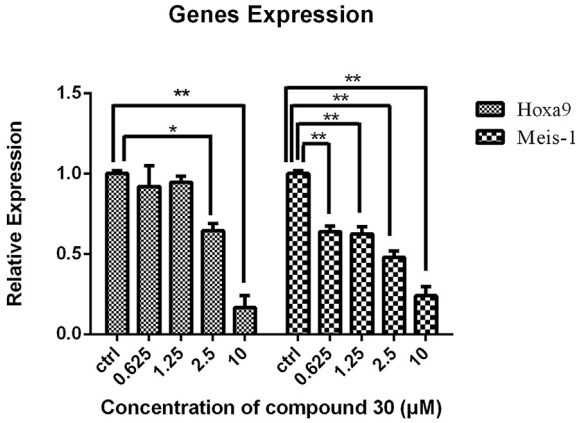


Fig. 7. Inhibition of *Hoxa9* and *Meis-1* genes expression in MV4-11 cells after treatment with DMSO and 0.625 µM, 1.25 µM, 2.5 µM and 10 µM compound **30** for 7 days assessed by RT-PCR. $^{*}p < 0.05$, $^{**}p < 0.01$, statistically significant difference from the nontreated blank control group.

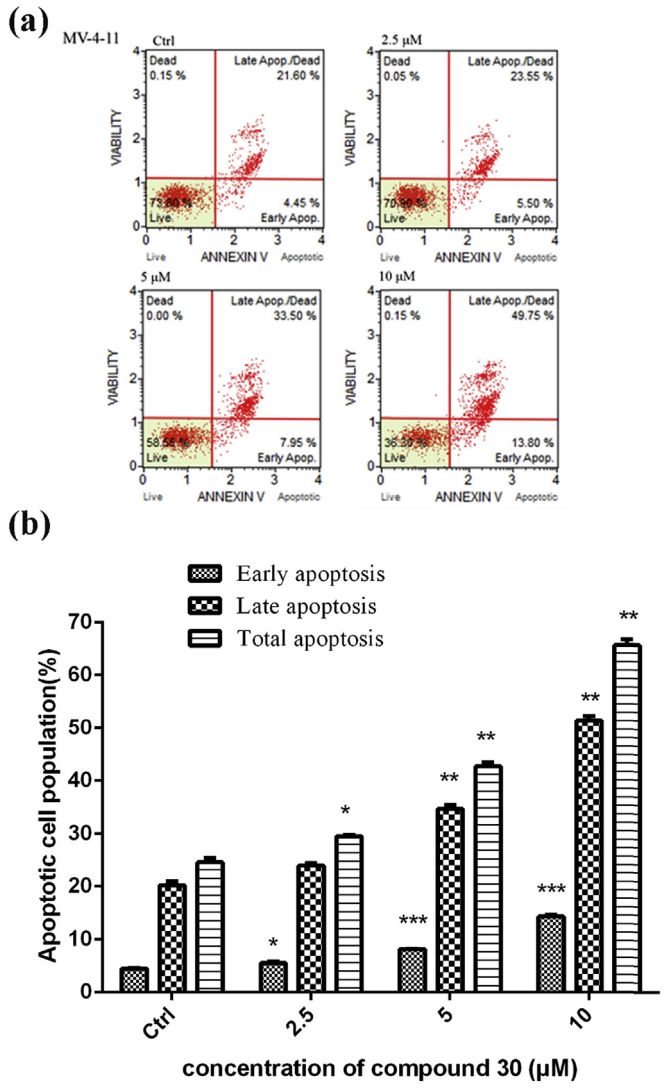


Fig. 8. Induction of apoptosis by DMSO and 2.5 μM, 5.0 μM, and 10 μM compound **30** in MV4-11 cells harboring MLL-AF4 protein. Concentration-dependent effects of compound **30** on apoptosis, analyzed by annexin V/propidium iodide (PI) staining. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, statistically significant difference from the non-treated blank control group.

of this structure, further modification was carried out at the 4-position of aromatic ring A that located in solvent area of WDR5 protein. From the docking model (Fig. 3a), the aromatic ring A was partially solvent exposed. But modification of this position did realize great gain in potency. The SAR study of aromatic ring A indicated that substitutes at 3-position resulted in twisting conformation of benzamide as well as the twist angle between the aromatic ring A and core phenyl ring B [19]. To maintain the π-π stacking interaction between the core phenyl ring B and Phe133, and to engage in an additional stacking interaction with Tyr191, optimization of aromatic ring A was selected at 4-position.

As shown in Table 4, almost all compounds substituted at 4-postion kept affinity to WDR5, except compounds **32**, **33** and **37B** (IC_{50} = 340.8, 421.9 and 1553 nM, respectively). The large hydrophobic groups of these compounds were located near to the solvent area of WDR5 protein, which may cause the potency loss. From the results, an aliphatic group seemed to be more suitable than an aromatic one (compounds **33**) in the 4-position of aromatic ring A. What's more, a hydrophilic tag substituent increased the activity of disturbing MLL1-WDR5 interaction (**34** versus **37**, **35** versus **38**, **36** versus **39**, and **32** versus **41**). When the amino was occluded with a *t*-butyloxy carbonyl (compounds **37B**, **38B**, **39B**, **40B** and **42B**), a slight decrease in potency was observed. That implied a hydrophobic group was not suitable for the solvent area of WDR5 protein and proved the vital effect of an exposed amino. The length of the linker between the amino and amide may have effect on the potency. Compounds **41** (IC_{50} = 8.5 nM) and **42** (IC_{50} = 7.6 nM), with a three carbon linker, were the most two potent inhibitors in blocking the MLL1-WDR5 interaction. Docking study (Fig. 3b) showed that the binding model of compound **41** was same as compound **30**, but the substituent group of aromatic A located in the solvent area.

Direct binding of compounds **41** and **42** to WDR5 protein were also assessed by ITC experiments. As the FP results, the affinity of optimized compounds **41** and **42** (Fig. 4, K_d = 7.5, 13.6 nM, respectively) were more 15-times improved than compound **30** (K_d = 202.0 nM). With the highest binding affinities to WDR5, compounds **41** and **42** also showed the most potent inhibitory activity in HMT assay with IC_{50} of 0.30 and 0.19 μM (Fig. 5b) in *vitro*.

In conclusion, modification of the 4-position of aromatic ring A with an amino tag and a linker with appropriate length, high-affinity inhibitors **41** (Kd = 7.5 nM) and **42** (Kd = 13.6 nM) were defined. These two compounds were the most potent small molecular inhibitors in blocking MLL1-WDR5 interaction reported to-date.

*2.3.2. Compounds **31**–**44** selectively inhibited proliferation of leukemia cells*

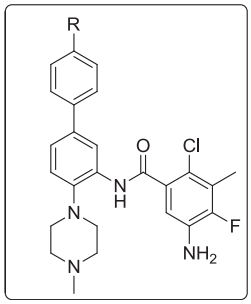
Compounds **31**–**44** optimized from compound **30** were evaluated their anti-proliferation activity of leukemia cell lines. As shown in Table 5, almost all compounds showed stronger inhibition in MV4-11 cells growth than compound **30**. Compounds (**32**, **34**, **35**, **36**, **37B**, **38B**, **39B** and **42B**) with a hydrophobic tag were more efficacious in growth inhibitory than those with a hydrophilic one (**41**, **37**, **38**, **39**, **37**, **38**, **39** and **42**). The polarity and cell membrane permeability of compounds may account for the difference in the activity. What's more, selective inhibition for leukemia cells harboring with or without MLL fusion proteins was clear when treating MV4-11, K562 and THP-1 cell lines with compounds **41** and **42** (Table 6). These data indicated that compounds optimized from **30** improved the anti-proliferation activity and specialty for leukemia cells carrying MLL fusion protein.

3. Conclusion

WDR5 was a critical protein for the integrity of MLL complex and MLL1 HMT activity [12,27,28]. Disturbing the interaction of MLL1-WDR5 with small molecular inhibitors represents a validated and attractive therapeutic strategy in acute leukemia with translocations of MLL genes [11,15,25,29].

In the present study, we designed and synthetized a series of potent compounds based on the SAR information and reported compound. A key amino was introduced into benzamide, which played a vital role in binding to WDR5 protein through a direct and an indirect hydrogen bonds with Asp107. With the additional amino, the binding affinity to WDR5 was great improved, as well as inhibition activity of MLL1 HMT in recombinant MLL complex (MLL1, WDR5, RbBP5, and ASH2L proteins) Alpha Screen assays in *vitro*. The representative compound **30** (**DDO-2084**) verified the inhibition activity of MLL1-dependent H3K4 methylation using western blotting experiment in MV4-11 leukemia cells. Down-regulation of expression level of *Hoxa9* and *Meis-1* genes, selective and effective inhibition of proliferation of leukemia cells harboring MLL1 fusion proteins and induction of apoptosis by disturbing MLL1-WDR5 interaction of **30** emphasized that our

Table 4
Activity of compounds optimized from **30** disturbing the interaction of MLL1 probe-WDR5.



Cpd.	R	IC_{50}/nM (FP)	Cpd.	R	IC_{50}/nM (FP)
31	$-NHCOCH_3$	70.1 ± 4.5	**39**	$-NHCOCH_2CH_2NH_2$	50.2 ± 5.3
32		340.8 ± 31.0	**39B**	$-NHCOCH_2CH_2NHBoc$	102.1 ± 14.8
33	−NHCOPh	421.9 ± 24.8	**40**	$-NHCOCH(i\text{-Pro})NH_2$	30.1 ± 2.6
34	$-NHCOCH_2CH_3$	81.5 ± 3.4	**40B**	$-NHCOCH(i\text{-Pro})NHBoc$	113.4 ± 10.3
35	$-NHCOCH(CH_3)_2$	95.6 ± 12.9	**41**		8.5 ± 0.7
36	$-NHCOCH_2CH_2CH_3$	76.0 ± 1.9	**42**	$-NHCO(CH_2)_3NH_2$	7.6 ± 0.1
37	$-NHCOCH_2NH_2$	62.5 ± 4.8	**42B**	$-NHCO(CH_2)_3NHBoc$	80.4 ± 16.9
37B	$-NHCOCH_2NHBoc$	1553 ± 122.3	**43**	$-NHCOCH_2CH(CH3)_2$	104.6 ± 4.6
38	$-NHCOCH(CH_3)NH_2$	15.0 ± 2.1	**44**		75.8 ± 4.3
38B	$-NHCOCH(CH_3)NHBoc$	77.9 ± 3.8	**MM-102**	–	1.7 ± 0.4

Table 5
Inhibition activity of compounds inhibited growth of MV4-11 cells harboring MLL-AF4 fusion protein.

Cpd.	IC_{50}/μM (MV:4–11)	Cpd.	IC_{50}/μM (MV:4–11)
30	17.7 ± 2.3	**39**	25.1 ± 11.1
31	12.5 ± 1.3	**39B**	4.6 ± 0.1
32	3.8 ± 0.5	**40**	2.8 ± 0.1
33	5.4 ± 0.2	**40B**	3.8 ± 0.1
34	6.5 ± 0.2	**41**	9.2 ± 0.9
35	4.5 ± 0.2	**42**	7.4 ± 1.4
36	2.3 ± 0.1	**42B**	4.8 ± 0.4
37	13.2 ± 1.2	**43**	3.9 ± 0.2
37B	4.1 ± 0.5	**44**	4.8 ± 0.2
38	11.1 ± 0.1	**MM-102**	20.7 ± 1.5
38B	4.3 ± 0.1		

Table 6
Selective inhibition of growth of leukemia cell lines with or without MLL fusion proteins.

Cpd.	IC_{50}/μM (MV:4–11)	activity@35 μM (K562)	activity@35 μM (THP-1)
30	17.7 ± 2.3	89.1%	84.2%
41	9.2 ± 0.9	113.7%	84.6%
42	7.4 ± 1.4	84.2%	86.8%
MM-102	20.7 ± 1.5 (25 μM reported)	37.8 ± 1.4 μM (IC_{50})	ND[a]

[a] ND = not determined.

compounds were on-target.

Modification of **30** with a 4-aminobutanamido group afforded to compounds **42** (**DDO-2117**). Especially, **42** was the most potent inhibitor with IC_{50} of 7.6 nM, and K_d of 13.6 nM reported to-date, and showed the most potent inhibitory activity in HMT assay with IC_{50} of 0.19 μM in *vitro*. It was the breakthrough achieving in MLL1-WDR5 PPI small molecular blockers in low nanomolar range, which may stimulate druggable compounds on this target. And our studies will pave the way toward further optimization of this structure into chemical probes for in *vivo* studies in MLL leukemia models and for potential therapeutic applications.

Conflicts of interest

The authors declare no other conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2016.08.036.

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