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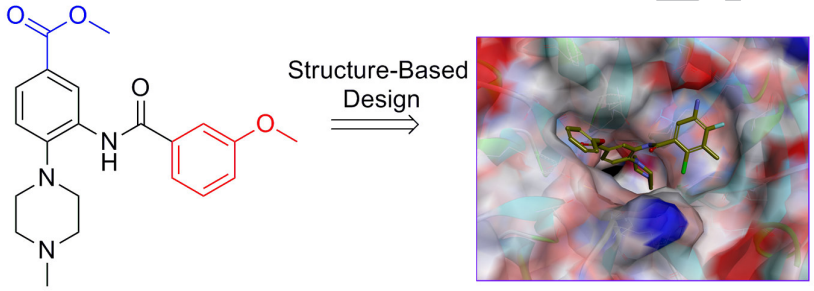

WDR5-0103
FP Kd = 3.0 μM

WL-15
FP IC_{50} = 26.4 nM
HMT IC_{50} = 5.4 μM

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ABSTRACT

WDR5 is an essential protein for enzymatic activity of MLL1. Targeting the protein-protein interaction (PPI) between MLL1 and WDR5 represents a new potential therapeutic strategy for MLL leukemia. Based on the structure of reported inhibitor **WDR5-0103**, a class of ester compounds were designed and synthetized to disturb MLL1-WDR5 PPI. These inhibitors efficiently inhibited the histone methyltransferase activity in *vitro*. Especially, **WL-15** was one of the most potent inhibitors, blocking the interaction of MLL1-WDR5 with IC_{50} value of 26.4 nM in competitive binding assay and inhibiting the catalytic activity of MLL1 complex with IC_{50} value of 5.4 μM. Docking model indicated that ester compounds suitably occupied the central cavity of WDR5 protein and recapitulated the interactions of **WDR5-0103** and the hydrophobic groups and key amino greatly increased the activity in blocking MLL1-WDR5 PPI.

1. Introduction

Histone lysine methyltransferases (HMTs) play a critical role in the regulation of gene expression, cell cycle, genome stability, and nuclear architecture [1]. Dysregulation or mutation of HMTs involved in the development of a wide range of human diseases, including cancer [2-3].

MLL1 is one member of six known SET1 family that catalyzes H3K4 mono-, di-, and trimethylation through its evolutionarily conserved SET domain [4]. In normal hematopoiesis, MLL1 regulates the expression level of *Hoxa9* and *Meis-1* genes, which are important for self-renewal of hematopoietic stem cells [5-7]. Dysregulation of MLL1 is associated with acute lymphoid leukemia (ALL) and acute myeloid leukemia (AML) [8]. MLL fusion proteins (MLL-FPs) were observed in leukemia resulted from MLL allele translocating, and MLL1-N terminal fusing in frame with one of more than 70 partners [9]. Lacking C-terminal SET domain, MLL-FPs cooperated with wild-type MLL1 complex to activate MLL1 targeted *Hox* and *Meis-1* genes, leading to leukemogenesis [10-11].

H3K4me2/3 markers were necessary for MLL1 and MLL-FPs to be recruited stably to the targeted *Hox* gene in leukemogenesis [12]. MLL1 alone can partially catalyze monomethylation of H3K4, but the enzymatic activity is weak [13]. In complex with WDR5, RbBP5 and Ash2L, MLL1 greatly increased the HMTs activity and the complex is necessary for dimethylation of H3K4 [14]. As a bridge between MLL1 and the reminder of the complex, WDR5 is required to maintain the integrity and the catalytic activity of MLL complex [15]. Thus, targeting MLL1-WDR5 PPI to inhibit MLL1 H3K4 HMT activity represents a new strategy for the treatment of leukemia carrying MLL-FPs [10].

Recently, series of small molecule inhibitors and peptidomimetics were identified to disturb MLL1-WDR5 PPI [16-22]. Three inhibitors were disclosed through screening compounds libraries using fluorescence polarization assay (FP assay) [16]. Based on the co-crystal structure of inhibitor-WDR5 protein, a more potent antagonist **WDR5-47** (**Figure 1a**) was acquired from optimization of **WDR5-0102** [17]. Then an aromatic ring was introduced at the 5-position of *N*-(2-(4-methylpiperazin-1-yl) phenyl) benzamide to defined compound **W-23** [18]. Further modification of this structure, **OICR-9429** was obtained to explore the mechanism of p30-dependent

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transformation in *CEBPA*-mutant AML [19-20]. With high affinity binding to WDR5, the minimal motif of MLL1 protein, 3-mer peptide **Ac-ARA-NH$_2$**, was determined based upon MLL1 sequences [21]. Then linear peptidomimetic **MM-102** and cyclic peptidomimetic **MM-401** were acquired by modification of **Ac-ARA-NH$_2$** [10, 22] (**Figure 1b**).

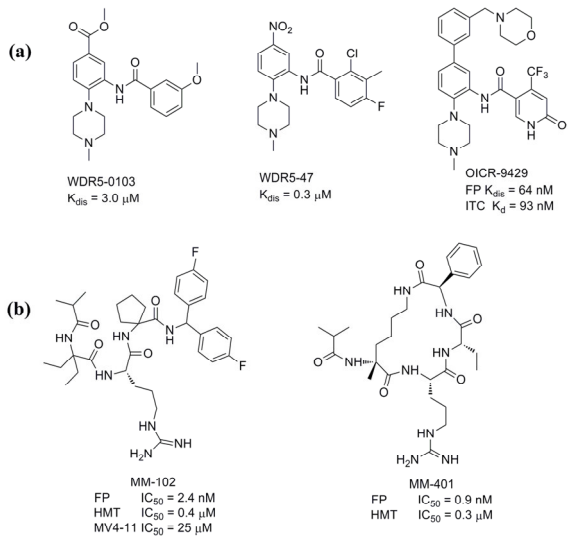


Figure 1. Inhibitors of MLL1-WDR5 PPI

Here, we reported a series of ester compounds optimized from **WDR5-0103** based on the co-crystal structure of **WDR5-0103** and WDR5 protein (**Figure 2**). These compounds designed to block MLL1-WDR5 PPI effectively inhibited the histone methyltransferase activity in *vitro*. Especially, **WL-15** (IC_{50} = 26.4 nM) was one of the most potent inhibitors. Docking study was applied to elucidate the binding model of ester compounds, which may stimulate more potent inhibitors in future.

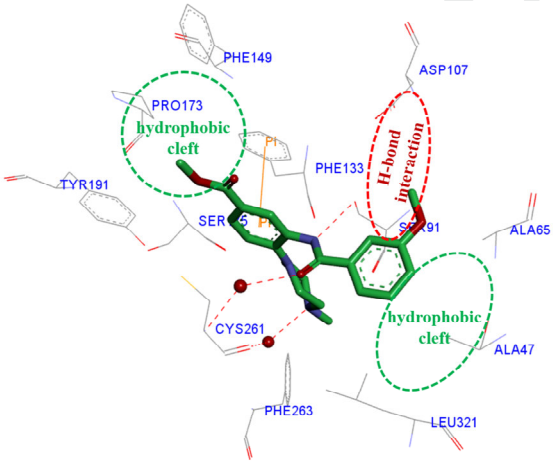
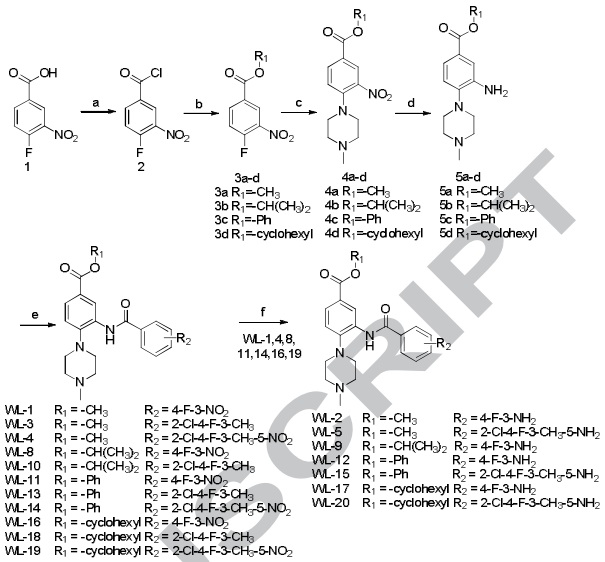


Figure 2. The design of ester compounds based on the co-crystal structure of inhibitor **WDR5-0103** and WDR5 protein (PDB code: 3UR4): **WDR5-0103** formed direct and water mediated hydrogen bonds interaction (red dashed lines) with Ser91 and Cys261 and π-π stacking (orange line) with Phe133. But the hydrophobic pocket surrounded by Tyr 191, Phe149 and Pro173 was only occupied by a methyl group and another pocket formed by Ala65, Ala47 and Leu321 was unoccupied. In addition, with a close distance to the benzamide, Asp107 was not involved in any interaction with **WDR5-0103**. So hydrophobic groups were introduced to occupy this two hydrophobic pockets and an amino was introduced to explore the H-bond interaction with Asp107.

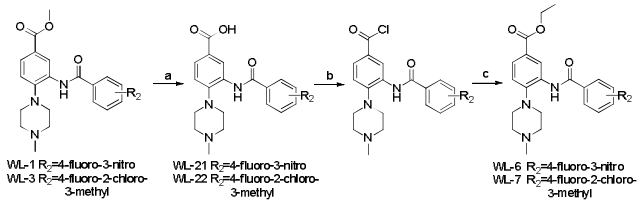
2. Results and discussion

2.1. Chemistry



Scheme 1. Synthesis of compounds **WL-1~5** and **WL-8~20**. Reagents and conditions: a. $SOCl_2$, reflux, 6h; b. R_1-OH, TEA, r.t., 0.5h; c. *N*-methyl piperazine, DIPEA, DMF, r.t., 0.5h; d. $SnCl_2.2H_2O$, EA, reflux, 4~8h; e. acyl chlorides, pyridine, DCM, r.t., 6h; f. $SnCl_2.2H_2O$, EA, reflux, 4~8h.

Ester derivatives (**WL-1~5** and **WL-8~20**) were synthetized following the synthetic route depicted in Scheme 1. 4-Fluoro-3-nitrobenzoyl chloride reacted with different alcohols giving ester intermediates **3a~d**. Compounds **4a~d** were formed by substituting the fluoro of **3a~d** with *N*-methyl piperazine. Reducing **4a~d** to provide amines **5a~d**. **5a~d** were treated with substituted acyl chlorides to afford target compounds. Nitro compounds **WL-1**, **4**, **8**, **11**, **14**, **16** and **19** were reduced by $SnCl_2.2H_2O$ to access compounds **WL-2**, **5**, **9**, **12**, **15**, **17** and **20**, respectively. In Scheme 2, 3-benzamido-4-(4-methylpiperazin-1-yl) benzoic acids **WL-21** and **WL-22** were generated by hydrolysing methyl ester of compounds **WL-1** and **WL-3**, respectively. Then benzoyl chlorides reacted with ethyl alcohol to provide ethyl ester compounds **WL-6** and **WL-7**. All of the target compounds were verified by ^{1}H NMR, HRMS, which in accordance with their depicted structures.



Scheme 2. Synthesis of compounds **WL-6~7** and **WL-21~22**. Reagents and conditions: a. 1M/LiOH, THF, r.t. 8h; $SOCl_2$, reflux, 6h; EtOH, TEA, r.t., 0.5h.

2.2. Competitive Binding Assay.

All synthetized compounds were determined the inhibition of MLL1-WDR5 PPI with FP assay, and the reported compound **MM-102** was selected as positive control. As illustrated in **Table1**, compound **WL-3**, the combination of **WDR5-0103** and **WDR5-47**, kept the activity in blocking MLL1-WDR5 PPI. Substituting the methyl of **WL-3** with various ester groups (**WL-7**, **10**, **13**) were tolerated except the cyclohexyl ester (**WL-18**).

Introduction of larger hydrophobic substitutes seemed to be more potent than a small one (**WL-13**, **10**, **7**, **3**), while removing of ester groups (**WL-21**, **22**) leading to complete loss in activity. It may because that the hydrophobic ester groups occupy the hydrophobic cleft surrounded by Phe149, Pro173and Tyr191, but the polar group such as carboxyl was not tolerated within the pocket (**Figure 4**). Phenyl substitute may form an additional π-π stacking interaction with the aromatic ring of residues such as Tyr191 of WDR5 protein, which resulted in higher activity of compounds with a phenol ester (**WL-13** versus **WL-3**, **7** and **10**, **WL-15** versus **WL-5**).

To explore whether electron withdrawing groups strengthened the amide-to-Ser91 hydrogen bond interaction [17], another electron withdrawing group-nitro was introduced at the *o*-position of F of the benzamide moiety. The effects of nitro were various in different esters. The nitro led to a slight increase of activity in compounds bearing a methyl (**WL-4** versus **WL-3**), while resulting in 4-fold loss of potency in phenol ester (**WL-14** versus **WL-13**). Introduction of nitro decreased the electron density of benzamide to strengthen the amide-to-Ser91 hydrogen bond interaction, but the hydrophobic effect may account for the loss of activity. Such a query required further investigation.

Removing methyl and chloro group form corresponding compounds, **WL-1** and **11** with 4-fluoro-3-nitro group kept potency, but compounds **WL-2** and **12** only with amino showed loss in activity. That proved the criticality of hydrophobic groups in benzamide moiety.

Great gain in potency was achieved when reducing the nitro compounds (**WL-4**, **14** and **19**) to amino compounds (**WL-5**, **15** and **20**). The key amino of compounds **WL-5**, **15** and **20** played an important role in increasing activity versus parent compounds **WL-3**, **13** and **18**, respectively. Asp107 of WDR5 was vital in driving binding MLL1 to WDR5 through interacting with Arg3765 of MLL peptide [23]. Compounds with an amino may form strong hydrogen bonds interaction with the residue of Asp107 of WDR5 protein, which led to the great gain of potency (**Figure 4b** and **4d**). Overall, combining the modification with a phenol ester and 5-amino-2-chloro-4-fluoro-3-methyl benzamide, **WL-15** effectively blocked MLL1-WDR5 interaction (IC_{50} = 26.4 nM) in FP assay (**Figure 3**).

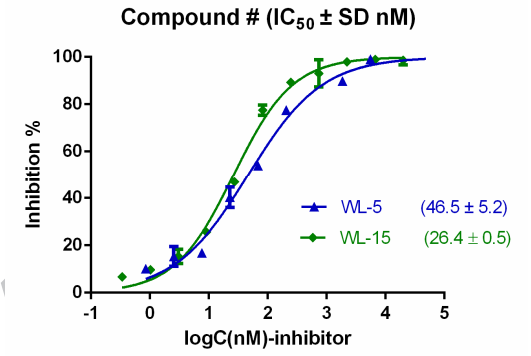


Figure 3. Competitive binding curves of compounds **WL-5** and **WL-15** as evaluated using FP-based assay. Data presented is the mean ± SD value of three independent determinations.

2.3. Molecular docking

To better understand the binding model of these compounds, we docked **WL-5** and **WL-15** into WDR5 protein by GOLD 5.1. The binding model of compounds **WL-5**, **15** and WDR5 protein was depicted in **Figure 4**. The best fitness scores of compounds **WL-5** and **WL-15** were 83.8 and 89.2, respectively. From the docking model, **WL-5** and **15** recapitulated interactions of **WDR5-0103** with WDR5 protein, including direct and water mediated hydrogen bonds interaction with Ser91 and Cys261 and π-π stacking with Phe133. But the 2-chloro-4-fluoro-3-methyl benzamide moiety of both compounds occupied the hydrophobic groove surrounded by side-chains of Ala 65, Ala47 and Ile90, and the additional amino formed a direct and a water mediated hydrogen bonds with Asp107, which may account for the great increase in potency. What's more, in the binding model of **WL-5** and **WL-15**, the hydrophobic cleft surround by Phe149, Pro173and Tyr191 was occupied by methyl and phenyl. Interestingly, the phenyl of **WL-15** form an additional π-π stacking with Tyr191 (**Figure 4d**), which may lead to an appreciable potency gain of **WL-15**. The molecular docking results, along with the competitive binding assay data, suggested that compounds **WL-5** and **WL-15** were potential inhibitors in disturbing MLL1-WDR5 protein-protein interaction.

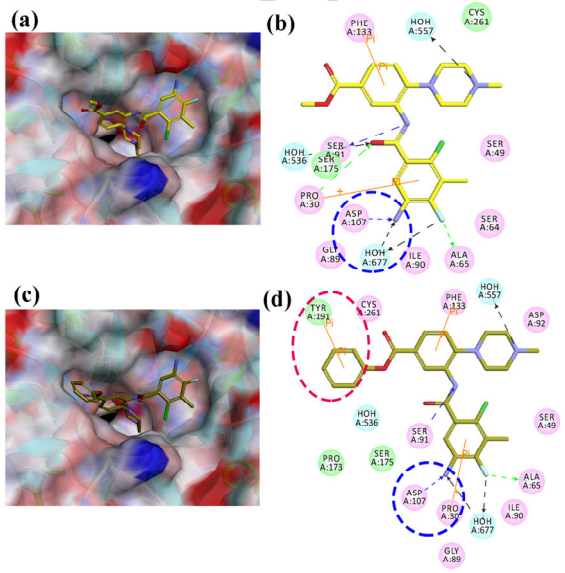
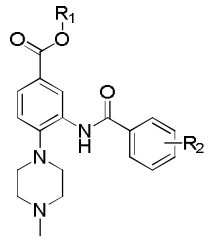


Figure 4. Docking compounds **WL-5** and **WL-15** into the binding pocket of **WDR5-0103** in WDR5 protein (PDB code: 3UR4). Left: 3D models of compounds **WL-5** and **WL-15** inserted into the cavity of WDR5, respectively; Right: 2D diagram of the interaction between **WL-5**, **WL-15** and WDR5. The amino acid residues were shown as balls. The carbon atoms of compounds **WL-5** and **WL-15** were colored yellow and green, respectively. The oxygen atoms and nitrogen atoms of both compounds were colored red and light blue, respectively. Hydrogen bonds were represented as dashed arrows and π-π stacking interaction were plotted in orange lines.

2.4. Inhibition of MLL complex methyltransferase activity in vitro

WDR5 was essential for the integrity of MLL core complex and the catalytic activity of methyltransferase [15]. Disturbing the interaction of MLL1-WDR5 with small molecule should inhibit the histone methyltransferase activity. To explore the inhibition of ester compounds for MLL complex methyltransferase activity, the most two potent compounds (**WL-5** and **15**, **Figure 3**) were determined the IC_{50} value in a recombinant MLL complex Alpha Screen assays in *vitro*. **MM-102** was selected as the positive control. As shown in **Figure 5**, compounds **WL-5** and **WL-15** designed to block MLL1-WDR5 PPI efficiently inhibited the catalytic activity of the MLL1 complex (IC_{50} = 4.6 and 5.4 μM, respectively).

Table1. The IC_{50} values of ester compounds in disturbing the interaction of MLL1-WDR5 evaluated in FP assay.



Compd.	R_1	R_2	IC_{50}[a] (nM)
WL-1	$-CH_3$	4-fluoro-3-nitro	454.0 ± 15.8
WL-2	$-CH_3$	3-amino-4-fluoro	>20 μM
WL-3	$-CH_3$	2-chloro-4-fluoro-3-methyl	433.2 ± 25.6
WL-4	$-CH_3$	2-chloro-4-fluoro-3-methyl-5-nitro	244.9 ± 13.2
WL-5	$-CH_3$	5-amino-2-chloro-4-fluoro-3-methyl	46.5 ± 5.2
WL-6	$-CH_2CH_3$	4-fluoro-3-nitro	>20 μM
WL-7	$-CH_2CH_3$	2-chloro-4-fluoro-3-methyl	283.9 ± 19.6
WL-8	$-CH(CH_3)_2$	4-fluoro-3-nitro	1171 ± 100
WL-9	$-CH(CH_3)_2$	3-amino-4-fluoro	>20 μM
WL-10	$-CH(CH3)_2$	2-chloro-4-fluoro-3-methyl	263.5 ± 25.5
WL-11	-Ph	4-fluoro-3-nitro	483.5 ± 25.6
WL-12	-Ph	3-amino-4-fluoro	>20 μM
WL-13	-Ph	2-chloro-4-fluoro-3-methyl	162.4 ± 12.6
WL-14	-Ph	2-chloro-4-fluoro-3-methyl-5-nitro	811.6 ± 50.2
WL-15	-Ph	5-amino-2-chloro-4-fluoro-3-methyl	26.4 ± 0.5
WL-16	-cyclohexyl	4-fluoro-3-nitro	>20 μM
WL-17	-cyclohexyl	3-amino-4-fluoro	>20 μM
WL-18	-cyclohexyl	2-chloro-4-fluoro-3-methyl	>20 μM
WL-19	-cyclohexyl	2-chloro-4-fluoro-3-methyl-5-nitro	>20 μM
WL-20	-cyclohexyl	5-amino-2-chloro-4-fluoro-3-methyl	58.8 ± 0.9
WL-21	-H	4-fluoro-3-nitro	>20 μM
WL-22	-H	2-chloro-4-fluoro-3-methyl	>20 μM
MM-102	-	-	1.7 ± 0.4

[a] Data presented is the mean ± SD value of three independent determinations.

2.5. Anti-proliferative activity

Active compounds in FP assay were selected to test their ability in inhibiting proliferation of two leukemia cell lines with or without MLL1 fusion protein.

From **Table2**, most of these compounds kept the anti-proliferative activity in MV4-11 cells carrying MLL-AF4 fusion protein. Compounds (**WL-1**, **4**, **8**, **11**, **14**) with a nitro showed more potent in anti-proliferative activity. But compounds **WL-5**, **15** and **20** with an amino almost lost potency in inhibiting cell growth, which was not consistent with their target-based data. Permeability is an important property that reflects the ability of molecule to diffuse through the cell membrane. Permeability test indicated that amino compounds had poor permeability compared with the nitro analogues. Poor cellular membrane permeability might account for the weak cell growth inhibition of **WL-20**. To our delight, compounds **WL-1** and **WL-4** with moderate activity in FP assay, effectively inhibited leukemia cells growth in MV4-11 (IC_{50} = 4.7, 3.4 μM, respectively).

Active compounds **WL-1**, **4** and **8** showed selective inhibition of cells growth in MV4-11 and K562. Especially, compound **WL-1** was 10-fold more potent in MV4-11 than K562. However, phenol ester compounds also showed activity in inhibiting growth of K562 cells harbouring BCR-ABL fusion protein, it was worth exploring to improve selectivity in future.

Table2. Inhibition activity of leukemia cell lines and permeability of compounds.

Compd.	MV:4-11(IC_{50}[a]/μM)	K562(IC_{50}[a]/μM)	*Pe* pH 7.4 (10^{-6} cm/s)
WL-1	4.7 ± 1.4	57.1 ± 1.3	52.4 ± 8.6
WL-3	>100	>100	59.8 ± 6.1
WL-4	3.4 ± 0.1	17.2 ± 4.6	74.8 ± 9.1
WL-5	>100	>100	52.9 ± 5.3
WL-7	49.8 ± 10.4	90.7 ± 4.2	44.9 ± 4.0
WL-8	13.3 ± 3.0	58.0 ± 4.5	61.4 ± 2.3
WL-10	57.1 ± 1.6	>100	49.8 ± 1.5
WL-11	10.8 ± 0.2	11.6 ± 1.0	60.0 ± 6.6
WL-13	45.2 ± 3.8	53.9 ± 3.7	48.0 ± 3.4
WL-14	9.9 ± 0.8	15.7 ± 1.5	56.3 ± 6.0
WL-15	66.5 ± 14.8	33.4 ± 0.7	38.0 ± 2.7
WL-20	>100	>100	5.6 ± 6.6
MM-102	20.7 ± 1.5	37.8 ± 1.4	ND [b]

[a] Data presented is the mean ± SD value of three independent determinations.

[b] ND = not determined.

3. Conclusion

Based on the co-crystal structure of **WDR5-0103** and WDR5 protein, a series of ester compounds were designed and synthetized to disturb the interaction of MLL1-WDR5. With ester groups occupied the hydrophobic groove surrounded by Phe149, Pro173 and Tyr191, compounds **WL-3**, **7**, **10**, and **13** kept the affinity to WDR5 and the anti-proliferation activity of MV4-11 cells. More potent inhibitors were acquired by introducing a nitro or an amino into benzamide moiety. The binding models of **WL-5** and **15** to WDR5 protein were elucidated by docking study. **WL-5** and **15** recapitulated interactions of **WDR5-0103** with WDR5 protein, but the additional amino formed hydrogen bonds interaction with Asp107, which increased activity greatly. Further, disturbing MLL1-WDR5 PPI with nanomolar activity (IC_{50} = 46.5 and 26.4 nM, respectively) in FP assay, **WL-5** and **15** efficiently inhibited MLL complex HMT activity in *vitro* (IC_{50} = 4.6 and 5.4 μM, respectively). Introducing a nitro at the benzamide moiety, **WL-1**, **4**, and **14** kept the affinity to WDR5, but effectively and selectively inhibited the growth of MV4-11 cells harboring MLL-AF4 fusion protein. However, the anti-proliferation activity of amino compounds were not consistent with the high affinity, which reminded that the physicochemical properties of ester compounds would be promoted in future study.

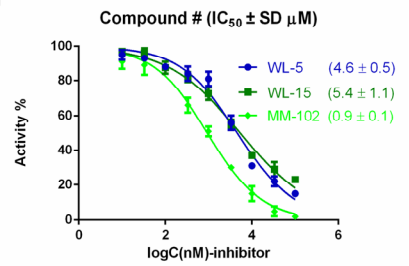


Figure 5. Effect of **WL-5** and **WL-15** on MLL complex activity as measured with Alpha Screen assays in *vitro*. **MM-102** was selected as positive control.

4. Experiments

4.1. Synthesis

Scheme 1. Synthesis of compounds WL-1~5 and WL-8~20:

Step a: Preparation of 4-fluoro-3-nitrobenzoyl chloride (2)

4-Fluoro-3-nitrobenzoic acid **1** (3.0 g, 16.2 mmol) was added to 15ml $SOCl_2$ as solvent and acylation reagent, refluxing for 6h. Then the solution was evaporated to give yellow oil **2**, which was used in the subsequent reaction.

Step b: General Method for the Preparation of Compounds 3a-d by Addition of Different Alcohols to 4-fluoro-3-nitrobenzoyl chloride

4-Fluoro-3-nitrobenzoyl chloride (16.2 mmol, got from **step a)** dissolved in anhydrous dichloromethane (20 mL) was added to different alcohols under N_2 atmosphere at room temperature. This was followed by the addition of TEA (2.7 mL, 19.4 mmol), and the resulting solution was stirred at room temperature for 0.5h. The solution was subsequently removed to provide **3a-d** as solid.

Methyl 4-fluoro-3-nitrobenzoate (3a)

Yield: 94.3%. m.p. 62-65 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.74 (dd, *J* = 7.2, 2.2 Hz, 1H), 8.34-8.29 (m, 1H), 7.41-7.35 (m, 1H), 3.98 (s, 3H). m/z (ESI-MS): 200.2013 $[M+H]^+$.

Isopropyl 4-fluoro-3-nitrobenzoate (3b)

Yield: 95.0%. Light yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.72 (dd, *J* = 7.2, 2.2 Hz, 1H), 8.35-8.29 (m, 1H), 7.41-7.35 (m, 1H), 5.31-5.27 (m, 1H), 1.40 (d, *J* = 6.3 Hz, 6H). m/z (ESI-MS): 238.0627 $[M+H]^+$.

Phenyl 4-fluoro-3-nitrobenzoate (3c)

Yield: 84.7%. m.p. 100-103 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.93 (dd, *J* = 7.1, 2.1 Hz, 1H), 8.52-8.47 (m, 1H), 7.50-7.45 (m, 3H), 7.35-7.30 (m, 1H), 7.27-7.22 (m, 2H). m/z (ESI-MS): 262.0471 $[M+H]^+$.

Cyclohexyl 4-fluoro-3-nitrobenzoate (3d)

Yield: 76.6%. Light yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.72 (dd, *J* = 7.2, 2.1 Hz, 1H), 8.35-8.31 (m, 1H), 7.41-7.35 (m, 1H), 5.05 (m, 1H), 2.29 (m, 2H), 1.99-1.96 (m, 2H), 1.84-1.79 (m, 2H), 1.63-1.43 (m, 4H). m/z (ESI-MS): 290.2695 $[M+Na]^+$.

Step c: General Method for the Preparation of Compounds 4a-d by Addition of *N*-methyl piperazine to Compounds 3a-d

To a solution of compounds **3a-d** (16.0 mmol) in DMF (30 mL) at room temperature was added *N*-methyl piperazine (2.08g, 2.3mL, 20.8 mmol), followed by *N*, *N*-diisopropylethylamine (3.65 mL, 20.8 mmol). The resulting solution was reacted in room temperature for 0.5 h. Following dilution with EA (100 mL), the mixture was washed with water (3 x 50 mL), then the organic phase was dried (Na_2SO_4), filtered, and concentrate to afford product **4a-d**.

Methyl 4-(4-methylpiperazin-1-yl)-3-nitrobenzoate (4a)

Yield: 79.3%. Brown red oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.42 (d, *J* = 2.1 Hz, 1H), 8.05 (dd, *J* = 8.8, 2.1 Hz, 1H), 7.06 (d, *J* = 8.8 Hz, 1H), 3.90 (s, 3H), 3.20 (t, *J* = 4.7 Hz, 4H), 3.56 (t, *J* = 4.7 Hz, 4H), 2.35 (s, 3H). m/z (ESI-MS): 280.1299 $[M+H]^+$.

Isopropyl 4-(4-methylpiperazin-1-yl)-3-nitrobenzoate (4b)

Yield: 86.3%. Brown red oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.40 (d, *J* = 2.1 Hz, 1H), 8.06 (dd, *J* = 8.8, 2.1 Hz, 1H), 7.07 (d, *J* = 8.8 Hz, 1H), 5.23 (m, 1H), 3.19 (t, *J* = 4.9 Hz, 4H), 2.57 (t, *J* =

4.9 Hz, 4H), 2.35 (s, 3H), 1.35 (d, *J* = 6.3, 6H). m/z (ESI-MS): 308.1603 $[M+H]^+$.

Phenyl 4-(4-methylpiperazin-1-yl)-3-nitrobenzoate (4c)

Yield: 41.1%. m.p. 103-106 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.61 (d, *J* = 2.1 Hz, 1H), 8.20 (dd, *J* = 8.8, 2.1 Hz, 1H), 7.47-7.41 (m, 2H), 7.31-7.28 (m, 1H), 7.22-7.19 (m, 2H), 7.14 (d, *J* = 8.8 Hz, 1H), 3.26 (t, *J* = 4.9 Hz, 4H), 2.59 (t, *J* = 4.9 Hz, 4H), 2.38 (s, 3H). m/z (ESI-MS): 342.1443 $[M+H]^+$.

Cyclohexyl 4-(4-methylpiperazin-1-yl)-3-nitrobenzoate (4d)

Yield: 80.7%. Brown red oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.41 (d, *J* = 2.1 Hz, 1H), 8.07 (dd, *J* = 8.7, 2.1 Hz, 1H), 7.08 (d, *J* = 8.8 Hz, 1H), 5.02-4.96 (m, 1H), 3.19 (t, *J* = 4.9 Hz, 4H), 2.57 (t, *J* = 4,9 Hz, 4H), 2.36 (s, 3H), 1.92-1.91 (m, 2H), 1.79-1.76 (m, 4H), 1.62-1.54 (m, 4H). m/z (ESI-MS): 348.1910 $[M+H]^+$.

Step d: General Method for the Preparation of Compounds 5a-d by Reduction of the nitro of Compounds 4a-d

To a solution of compounds **4a-d** (13.2 mmol) in EA (100 mL) at room temperature was added $SnCl_2.2H_2O$ (8.9 g, 39.7 mmol). The resulting solution was then refluxed with vigorous stirring for 8 h, and subsequently cooled to room temperature. Following dilution with EA (150 mL) and the mixture was neutralize with saturated sodium bicarbonate solution. Then filtered and washed residue with EA (5 x 40 mL), and the organic phase was dried (Na_2SO_4), filtered, and concentrated to afford compounds **5a-d**.

Methyl 3-amino-4-(4-methylpiperazin-1-yl) benzoate (5a)

Yield: 82.1%. m.p. 172-175 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.44 (dd, *J* = 8.2, 2.0 Hz, 1H), 7.40 (d, *J* = 2.0 Hz, 1H), 6.99 (d, *J* = 8.2 Hz, 1H), 3.96 (s, 2H), 3.00 (br s, 4H), 2.59 (br s, 4H), 2.37 (s, 3H). m/z (ESI-MS): 250.1542 [M+H]+.

Isopropyl 3-amino-4-(4-methylpiperazin-1-yl) benzoate (5b)

Yield: 91.5%. m.p. 134-136 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.44 (dd, *J* = 8.2, 1.9 Hz, 1H), 7.39 (d, *J* = 1.9 Hz, 1H), 6.99 (d, *J* = 8.2, 1H), 5.20 (m, 1H), 3.95 (s, 2H), 2.99 (br s, 4H), 2.58 (br s, 4H), 2.36 (s, 3H), 1.33 (d, *J* = 6.3, 6H). m/z (ESI-MS): 278.1855 $[M+H]^+$.

Phenyl 3-amino-4-(4-methylpiperazin-1-yl) benzoate (5c)

Yield: 85.2%. m.p. > 250 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.65 (dd, *J* = 8.2, 2.0 Hz, 1H), 7.57 (d, *J* = 2.0 Hz, 1H), 7.47-7.42 (m, 2H), 7.28-7.20 (m, 3H), 7.08 (d, *J* = 8.3 Hz, 1H), 4.03 (s, 2H), 3.07 (br s, 4H), 2.64 (br s, 4H), 2.41 (s, 3H). m/z (ESI-MS): 312.1696 $[M+H]^+$.

Cyclohexyl 3-amino-4-(4-methylpiperazin-1-yl) benzoate (5d)

Yield: 70.8%. m.p. 102-104 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.49 (dd, *J* = 8.2, 2.0 Hz, 1H), 7.43 (d, *J* = 2.0 Hz, 1H), 7.02 (d, *J* = 8.2 Hz, 1H), 5.01 (m, 1H), 3.99 (s, 2H), 3.02 (br s, 4H), 2.62 (br s, 4H), 2.40 (s, 3H), 1.95-1.92 (m, 2H), 1.83-1.75 (m, 4H), 1.62-1.54 (m, 4H). m/z (ESI-MS): 318.2169 $[M+H]^+$.

Step e: General Method for the Preparation of Compounds WL-1, 3, 4, 8, 10, 11, 13, 14, 16, 18, 19 by Addition of the Acyl Chloride of Different Acids

To a solution of compounds **5a-d** (0.9 mmol) in anhydrous dichloromethane (20 mL) were added acyl chloride of different acids (1.1 mmol) dissolved in anhydrous dichloromethane (10 mL) under N_2 atmosphere at room temperature. Reactions were followed by the addition of pyridine (0.1 mL, 1.1 mmol), and the resulting solutions were stirred at room temperature for 6h. Then the reactions were subsequently diluted with dichloromethane (50 mL), and washed with saturated sodium bicarbonate solution (3 x 50 mL), then saturated sodium chloride solution (50 mL). The organic phase was then separated, dried (Na_2SO_4), filtered, and concentrated to provide target products **WL-1, 3, 4, 8, 10, 11, 13, 14, 16, 18, 19**.

Methyl 3-(4-fluoro-3-nitrobenzamido)-4-(4-methylpiperazin-1-yl) benzoate (WL-1)

m.p. 222-225 °C. ^{1}H NMR (300 MHz, DMSO- d_6) δ 10.12 (s, 1H), 8.73 (dd, *J* = 7.2, 2.1 Hz, 1H), 8.32 (d, *J* = 1.8 Hz, 1H), 8.48-8.43 (m, 1H), 7.83-7.78 (m, 2H), 7.29 (d, *J* = 8.4 Hz, 1H), 3.83 (s, 3H), 3.32 (br s, 8H), 2.75 (s, 3H). HRMS (ESI): calcd. for m/z $C_{20}H_{22}FN_4O_5$ $[M + H]^+$ 417.1569, found 417.1568. HPLC (90% methanol in water) t_R = 4.119 min, 96.37%.

Methyl 3-(2-chloro-4-fluoro-3-methylbenzamido)-4-(4-methylpiperazin-1-yl) benzoate (WL-3)

m.p. 169-172 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.16 (s, 1H), 8.95 (s, 1H), 7.85 (dd, *J* = 8.3, 2.0 Hz, 1H), 7.62-7.56 (m, 1H), 7.28 (s, 1H), 7.12 (t, *J* = 8.6 Hz, 1H), 3.93 (s, 3H), 3.02 (t, *J* = 4.8 Hz, 4H), 2.63 (s, 3H), 2.56 (br s, 2H), 2.41 (d, *J* = 2.4 Hz, 3H), 2.40 (br s, 2H). HRMS (ESI): calcd. for m/z $C_{21}H_{24}ClFN_3O_3$ $[M + H]^+$ 420.1485, found 420.1484. HPLC (80% methanol in water) t_R = 6.830 min, 97.68%.

Methyl 3-(2-chloro-4-fluoro-3-methyl-5-nitrobenzamido)-4-(4-methylpiperazin-1-yl) benzoate (WL-4)

m.p. 237-239 °C. ^{1}H NMR (300 MHz, DMSO- d_6) δ 9.99 (s, 1H), 8.53 (d, *J* = 1.5 Hz, 1H), 8.45 (d, *J* = 7.7 Hz, 1H), 7.79 (dd, *J* = 8.3, 1.7 Hz, 1H), 7.26 (d, *J* = 8.4 Hz, 1H), 3.85 (s, 3H), 3.28 (br s, 8H), 2.72 (s, 3H), 2.42 (d, *J* = 2.0 Hz, 3H). HRMS (ESI): calcd. for m/z $C_{21}H_{23}ClFN_4O_5$ $[M + H]^+$ 465.1336, found 465.1336. HPLC (80% methanol in water) t_R = 5.757 min, 98.63%.

Isopropyl 3-(4-fluoro-3-nitrobenzamido)-4-(4-methylpiperazin-1-yl) benzoate (WL-8)

m.p. 242-247 °C. ^{1}H NMR (300 MHz, DMSO- d_6) δ 10.12 (s, 1H), 8.75 (dd, *J* = 7.2, 2.2 Hz, 1H), 8.48-8.45 (m, 1H), 8.28 (d, *J* = 1.7 Hz, 1H), 7.83-7.76 (m, 2H), 7.30 (d, *J* = 8.4 Hz, 1H), 5.14 (m, 1H), 3.34 (br s, 8H), 2.75 (s, 3H), 1.31 (d, *J* = 6.0 Hz, 6H). HRMS (ESI): calcd. for m/z $C_{22}H_{26}FN_4O_5$ $[M + H]^+$ 445.1882, found 445.1887. HPLC (80% methanol in water) t_R = 7.208 min, 96.25%.

Isopropyl 3-(2-chloro-4-fluoro-3-methylbenzamido)-4-(4-methylpiperazin-1-yl) benzoate (WL-10)

m.p. 120-123 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.99 (s, 1H), 8.69 (s, 1H), 7.75 (dd, *J* = 8.3, 1.7 Hz, 1H), 7.52 (t, *J* = 6.7 Hz, 1H), 7.28 (d, *J* = 8.4 Hz, 1H), 7.02 (t, *J* = 8.5 Hz, 1H), 5.21-5.11 (m, 1H), 3.55 (br s, 8H), 2.88 (s, 3H), 2.30 (d, *J* = 2.0 Hz, 3H), 1.28 (d, *J* = 6.2 Hz, 6H). ^{13}C NMR (75 MHz, DMSO- d_6) δ 11.8, 21.6, 42.2, 47.4, 52.2, 68.0, 113.8, 114.1, 119.9, 123.8, 124.1, 125.2, 126.8, 127.1, 131.0, 131.3, 133.6, 147.6, 164.8, 165.2. HRMS (ESI): calcd. for m/z $C_{23}H_{28}ClFN_3O_3$ $[M + H]^+$ 448.1798, found 448.1803. HPLC (90% methanol + 0.3% TEA in water) t_R = 5.015 min, 95.18%.

Phenyl 3-(4-fluoro-3-nitrobenzamido)-4-(4-methylpiperazin-1-yl) benzoate (WL-11)

m.p. >250 °C. ^{1}H NMR (300 MHz, DMSO- d_6) δ 10.19 (s, 1H), 8.76 (dd, *J* = 7.3, 2.3 Hz, 1H), 8.47-8.46 (m, 2H), 8.00 (dd, *J* = 8.5, 2.1 Hz, 1H), 7.83-7.77 (m, 1H), 7.49 (m, 2H), 7.39-7.27 (m, 4H), 3.48 (br s, 4H), 3.25 (br s, 4H), 2.81 (s, 3H). HRMS (ESI): calcd. for m/z $C_{25}H_{24}FN_4O_5$ $[M + H]^+$ 479.1725, found

479.1729. HPLC (80% methanol in water) t_R = 7.724 min, 98.25%.

Phenyl 3-(2-chloro-4-fluoro-3-methylbenzamido)-4-(4-methylpiperazin-1-yl) benzoate (WL-13)

m.p. 70-73 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.20 (s, 1H), 8.75 (s, 1H), 7.91 (dd, *J* = 8.3, 2.0 Hz, 1H), 7.60-7.51 (m, 1H), 7.36-7.31 (m, 3H), 7.20-7.11 (m, 3H), 7.03 (t, *J* = 8.5 Hz, 1H), 3.29 (br s, 4H), 3.11 (br s, 4H), 2.70 (s, 3H), 2.31 (d, *J* = 2.2 Hz, 3H). HRMS (ESI): calcd. for m/z $C_{26}H_{26}ClFN_3O_3$ $[M + H]^+$ 482.1641, found 482.1644. HPLC (90% methanol + 0.3% TEA in water) t_R = 4.925 min, 95.58%.

Phenyl 3-(2-chloro-4-fluoro-3-methyl-5-nitrobenzamido)-4-(4-methylpiperazin-1-yl) benzoate (WL-14)

m.p. 192-195 °C. ^{1}H NMR (300 MHz, DMSO- d_6) δ 9.98 (s, 1H), 8.53 (d, *J* = 1.7 Hz, 1H), 8.37 (d, *J* = 7.7 Hz, 1H), 7.94 (d, *J* = 1.7 Hz, 1H), 7.50-7.45 (m, 2H), 7.31-7.25 (m, 4H), 3.32 (br s, 4H), 3.05 (br s, 4H), 2.42 (d, *J* = 2.3 Hz, 3H), 2.21 (s, 3H). HRMS (ESI): calcd. for m/z $C_{26}H_{25}ClFN_4O_5$ $[M + H]^+$ 527.1492, found 527.1492. HPLC (80% methanol in water) t_R = 9.214 min, 97.93%.

Cyclohexyl 3-(4-fluoro-3-nitrobenzamido)-4-(4-methylpiperazin-1-yl) benzoate (WL-16)

m.p. 245-248 °C. ^{1}H NMR (300 MHz, DMSO- d_6) δ 10.09 (s, 1H), 8.74 (dd, *J* = 7.2, 2.2 Hz, 1H), 8.47-8.43 (m, 1H), 8.32 (d, *J* = 2.0 Hz, 1H), 7.85-7.76 (m, 2H), 7.31 (d, *J* = 8.5 Hz, 1H), 4.93 (m, 1H), 3.34 (br s, 8H), 2.79 (s, 3H), 1.86 (m, 2H), 1.74-1.72 (m, 2H), 1.56-1.50 (m, 2H), 1.43-1.36 (m, 4H). HRMS (ESI): calcd. for m/z $C_{25}H_{29}FN_4O_5$ $[M + H]^+$ 485.2195, found 485.2197. HPLC (80% methanol in water) t_R = 13.702 min, 98.11%.

Cyclohexyl 3-(2-chloro-4-fluoro-3-methylbenzamido)-4-(4-methylpiperazin-1-yl) benzoate (WL-18)

m.p. 146-148 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.14 (s, 1H), 8.91 (s, 1H), 7.84 (dd, *J* = 8.3, 1.9 Hz, 1H), 7.61-7.56 (m, 1H), 7.29 (s, 0.5H), 7.10 (t, *J* = 8.6 Hz, 1H), 6.95 (m, 0.5H), 5.02 (m, 1H), 3.09 (br s, 4H), 2.80 (br s, 4H), 2.50 (s, 3H), 2.39 (d, *J* = 2.2 Hz, 3H), 1.96-1.92 (m, 2H), 1.81-1.79 (m, 2H), 1.63-1.56 (m, 3H), 1.46-1.42 (m, 3H). HRMS (ESI): calcd. for m/z $C_{26}H_{32}ClFN_3O_3$ $[M + H]^+$ 488.2111, found 488.2117. HPLC (80% methanol in water) t_R = 19.250 min, 98.01%.

Cyclohexyl 3-(2-chloro-4-fluoro-3-methyl-5-nitrobenzamido)-4-(4-methylpiperazin-1-yl) benzoate (WL-19)

m.p. 149-152 °C. ^{1}H NMR (300 MHz, DMSO- d_6) δ 9.94 (s, 1H), 8.51 (s, 1H), 8.43 (d, *J* = 7.4 Hz, 1H), 7.79 (d, *J* = 8.6 Hz, 1H), 7.26 (d, *J* = 8.4 Hz, 1H), 4.92 (m, 1H), 3.21 (br s, 4H), 3.16 (br s, 4H), 2.66 (s, 3H), 2.42 (s, 3H), 1.85 (m, 2H), 1.71-1.70 (m, 2H), 1.55-1.22 (m, 6H). HRMS (ESI): calcd. for m/z $C_{26}H_{31}ClFN_4O_5$ $[M + H]^+$ 533.1962, found 533.1964. HPLC (80% methanol in water) t_R = 15.279 min, 98.40%.

Step f: General Method for the Preparation of Compounds WL-2, 5, 9, 12, 15, 17, 20 by Reduction of the nitro of Compounds WL-1, 4, 8, 11, 14, 16, 19.

To a solution of compounds **WL-1, 4, 8, 11, 14, 16, 19** (0.5 mmol) in EA (50 mL) at room temperature was added $SnCl_2.2H_2O$ (0.46g, 2.0 mmol). The resulting solution was then refluxed with vigorous stirring for 4~8 h, and subsequently cooled to room temperature. Following dilution with EA (100 mL) and the mixture was neutralize with saturated sodium bicarbonate solution. Then filtered and washed residue with EA (3 x 50 mL), and the organic phase was dried (Na_2SO_4), filtered, and concentrated to afford compounds **WL-2, 5, 9, 12, 15, 17, 20**.

Methyl 3-(3-amino-4-fluorobenzamido)-4-(4-methylpiperazin-1-yl) benzoate (WL-2)

m.p. 218-222 °C. ^{1}H NMR (300 MHz, DMSO- d_6) δ 9.29 (s, 1H), 8.51 (d, *J* = 2.0 Hz, 1H), 7.73 (dd, *J* = 8.4, 2.1 Hz, 1H), 7.36 (dd, *J* = 8.7, 2.0 Hz, 1H), 7.27 (d, *J* = 8.4 Hz, 1H), 7.20-7.09 (m, 2H), 5.48 (s, 2H), 3.83 (s, 3H), 2.92 (t, *J* = 4.4 Hz, 4H), 2.49 (t, *J* = 4.4 Hz, 4H), 2.22 (s, 3H). HRMS (ESI): calcd. for m/z $C_{20}H_{24}FN_4O_3$ $[M + H]^+$ 387.1827, found 387.1818. HPLC (90% methanol in water) t_R = 3.708 min, 96.58%.

Methyl 3-(5-amino-2-chloro-4-fluoro-3-methylbenzamido)-4-(4-methylpiperazin-1-yl) benzoate (WL-5)

m.p. 223-225 °C. ^{1}H NMR (300 MHz, DMSO- d_6) δ 9.40 (s, 1H), 8.40 (s, 1H), 7.74 (d, *J* = 8.3 Hz, 1H), 7.22 (d, *J* = 8.4 Hz, 1H), 6.81 (d, *J* = 9.2 Hz, 1H), 5.48 (s, 2H), 3.83 (s, 3H), 2.96 (br s, 4H), 2.45 (br s, 4H), 2.24-2.20 (m, 6H). ^{13}C NMR (75 MHz, DMSO- d_6) δ 11.9, 45.7, 50.1, 51.9, 54.5, 112.6, 112.7, 115.6, 119.7, 123.9, 124.4, 126.7, 131.0, 132.6, 135.3, 135.5, 148.9, 165.3, 165.8. HRMS (ESI): calcd. for m/z $C_{21}H_{25}ClFN_4O_3$ $[M + H]^+$ 435.1594, found 435.1597. HPLC (90% methanol + 0.3% TEA in water) t_R = 3.868 min, 95.00%.

Isopropyl 3-(3-amino-4-fluorobenzamido)-4-(4-methylpiperazin-1-yl) benzoate (WL-9)

m.p. 209-212 °C. ^{1}H NMR (300 MHz, DMSO- d_6) δ 9.31 (s, 1H), 8.46 (d, *J* = 2.0 Hz, 1H), 7.71 (dd, *J* = 8.4, 2.0 Hz, 1H), 7.36 (dd, *J* = 8.9, 2.1 Hz, 1H), 7.26 (d, *J* = 8.4 Hz, 1H), 7.17-7.10 (m, 2H), 5.47 (s, 2H), 5.11 (m, 1H), 2.94 (t, *J* = 4.5 Hz, 4H), 2.49 (t, *J* = 4.5 Hz, 4H), 2.22 (s, 3H), 1.30 (d, *J* = 6.2, 6H). HRMS (ESI): calcd. for m/z $C_{22}H_{28}FN_4O_3$ $[M + H]^+$ 415.214, found 415.214. HPLC (80% methanol in water) t_R = 5.699 min, 98.54%.

Phenyl 3-(3-amino-4-fluorobenzamido)-4-(4-methylpiperazin-1-yl) benzoate (WL-12)

m.p. 221-223 °C. ^{1}H NMR (300 MHz, DMSO- d_6) δ 9.37 (s, 1H), 8.62 (s, 1H), 7.90 (dd, *J* = 8.4, 2.1 Hz, 1H), 7.49-7.46 (m, 2H), 7.39-7.27 (m, 4H), 7.18-7.12 (m, 2H), 5.47 (s, 2H), 3.48 (br s, 4H), 2.99 (t, *J* = 4.2, 4H), 2.49 (t, *J* = 4.2, 4H), 2.22 (s, 3H). HRMS (ESI): calcd. for m/z $C_{25}H_{26}FN_4O_3$ $[M + H]^+$ 449.1983, found 449.1984. HPLC (80% methanol in water) t_R = 6.112 min, 97.72%.

Phenyl 3-(5-amino-2-chloro-4-fluoro-3-methylbenzamido)-4-(4-methylpiperazin-1-yl) benzoate (WL-15)

m.p. 212-214 °C. ^{1}H NMR (300 MHz, DMSO- d_6) δ 9.50 (s, 1H), 8.51 (s, 1H), 7.91 (d, *J* = 7.3 Hz, 1H), 7.49-7.44 (m, 2H), 7.33-7.25 (m, 4H), 6.83 (d, *J* = 9.1 Hz), 5.48 (s, 2H), 3.02 (br s, 4H), 2.49 (br s, 4H), 2.24-2.21 (m, 6H). ^{13}C NMR (75 MHz, DMSO- d_6) δ 11.9, 45.7, 50.4, 54.5, 112.7, 115.7, 119.8, 121.9, 122.8, 123.3, 125.3, 125.9, 127.5, 129.5, 131.0, 132.6, 135.3, 135.5, 149.9, 150.7, 164.2, 165.4. HRMS (ESI): calcd. for m/z $C_{26}H_{27}ClFN_4O_3$ $[M + H]^+$ 497.175, found 497.1748. HPLC (80% methanol in water) t_R = 8.750 min, 95.05%.

Cyclohexyl 3-(3-amino-4-fluorobenzamido)-4-(4-methylpiperazin-1-yl) benzoate (WL-17)

m.p. 219-221 °C. ^{1}H NMR (300 MHz, DMSO- d_6) δ 9.31 (s, 1H), 8.50 (d, *J* = 2.0 Hz, 1H), 7.72 (dd, *J* = 8.4, 2.1 Hz, 1H), 7.37 (dd, *J* = 8.9, 2.1 Hz, 1H), 7.27 (d, *J* = 8.4 Hz, 1H), 7.19-7.10 (m, 2H), 5.47 (s, 2H), 4.91 (m, 1H), 2.92 (t, *J* = 4.4 Hz, 4H), 2.49 (t, *J* = 4.4 Hz, 4H), 2.22 (s, 3H), 1.88-1.83 (m, 2H), 1.70 (m, 2H), 1.55-1.35 (m, 6H). HRMS (ESI): calcd. for m/z $C_{25}H_{32}FN_4O_3$ [M

+ H]$^+$ 455.2453, found 455.2449. HPLC (80% methanol in water) t_R = 9.796 min, 98.47%.

Cyclohexyl 3-(5-amino-2-chloro-4-fluoro-3-methylbenzamido)-4-(4-methylpiperazin-1-yl) benzoate (WL-20)

m.p. 226-229 °C. ^{1}H NMR (300 MHz, DMSO- d_6) δ 9.40 (s, 1H), 8.40 (s, 1H), 7.74 (d, J = 8.4 Hz, 1H), 7.22 (d, J = 8.5 Hz, 1H), 6.82 (m, 1H), 5.48 (s, 2H), 4.91 (m, 1H), 2.95 (br s, 4H), 2.45 (br s, 4H), 2.24-2.20 (m, 6H), 1.84 (m, 2H), 1.70 (m, 2H), 1.54-1.29 (m, 6H). ^{13}C NMR (75 MHz, DMSO- d_6) δ 11.9, 23.0, 24.9, 31.0, 45.7, 50.6, 54.6, 72.2, 112.7, 115.6, 119.7, 123.3, 124.4, 124.7, 126.6, 131.1, 132.6, 135.3, 135.5, 148.8, 164.7, 165.2. HRMS (ESI): calcd. for m/z $C_{26}H_{33}ClFN_4O_3$ [M + H]$^+$ 503.222, found 503.2227. HPLC (80% methanol in water) t_R = 16.342 min, 96.54%.

Scheme 2. Synthesis of compounds WL6-7 and WL21-22:

Step a: General Method for the Preparation of 3-benzamido-4-(4-methylpiperazin-1-yl) benzoic acids (WL-21 and WL-22).

Li (OH) (1 M, 20 mL) was added dropwise to a solution of compound **WL-3** (0.5 g, 1.2 mmol) in THF (25 mL) at room temperature. THF was removed after the mixture was stirred for 8h. The residue was acidified with 1M HCl and filtered to give white solid.

3-(4-fluoro-3-nitrobenzamido)-4-(4-methylpiperazin-1-yl) benzoic acid (WL-21)

m.p. > 250 °C. ^{1}H NMR (300 MHz, DMSO- d_6) δ 11.99 (s, 1H), 9.69 (s, 1H), 8.50-8.39 (m, 2H), 8.15 (d, J = 8.6 Hz, 1H), 7.76 (d, J = 6.4 Hz, 1H), 7.36-7.26 (m, 2H), 3.47 (br s, 4H), 3.19 (br s, 4H), 2.80 (s, 3H). HRMS (ESI): calcd. for m/z $C_{19}H_{20}FN_4O_5$ [M + H]$^+$ 401.1456, found 401.1449. HPLC (80% methanol in water) t_R = 2.817 min, 98.95%.

3-(2-chloro-4-fluoro-3-methylbenzamido)-4-(4-methylpiperazin-1-yl) benzoic acid (WL-22)

Yield: 70.8%. m.p. > 250 °C. ^{1}H NMR (300 MHz, DMSO- d_6) δ 9.57 (s, 1H), 8.39 (s, 1H), 7.74 (d, J = 7.6 Hz, 1H), 7.57-7.47 (m, 1H), 7.36-7.30 (m, 1H), 7.19 (d, J = 8.1 Hz, 1H), 2.98 (br s, 4H), 2.52 (br s, 4H), 2.25 (s, 3H), 2.07 (s, 3H). HRMS (ESI): calcd. for m/z $C_{20}H_{21}ClFN_3O_3$ [M + H]$^+$ 406.1328, found 406.1335. HPLC (90% methanol in water) t_R = 3.228 min, 99.08%.

Step b: General Method for the Preparation of 3-benzamido-4-(4-methylpiperazin-1-yl) benzoyl chlorides.

WL-22 (0.15 g, 0.37 mmol) was added to 3ml $SOCl_2$ as solvent and acylation reagent, refluxing for 6h. Then the solution was evaporated to give yellow oil, which was used in the subsequent reaction.

Step c: General Method for the Preparation of ethyl 3-benzamido-4-(4-methylpiperazin-1-yl) benzoates.

3-Benzamido-4-(4-methylpiperazin-1-yl) benzoyl chlorides (0.37 mmol, got from **step b)** dissolved in anhydrous dichloromethane (10 mL) was added to ethanol (20 mL). This was followed by the addition of TEA (0.08 mL, 0.56 mmol), and the resulting solution was stirred at room temperature for 0.5h. The solution was subsequently removed to provide **WL-6** and **WL-7** as solid.

Ethyl 3-(4-fluoro-3-nitrobenzamido)-4-(4-methylpiperazin-1-yl) benzoate (WL-6)

m.p. > 250 °C. ^{1}H NMR (300 MHz, DMSO- d_6) δ 9.74 (s, 1H), 8.51 (d, J = 2.2 Hz, 1H), 8.38 (d, J = 2.0 Hz, 1H), 8.15 (dd, J = 8.8, 2.2 Hz, 1H), 7.78 (dd, J = 8.4, 2.0 Hz, 1H), 7.36-7.28 (m, 2H), 4.30 (q, J = 7.1 Hz, 2H), 3.34 (br s, 4H), 3.15 (br s, 4H), 2.81 (s, 3H), 1.30 (t, J = 7.1 Hz, 3H). HRMS (ESI): calcd. for m/z $C_{21}H_{24}FN_4O_5$ [M + H]$^+$ 429.178, found 429.1781. HPLC (90% methanol in water) t_R = 3.425 min, 99.55%.

Ethyl 3-(2-chloro-4-fluoro-3-methylbenzamido)-4-(4-methylpiperazin-1-yl) benzoate (WL-7)

m.p. 123-125 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.16 (s, 1H), 8.96 (s, 1H), 7.85 (dd, J = 8.3, 1.9 Hz, 1H), 7.61-7.56 (m, 1H), 7.26 (d, J = 8.3 Hz, 1H), 7.11 (t, J = 8.6 Hz, 1H), 4.40 (q, J = 7.0 Hz, 2H), 2.97 (t, J = 4.7 Hz, 4H), 2.55 (br s, 2H), 2.42 (d, J = 2.2 Hz, 3H), 2.35 (s, 3H), 1.73 (br s, 2H), 1.41 (t, J = 7.0 Hz, 3H). HRMS (ESI): calcd. for m/z $C_{22}H_{26}ClFN_3O_3$ [M + H]$^+$ 434.1641, found 434.1637. HPLC (80% methanol in water) t_R = 8.011 min, 96.12%.

4.2 Competitive Binding assay.

The binding affinities of all synthesized compounds were tested using a fluorescence polarization (FP) based competitive binding assay. 10 Peptidomimetic of Win motif (ARTEVHLRKS) for WDR5 was synthesized, C-terminal-labeled with 5-carboxy fluorescein (5-FAM) tagged tracers linked through the side chain of a lysine residue next to the two 6-amino hexanoic acid with the serine [13]. Competitive binding assays were performed in a 60 μL volume at a constant labeled peptide concentration of 3 nM and WDR5 concentration of 8 nM in 100 mM potassium phosphate (pH 6.5), 25 mM KCl and 0.01% Triton X-100. Fluorescence polarization assays were performed in 384-well Corning plates using a SpectraMax paradigm reader (Molecular Device). An excitation wavelength of 485 nm and an emission wavelength of 535 nm were used. IC_{50} values were calculated by nonlinear regression analysis using Graphpad software.

4.3 Molecular docking

Docking study was carried out using GOLD5.1. The protein structure of WDR5 was downloaded from PDB (3UR4) and was edited by adding hydrogen, deleting unnecessary waters and ligands. Then the binding sites were defined according the endogenous ligand **WDR5-0103**. Gold score was chosen as the score function of binding interaction energy for ranking. Compounds **WL-5** and **WL-15** were prepared by DS3.0 with CHARMm. The high fitness score model was selected to analyze binding model.

4.4 MLL complex Alpha Screen assays

MLL1 methyltransferase inhibition assays were performed by the HUAWEI PHARMA, Shanghai, China, using the Histone methyltransferase Assays platform (www.huajianpharma.com). The MLL1 enzymatic reactions were conducted in duplicate at room temperature for 60 minutes in a 50 μL mixture containing proper methyltransferase assay buffer (50 mM Tris, pH 8.8, 5 mM $MgCl_2$, 4M DTT), S-adenosylmethionine (1μM), recombinant enzyme (MLL1, WDR5, Ash2L and RbBP5, 150 ng), and the test compounds in wells of a Histone substrate pre-coated plate. Compounds and control compound MM-102 were dissolved in DMSO and tested in 10-dose IC_{50} mode with 3-fold serial dilution starting at 100 μM. After enzymatic reactions, the reaction mixtures were discarded and each of the wells was washed three times with TBST buffer, and slowly shaken with Blocking Buffer for 10 minutes. Wells were emptied, and 100 μL of diluted primary antibody was added. The plate was then slowly shaken for 60 minutes at room temperature. As before, the

plate was emptied and washed three times, and shaken with Blocking Buffer for 10 minutes at room temperature. After discarding the Blocking Buffer, 100 μL of diluted secondary antibody was added. The plate was then slowly shaken for 30 minutes at room temperature. As before, the plate was emptied and washed three times, and shaken with Blocking Buffer for 10 minutes at room temperature. Blocking Buffer was discarded and a mixture of the HRP chemiluminescent substrates was freshly prepared. 100 μL of this mixture was added to each empty well. Immediately, the luminescence of the samples was measured in a BioTek SynergyTM 2 microplate reader. Data were normalized to the no enzyme control and the IC_{50} values were calculated using nonlinear regression with normalized dose–response fit using Prism GraphPad software.

4.5 Anti-proliferative activity

MV4-11and K562 cells were bought from Cell Bank of Chinese Academy of Sciences. Cell viabilities of the synthetized compounds were evaluated using CCK8 (WST-8, 2-(2-methoxy 4-nitro phenyl)-3-(4-nitro phenyl)-5- (2, 4-disulfonic acid benzene)-2H-tetrazolium monosodium salt)-based colorimetric assay. Cell lines were cultured to log phase in IMDM supplemented with 10% fetal bovine serum, under a humidified atmosphere of 5% CO_2 at 37 ^{o}C. Cells were seeded in 96- well white opaque cell culture plates at a density of 5000 cells/ well in 100 μL of culture medium. The plates were returned to the incubator for 24 h to allow the cells to reattach. Subsequently, cells were treated with the target compounds diluted in 10 μL IMDM at increasing concentrations for 72 h. Then, cell viability was assessed by the conventional (CCK8) reduction assay and the absorption was measured at 450 nm using Thermo Multiskan Spectrum. IC_{50} was taken as the concentration that caused 50 % inhibition of cell viabilities and calculated by nonlinear regression analysis using Graphpad software.

4.6 Permeability determination

Permeability (Pe) was determined by a standard parallel artificial membrane permeability assay (PAMPA by pION). PAMPA was conducted on a PAMPA Explorer instrument (pION Inc., Woburn, MA) with PAMPA Explorer command software (Version 3.7.4.1) as follows: test compound stock was dissolved in DMSO at 10 mM concentration. Then 5μL DMSO diluted with compounds was added to 1 mL Prisma HT buffer (pH 7.4) in deep well plate to make sample solution. Then 150 μL of diluted test compound was transferred to a UV plate (pION Inc., Woburn, MA), and the UV spectrum was read as the reference plate. The membrane on a preloaded PAMPA sandwich (pION Inc., Woburn, MA) was painted with 5 μL of GIT lipid (pION Inc., Woburn, MA). The acceptor chamber was then filled with 200 μL of acceptor solution buffer (pION Inc., Woburn, MA), and the donor chamber was filled with 200 μL of diluted test compound. The PAMPA sandwich was assembled at 25 °C for 4 hours. The UV spectrum (240–500 nm) of the donor and the acceptor were read. The permeability coefficient was calculated using PAMPA Explorer command software (Version 3.7.4.1) based on the AUC of the reference plate, the donor plate, and the acceptor plate. All compounds were tested in quadruplicate, and the data were presented as an average value. Standards for this assay included Ketoprofen (2.2 × 10−6 cm/s) and propranolol (98.7 × 10−6 cm/s).

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Notes and Reference

[1] Black, J. C.; Van Rechem, C.; Whetstine, J. R. *Mol. Cell* **2012**, *48*, 491.
[2] Chi, P.; Allis, C. D.; Wang, G. G. *Nat. Rev. Cancer* **2010**, *10*, 457.
[3] Wang, G. G.; Allis, C. D.; Chi, P. *Trends Mol. Med.* **2007**, *13*, 363.

[4] Dou, Y. L.; Milne, T. A.; Tackett, A. J.; Smith, E. R.; Fukuda, A.; Wysocka, J.; Allis, C. D.; Chait, B. T.; Hess, J. L.; Roeder, R. G. *Cell* **2005**, *121*, 873.

[5] McMahon, K. A.; Hiew, S. Y.; Hadjur, S.; Veiga-Fernandes, H.; Menzel, U.; Price, A. J.; Kioussis, D.; Williams, O.; Brady, H. J. *Cell stem cell* **2007**, *1*, 338.

[6] Jude, C. D.; Climer, L.; Xu, D.; Artinger, E.; Fisher, J. K.; Ernst, P. *Cell stem cell* **2007**, *1*, 324.

[7] Argiropoulos, B.; Humphries, R. K. *Oncogene* **2007**, *26*, 6766.

[8] Marschalek, R. *Br. J. Haematol.* **2011**, *152*, 141.

[9] Dou, Y.; Hess, J. L. *Int. J. Hematol.* **2008**, *87*, 10.

[10] Karatas, H.; Townsend, E. C.; Cao, F.; Chen, Y.; Bernard, D.; Liu, L.; Lei, M.; Dou, Y.; Wang, S. *J. Am. Chem. Soc.* **2012**, *135*, 669.

[11] Thiel, A. T.; Blessington, P.; Zou, T.; Feather, D.; Wu, X.; Yan, J.; Zhang, H.; Liu, Z.; Ernst, P.; Koretzky, G. A.; Hua, X. *Cancer cell* **2010**, *17*, 148.

[12] Milne, T. A.; Kim, J.; Wang, G. G.; Stadler, S. C.; Basrur, V.; Whitcomb, S. J.; Wang, Z. X.; Ruthenburg, A. J.; Elenitoba-Johnson, K. S. J.; Roeder, R. G.; Allis, C. D. *Mol. Cell* **2010**, *38*, 853.

[13] Patel, A.; Dharmarajan, V.; Vought, V. E.; Cosgrove, M. S. *J. Biol. Chem.* **2009**, *284*, 24242.

[14] Dou, Y. L.; Milne, T. A.; Ruthenburg, A. J.; Lee, S.; Lee, J. W.; Verdine, G. L.; Allis, C. D.; Roeder, R. G. *Nat. Struct. Mol. Biol.* **2006**, *13*, 713.

[15] Steward, M. M.; Lee, J. S.; O'Donovan, A.; Wyatt, M.; Bernstein, B. E.; Shilatifard, A. *Nat. Struct. Mol. Biol.* **2006**, *13*, 852.

[16] Senisterra, G.; Wu, H.; Allali-Hassani, A.; Wasney, G. A.; Barsyte-Lovejoy, D.; Dombrovski, L.; Dong, A.; Nguyen, K. T.; Smil, D.; Bolshan, Y.; Hajian, T.; He, H.; Seitova, A.; Chau, I.; Li, F.; Poda, G.; Couture, J. F.; Brown, P. J.; Al-Awar, R.; Schapira, M.; Arrowsmith, C. H.; Vedadi, M. *Biochem. J.* **2013**, *449*, 151.

[17] Bolshan, Y.; Getlik, M.; Kuznetsova, E.; Wasney, G. A.; Hajian, T.; Poda, G.; Nguyen, K. T.; Wu, H.; Dombrovski, L.; Dong, A.; Senisterra, G.; Schapira, M.; Arrowsmith, C. H.; Brown, P. J.; Al-awar, R.; Vedadi, M.; Smil, D. *ACS Med. Chem. Lett.* **2013**, *4*, 353.

[18] Li, D.-D.; Chen, W.-L.; Xu, X.-L.; Jiang, F.; Wang, L.; Xie, Y.-Y.; Zhang, X.-J.; Guo, X.-K.; You, Q.-D.; Sun, H.-P. *Eur. J. Med. Chem.* **2016**, *118*, 1.

[19] Grebien, F.; Vedadi, M.; Getlik, M.; Giambruno, R.; Grover, A.; Avellino, R.; Skucha, A.; Vittori, S.; Kuznetsova, E.; Smil, D.; Barsyte-Lovejoy, D.; Li, F.; Poda, G.; Schapira, M.; Wu, H.; Dong, A.; Senisterra, G.; Stukalov, A.; Huber, K. V.; Schonegger, A.; Marcellus, R.; Bilban, M.; Bock, C.; Brown, P. J.; Zuber, J.; Bennett, K. L.; Al-Awar, R.; Delwel, R.; Nerlov, C.; Arrowsmith, C. H.; Superti-Furga, G. *Nat. Chem. Biol.* **2015**, *11*, 571.

[20] Getlik, M.; Smil, D.; Zepeda-Velazquez, C.; Bolshan, Y.; Poda, G.; Wu, H.; Dong, A.; Kuznetsova, E.; Marcellus, R.; Senisterra, G.; Dombrovski, L.;

Hajian, T.; Kiyota, T.; Schapira, M.; Arrowsmith, C. H.; Brown, P. J.; Vedadi, M.; Al-Awar, R. *J. Med. Chem.* **2016**, *59*, 2478.

[21] Karatas, H.; Townsend, E. C.; Bernard, D.; Dou, Y.; Wang, S. *J. Med. Chem.* **2010**, *53*, 5179.

[22] Cao, F.; Townsend, E. C.; Karatas, H.; Xu, J.; Li, L.; Lee, S.; Liu, L.; Chen, Y.; Ouillette, P.; Zhu, J.; Hess, J. L.; Atadja, P.; Lei, M.; Qin, Z. S.; Malek, S.; Wang, S.; Dou, Y. *Mol. Cell* **2014**, *53*, 247.

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

- A series of ester compounds were designed and synthetized to disturb MLL1-WDR5 interaction.
- **WL-5** and **WL-15** effectively inhibited MLL1-dependent H3K4 methylation in *vitro*.
- Representative ester compounds selectively inhibited the proliferation of MV4-11 cells.
- Docking study was applied to elucidate the binding model of ester compounds.