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Design, synthesis, and initial evaluation of affinity-based small molecular probe for detection of WDR5

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ABSTRACT

WDR5, a subunit of the SET/MLL complex, plays critical roles in various biological progresses and are abnormally expressed in many cancers. Here we report the design, synthesis, and biochemical characterization of a new chemical tool to capture WDR5 protein. The probe is a biotinylated version of compound **30** that is a potent WDR5 inhibitor we previously reported. Importantly, the probe displayed high affinity to WDR5 protein *in vitro* binding potency and showed the ability in specifically and real time monitoring WDR5 protein. Further, the biotinylated tag of the probe enabled selectively "chemoprecipitation" of WDR5 from whole cell lysates of MV4-11. This probe provided a new approach to identify the overexpressed WDR5 protein in different cancer cells and applications to proteomic analysis of WDR5 and WDR5-binding partners.

1. Introduction

WD repeat domain (WDR5) is an essential component of the human trithorax-like family of SET1 [Su(var)3–9 enhancer of zeste trithorax 1] methyltransferase complexes that carry out trimethylation of histone 3 Lys4 (H3K4me3) [1]. WDR5 plays key roles in various biological functions and development of many cancers. First and foremost, WDR5 preferentially recognizes arginine 3765 of the MLL1 *win* motif. WDR5 functions as a structural platform that bridges the interactions between the catalytic and regulatory subunits of MLL1 complexes. Therefore, it is required to maintain the integrity and H3K4 methyltransferase (HMT) activity of MLL1 complex as well as its homologous complexes containing MLL2, MLL3 and MLL4, whose expression is often abnormally in some cancers [2–5]. Mutation of key residues on WDR5 effectively dissociated the MLL1 core complex and resulted in dramatic decrease of the MLL1 H3K4 HMT activity [6]. These findings fully confirmed the predominant role of WDR5 in MLL-rearranged leukemias, and also inspired the development of MLL1-WDR5 interaction antagonists. Several small molecule inhibitors and peptidomimetics that exhibited tight binding to WDR5 to disturb WDR5-MLL1 interaction have been reported [6–13]. Recently, Mungamuri et al. reported that WDR5 silencing cooperated with Trastuzumab or chemotherapy specifically inhibited the growth of ErbB2 positive breast tumor cells [14]. In addition, Kim and his co-workers showed that WDR5 was a critical epigenomic integrator of histone phosphorylation and methylation and a major driver of androgen-dependent prostate cancer cell proliferation [15]. Besides, the other study indicated that WDR5 promoted proliferation, self-renewal and chemoresistance to cisplatin in bladder cancer cells *in vitro*, and tumor growth *in vivo* [16]. Considering the importance for further exploration of function and therapeutical potential of WDR5, the development of chemical tools targeting WDR5 is quite important. To this end, we report the design, synthesis and application of biotinylated probe of WDR5 to specifically detect WDR5.

Biotin is universally used to immobilise or conjugate bioactive compounds, normally via its high-affinity interaction with avidin or streptavidin [17]. The biotin-streptavidin system has proven to be particularly useful in the detection and localization of antigens, glycoconjugates, and nucleic acids by employing biotinylated antibodies, lectins, or nucleic acid probes. Herein, on the basis of our previous reported high-affinity small molecule blockers of MLL1-WDR5 interaction and their binding models to WDR5, we designed, synthetized and biochemically characterized the probe **LC001** [13]. **LC001** attached a biotin tag to compound **30** (IC_{50} = 88.7 nmol), which kept the affinity to WDR5 protein and showed equal MLL1 HMT activity (IC_{50} = 1.65 μmol) to the reported peptidomimetic **MM-102** (IC_{50} = 0.75 μmol) [13], though MM-102 was more potent

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in competitive assay. **LC001** also displayed low nanomolar binding affinity, and specifically and real time monitored WDR5 protein. Moreover, the probe could selectively precipitate WDR5 from whole cell lysates of MV4-11 leukemia cells, which can be applied in further research of WDR5.

2. Results and discussions

WDR5 substrate binding cavity that has been well studied, provided the parameters of moieties tolerated within the WDR5 binding site regions. Previously, we have investigated the binding model of compound **30** with WDR5 and found that the introduced aromatic ring A occupied the hydrophobic groove of WDR5, surrounded by sidechains of Phe133, Phe149 and Tyr191 and formed π-π stacking interaction with Tyr191 [13]. Compound **42**, the modification of **30** with a 4-aminobutanamido group still enhanced the binding affinity from an IC_{50} value of 88.7 nM to 7.6 nM [13] (Fig. 1). Docking study showed the 4 position substituent group of aromatic A located in the solvent area. Given the high binding potency and satisfactory match to WDR5, it can be used as a good reference to develop the functional small-molecule probe targeting WDR5.

Our design strategy was to obtain a biotin probe while minimally affecting the binding activity. On the basis of our previous study, we introduce a rational long linker and append a biotin tag to the solvent exposed region of compound **30**. The synthetic route is depicted in Scheme 1. The initial compound **30** was synthesized as the methods that previously reported [13]. Compound **30** was acylated with Boc-6-aminohexanoic acid catalyzed by Reagent Castros to afford **Boc-LD055**. Then the *t*-butyloxycarboryl group was removed with TFA to obtain **LD055**. Compared to **30**, which exhibited an IC_{50} value of 88.7 nM in a fluorescence polarization (FP) competition assay in our previous study [13], **LD055** showed an IC_{50} value of 33.8 nM, suggesting that the introduction of the linker further improved the WDR5 binding affinity. After that, introduction of biotin group into the amino of the linker gave the probe **LC001**.

We first examined the binding affinity of **LC001** with WDR5. The IC_{50} value of **LC001** in the FP competition assay was 17.0 nM (Fig. 2), still slightly improved the potency of **LD055** to WDR5, which indicated that the introduction of the biotin group maintained the WDR5 binding affinity. This result was supported by docking studies using WDR5 X-ray structure (Fig. 3). As expected, **LC001** docked in a nearly identical orientation to compound **30**, and the biotin solvent exposed, well away from the surface of the protein.

We next confirmed **LC001** inhibited the activity for MLL complex methyltransferase *in vitro*. The HMT inhibition of **LC001** was evaluated in a recombinant MLL complex (MLL1, WDR5, RbBP5, and ASH2L proteins) Alpha Screen assay. **LC001** indeed showed

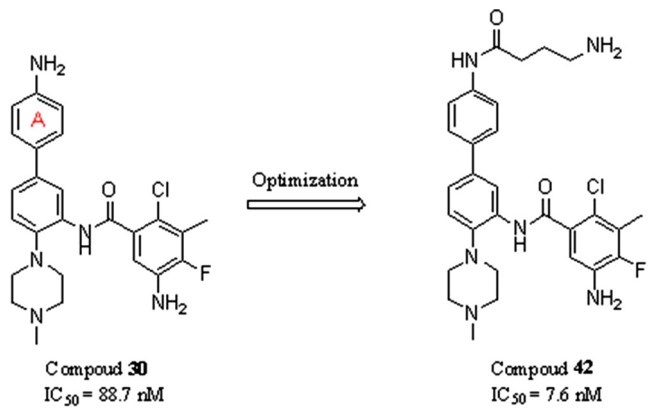


Fig. 1. The structure and activity of Compound **30** and Compound **42**.

potent inhibition, with an IC_{50} value of 1.2 μM (Fig. 4). The inhibition of MLL1 complex catalytic activity implied that **LC001** was on-target and would bind the protein tightly.

The binding between **LC001** and WDR5 protein was further investigated biophysical characterization. The biolayer interferometry assay (BLI), a label-free technology for measuring biomolecular interaction [18], was applied. In our present work, the biotin-labeled small molecule probe was immobilized on the Super Streptavidin Biosensors through biotin-streptavidin complex as the ligand and the WDR5 protein as the analyte. The interaction between the surface-bound LC001 and the WDR5 protein was observed in real time, which achieved the ability to monitor binding specificity and quantify the concentration of the WDR5 protein, with precision and accuracy (Fig. 5). The rate constants of k_{on} = 2.17E + 04 M^{-1} S^{-1} and k_{dis} = 6.61E−04 S^{-1} resulted in a K_d value of 30.4 nM (Fig. 5). The kinetic determinants showed very slow dissociation kinetics, which indicated a long residence time of **LC001** and a stable binding between the probe and WDR5 protein. This result demonstrated that the biotinylated probe could be used to specifically detect WDR5 and properly suggested the suitability of **LC001** for further research.

The tight binding between **LC001** and the WDR5 protein ensured that the probe could be a potent tool to explore the probe further applications. As we described before, recent reported studies revealed that WDR5 was also overexpressed in many cancer cells and played important roles in some cancers, such as bladder cancer, breast cancer and prostate cancer [14–16]. Despite recent progress related to WDR5, the biological function of WDR5 in more cancers remained to be further elucidated. Thus, we further validated the cellular interaction between **LC001** and WDR5 protein using affinity pull-down assay. The probe was used as the bait and the WDR5 is prey. As shown in Fig. 6, the cellular WDR5 was successfully preyed and enriched by **LC001**, confirming that the probe could tightly bind to the cellular WDR5 protein. However, pre-incubation of the lysate with **LD055** blocked the interaction. Therefore, **LC001** could selectively chemiprecipitates WDR5 from whole cell lysates of MV4-11 leukemia cells, which further confirmed that the biotin moiety of **LC001** did not interfere with its binding to WDR5 protein. This probe would future apply to capture WDR5 protein from other cell lines which may overexpress WDR5 protein. Future applications for the chemoprecipitation technique could include proteomic analysis of precipitated proteins to identify WDR5-binding partners.

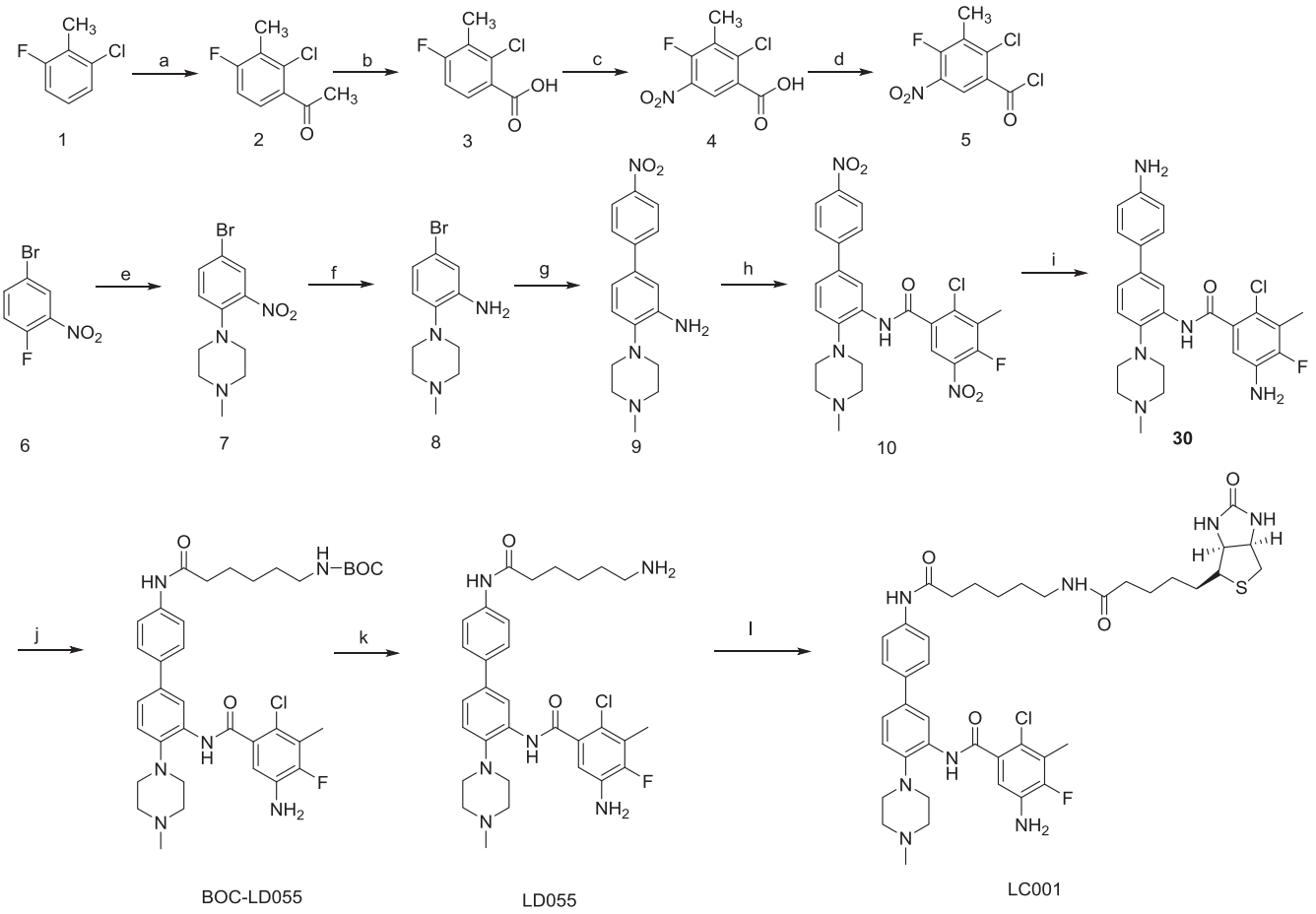
3. Conclusion

In this study, using our previously reported potent WDR5 small molecule inhibitor as the structure template, the biotin moiety was attached to the linker substituted on the 4 position of aromatic A, which afforded the biotinylated probe **LC001**. The probe exhibited high potency and on-target action, which provided the premise to explore the function of WDR5 protein. In addition, **LC001** could specifically and real time detect the WDR5 protein and selectively precipitates WDR5 from whole cell lysates. We look forward to future applications of the probe in studying the role of WDR5 in its interactome as well as in the progression of more cancers.

4. Experimental section

4.1. General chemistry

All reagents were purchased from commercial sources. Reactions were monitored by thin-layer chromatography (TLC) on 0.25 mm silica gel plates (GF254) and visualized under UV light. Melting points were determined with a Melt-Temp II apparatus. The ^{1}H NMR and ^{13}C NMR spectra were recorded with a Bruker



Scheme 1. Reaction: the conditions of step a to step i were seen in Ref. [14]. (j) Acid, BOP, TEA, DMF, r.t., 12 h. (k) TFA, DCM, r.t., 2 h. (l) BOP, TEA, DMF, r.t., 12 h.

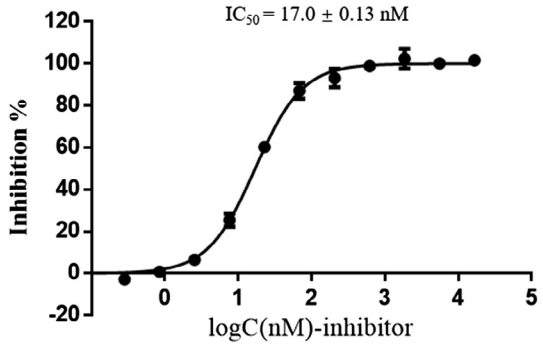


Fig. 2. Dose-response inhibition curve of **LC001** determined by the FP-based binding assay. Value was shown as mean ± SD (n = 3).

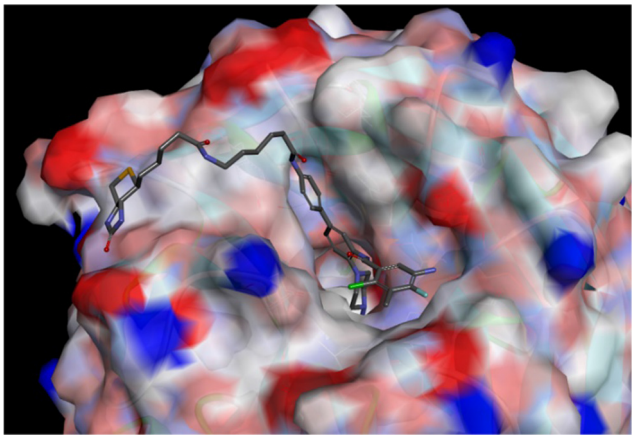


Fig. 3. Docking of **LC001** into the WDR5 crystal structure (PDB code: 4IA9) predicts a similar binding mode as compound **30**. Importantly, the biotin moiety of **LC001** is solvent exposed.

AV-300 instrument using deuterated solvents with tetramethylsilane (TMS) as internal standard. EI-MS was collected on shimadzu GCMS-2010 instruments. ESI-mass and high resolution mass spectra (HRMS) were recorded on a Water Q-Tofmicro mass spectrometer. Analytical results are within 0.4% of the theoretical values. The detailed experimental procedures of synthesis compound **30** can be found in Ref. [14]. The synthesis of **LC001** were described in the following section.

4.1.1. 5-amino-N-(4′-amino-4-(4-methylpiperazin-1-yl)-[1,1′-biphenyl]-3-yl)-2-chloro-4-fluoro-3-methylbenzamide (BOC-LD055)

To a solution of acid (0.65 mmol) in DMF (10 mL) at room temperature was added BOP reagent (0.28 g, 0.65 mmol), followed by TEA (0.18 mL, 1.29 mmol). The mixture was stirred for 0.5 h before compound **30** (0.20 g, 0.43 mmol) was added. Then the resulting solution was stirred at room temperature for 12 h. The aqueous solution was poured into 100 mL water and white precipitated solid was filtered off, washed with water, and dried to afford products **BOC-LD055**. m.p. 248–250 °C. ^{1}H NMR (300 MHz, DMSO d_6) δ 9.97 (s, 1H), 9.29 (s, 1H), 8.33 (s, 1H), 7.68 (m, 2H), 7.53 (d, J = 6.5 Hz, 2H), 7.38 (m, 1H), 7.29 (d, J = 7.8 Hz, 1H), 6.89 (d, J = 8.7 Hz,

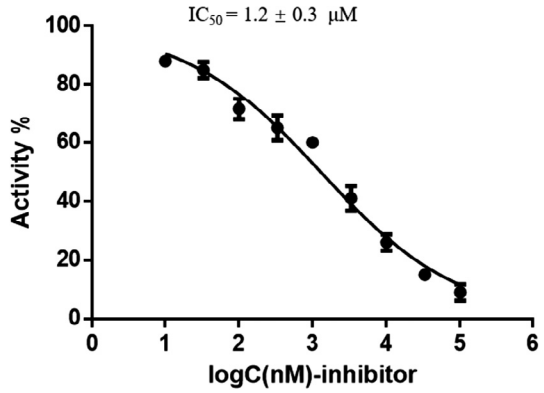


Fig. 4. Inhibition of HMT activity of reconstituted MLL1 core complex as measured with Alpha Screen assay. Value was shown as mean ± SD (n = 3).

1H), 6.79 (s, 1H), 5.51 (s, 2H), 2.89 (brs, 6H), 2.72 (m, 4H), 2.25 (m, 8H), 1.58 (brs, 2H), 1.29–1.22 (m, 13H). HRMS (ESI): calcd. for *m/z* $C_{36}H_{46}ClFN_6O_4$ $[M+H]^+$ 681.3253, found 681.3326.

4.1.2. 5-amino-N-(4′-(6-aminohexanamido)-4-(4-methylpiperazin-1-yl)-[1,1′-biphenyl]-3-yl)-2-chloro-4-fluoro-3-methylbenzamide (LD055)

TFA (1.7 mL, 23 mmol) was added to a solution of compound **Boc-LD055** (0.23 mmol) in DCM (20 mL) at room temperature. The mixture was stirred for 2 h. Then the aqueous solution was alkalified with saturated sodium bicarbonate solution to pH = 8. The organic phase was separated and water phase was extracted with DCM. Then the organic phase was dried (Na_2SO_4), filtered, and concentrated to provide target compound **LD055**. m.p. 218–220 °C. ^{1}H NMR (300 MHz, DMSO d_6) δ 9.29 (s, 1H), 8.34 (s, 1H), 7.75 (d, J = 8.5 Hz, 2H), 7.56 (d, J = 8.5 Hz, 2H), 7.40 (dd, J = 8.3, 2.0 Hz, 1H), 7.29 (d, J = 8.3 Hz, 1H), 6.89 (d, J = 9.1 Hz, 1H), 5.52 (s, 2H), 3.46 (q, J = 6.7 Hz, 1H), 2.88 (brs, 4H), 2.45 (brs, 4H), 2.25 (d, J = 2.5 Hz, 3H), 2.20 (s, 3H), 123 (d, J = 6.8 Hz, 3H). HRMS (ESI): calcd. for *m/z* $C_{31}H_{38}ClFN_6O_2$ $[M+H]^+$ 581.2729, found 581.2799.

4.1.3. 5-amino-2-chloro-4-fluoro-3-methyl-N-(4-(4-methylpiperazin-1-yl)-4′-(6-(5-((3aS,4R,6aR)-2-oxohexahydro-1H-thieno[3,4-d] imidazol-4 yl)pentanamido)hexanamido)-[1,1′-biphenyl]-3-yl) benzamide (LC001)

To a solution of d-biotin (0.65 mmol) in DMF (10 mL) at room temperature was added BOP reagent (0.28 g, 0.65 mmol), followed by TEA (0.18 mL, 1.29 mmol). The mixture was stirred for 0.5 h before compound **LD055** (0.25 g, 0.43 mmol) was added. Then the resulting solution was stirred at room temperature for 12 h. The aqueous solution was poured into 100 mL water and white precipitated solid was filtered off, washed with water, and dried to afford **LC001**. m.p. 225–227 °C. ^{1}H NMR (300 MHz, DMSO d_6) δ 9.98 (s, 1H), 9.29 (s, 1H), 8.32 (s, 1H), 7.76 (s, 1H), 7.68 (d, J = 8.3 Hz,2H), 7.53 (d, J = 8.25 Hz, 2H), 7.38 (d, J = 6.6 Hz, 2H), 7.28 (m, 1H), 6.88 (d, J = 9.1 Hz, 1H), 6.42 (s, 1H), 6.36 (s, 1H), 5.51 (s, 1H), 4.27 (brs, 2H), 4.11 (brs, 2H), 3.03–3.01 (m, 4H), 2.88 (brs, 4H), 2.82–2.78 (m, 2H), 2.57 (m, 2H), 2.30–2.24 (m, 6H), 2.02 (brs, 2H), 1.58 (m, 4H), 1.48–1.40 (m, 4H), 1.28–1.22 (m, 4H). HRMS (ESI): calcd. for *m/z* $C_{41}H_{52}ClFN_8O_4S$ $[M+H]^+$ 807.3505, found 807.3576.

4.2. Expression and purification of human WDR5

The expression and purification of WDR5 were described in Ref. [10].

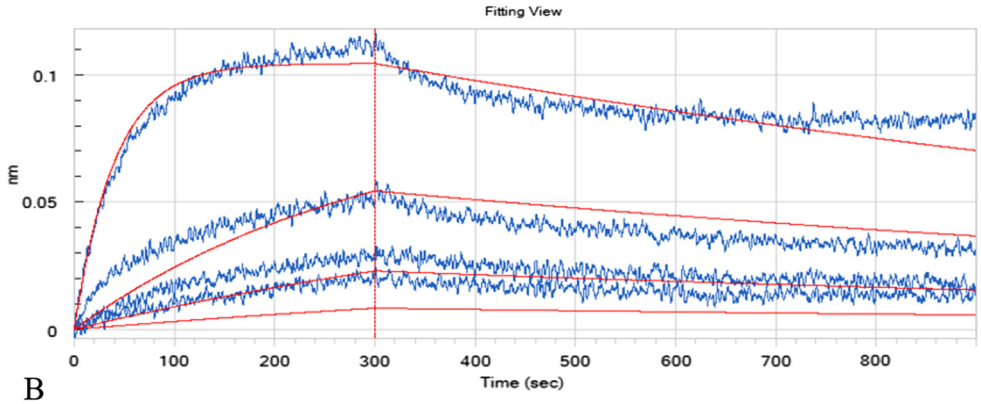
4.3. FP-based binding assay

The inhibition of MLL1-WDR5 interaction by **LD055** and **LC001** was evaluated by fluorescence polarization (FP) assay using the protocol described previously Ref. [10]. FP assays were performed in a 60 μL volume at a constant concentration of 3 nM 10mer-Thr-FAM probe and 8 nM WDR5 protein and incubated for 2 h in 384-well Corning plates using a SpectraMax paradigm reader (Molecular Device). For fluorescein, 485 nm excitation and 535 nm emission liters were used.

4.4. Biolayer interferometry

The interaction between the ligand and WDR5 was determined by biolayer interferometry using an Octet Red 96 instrument (For-

A



B

K_d (nM)	k_{on}(1/Ms)	k_{dis}(1/s)
30.4	2.17E+04	6.61E-04

Fig. 5. Biolayer interferometry curves of the binding of varying concentrations (1111, 123, 41, 14 nM) of the WDR5 protein to the immobilized probe.

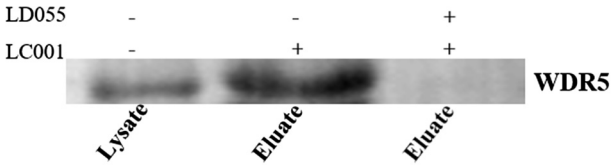


Fig. 6. Western blot analysis of **LC001** bound to the streptavidin coupled magnetic beads after incubation with MV4-11 (leukemia cell line) cell lysates. WDR5 was detected with an anti-WDR5 primary antibody. WDR5 western blot indicated that **LC001-**treated beads (lane 2) chemoprecipitated WDR5 compared to control lane 1. This interaction can be blocked by pretreating lysate with **LD055** (lane 3).

téBio Inc.). Super Streptavidin Biosensors tips (FortéBio Inc., Menlo Park, CA) were prewetted with buffer to establish a baseline before immobilization. Then **LC001** was immobilized onto Super Streptavidin Biosensors. All of the binding data were collected at 30 °C. The experiments comprised five steps: (1) baseline acquisition, (2) **LC001** loading onto sensor, (3) second baseline acquisition, (4) association of WDR5 for measurement of k_{on}, and (5) association of WDR5 for measurement of k_{dis}, Four concentrations of WDR5 were used for detection. The association and disassociation plot and kinetic constants were obtained with FortéBio data analysis software. Equilibrium dissociation constants (K_d) were calculated by the ratio of k_{dis} to k_{on}.

4.5. Affinity pull-down experiment

The streptavidin coupled magnetic beads ((Pierce™ Pull-Down Biotinylated Protein: Protein Interaction Kit 21115, Thermo Scientific) were loaded with **LC001** (50 μg/100 μL) and the mixture was incubated for 30 min at 4 °C with gentle rotation. Afterwards, beads were washed with 250 μL of Biotin Blocking Solution to block available streptavidin sites with free Biotin. The mixture was incubated for 5 min at room temperature with gentle rotation and washed with TBS again. Beads were suspended in 400 μL of the MV4-11 cell lysate. After incubation (3h, 4 °C, gentle rotation), beads were washed with TBS and suspended in 24 μL assay buffer containing 2% (w/v) SDS. The mixture was incubated at 95 °C for 5 min. Finally, beads were removed to yield the samples, which were further investigated by SDS-Page and western blot analysis.

4.6. Inhibition of methyltransferase activity of MLL complex in vitro

The inhibition of methyltransferase activity of MLL complex (MLL1, WDR5, RbBP5, and ASH2L proteins) was determined with Alpha Screen assays *in vitro*. The MLL1 enzymatic reactions were conducted in duplicate at room temperature for 60 min in a 50 μL mixture containing proper methyltransferase assay buffer (50 mM Tris, pH 8.8, 5 mM $MgCl_2$, 4 M DTT), S-adenosylmethionine (1μM), recombinant enzyme (MLL1, WDR5, Ash2L and RbBP5, 150 ng), and the probe in wells of a Histone substrate pre-coated plate. **LC001** was dissolved in DMSO and tested in 9-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 min. Wells were emptied, and 100 μL of diluted primary antibody was added. The plate was then slowly shaken for 60 min at room temperature. As before, the plate was emptied and washed three times, and shaken with Blocking Buffer for 10 min at room temperature. After discarding the Blocking Buffer, 100 μL of diluted secondary antibody was added. The plate was then slowly shaken for 30 min at room temperature. As before, the plate was emptied and washed three times, and shaken with Blocking Buffer for 10 min 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 were 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 sofeware.

4.7. Molecular docking

GOLD 5.1 was used to perform the docking of **LC001** to the catalytic site of WDR5. The protein structure of WDR5 was downloaded from PDB (4IA9) and was edited by adding hydrogen, deleting unnecessary waters and ligands. Water 536, 557, 667 and 703 were retained in protein, which played vital roles in **WDR5-47** binding to WDR5. Then the binding sites were defined according the endogenous ligand **WDR5-47**. Gold score was chosen as the score function of binding interaction energy for ranking. **LC001** was prepared by DS3.0 with CHARMm. The high fitness score model was selected to analyze binding model.

Conflict of interest

The authors confirm that this article content has no conflicts of interest.

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