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RAPID COMMUNICATION

Development of an orally bioavailable selective inhibitor of the menin-MLL

The protein–protein interaction between menin and mixed lineage leukemia (MLL) plays an important role in the development of human hepatocellular carcinogenesis (HCC) and is associated with poor prognosis of HCC patients.[1,2] Hence, interrupting the menin-MLL interaction is an attractive strategy in cancer treatment, particularly for liver cancer.[3,4] In this study, we identified complex **C1** as the first rhodium(III)-based orally bioavailable selective inhibitor of the menin-MLL interaction for HCC.

Nine rhodium(III)- and iridium(III)-based complexes (**C1**–**C9**) with general structure $[M(CN)_2(NN)]^+$ (M = Ir/Rh) were synthesized by coupling dichloride-bridged rhodium precursor compounds bearing the appropriate CN ligands (**C10**–**C12**) with a synthesized NN ligand **C13** (Fig. S1). The stability of **C1** in an aqueous buffer was confirmed by UV/Vis spectroscopy (Fig. S2). Complexes **C1**–**C9**, ligands **C10**–**C13**, and MI-2 were tested for menin-MLL inhibition using a biomolecular fluorescence complementation (BiFC) assay (Fig. S3, S4). In this assay, HepG2 cells were transfected with the plasmid pairs menin-VN and MLL-VC, which express the N-terminal and C-terminal fragments (VN and VC) of the fluorescent protein Venus fused to menin and MLL respectively. Compounds that disrupt the menin-MLL interaction *in cellulo* would be expected to reduce the fluorescence of the cells. The BiFC results showed that complex **C1** $[Rh(Brppy)_2(5,6\text{-dmphen})](PF_6)$ (where Brppy = 2-(4-bromophenyl)pyridine, 5,6-dmphen = 5,6-dimethyl-1,10-phenanthroline) exhibited the greatest inhibition of the menin-MLL interaction (Fig. 1A; Fig. S4). Encouragingly, **C1** displayed greater activity compared to the positive control MI-2,[5] without luminescence interference or impacting the total levels of menin and MLL (Fig. 1B, C; Fig. S4–6).

Complexes **C1**–**C6** all contain the 5,6-dmphen NN ligand (**C13**), but vary in the nature of their CN ligands. The rhodium(III) complex **C1** bearing the 2-(4-bromophenyl)pyridine (**C10**) CN ligand was the most effective of the series, and was significantly more potent than its iridium(III) congener **C4**, demonstrating the importance of the metal ion for menin-MLL inhibitory activity. Moreover, no inhibition was observed with the free ligands **C10**–**C13** (Fig. S4). These results highlight the importance of the rhodium(III) center in arranging the ligands in a bioactive configuration.

To verify the *in vitro* potency of **C1**, an AlphaScreen assay was performed. **C1** inhibited the binding of menin to MBM1 (menin-binding motif 1) with an IC_{50} value of 46 ± 9.47 nM (Fig. 1B). Fluorescence polarization (FP) is another common assay to evaluate *in vitro* potency. A fluorescein isothiocyanate (FITC)-labeled MBM1 peptide of MLL was used to assess the ability of **C1** to disrupt the menin-MLL interaction. **C1** dose-dependently inhibited the interaction of menin and MBM1 with an IC_{50} value of 0.398 ± 0.136 μM, while it bound to menin with a K_d value of 0.138 ± 0.088 μM *in vitro* (Fig. S7a). We further confirmed that **C1** does not interfere with the signal output of the FP assay (Fig. S7b).

We next investigated the ability of **C1** to disrupt the menin-MLL interaction in cells using the co-IP assay. As shown in Figure 1D and Figure S8, **C1** disrupted the menin-MLL interaction in a dose-dependent manner, with a more potent effect compared with MI-2. Subsequently, a cellular thermal shift assay (CETSA) experiment was performed to monitor the cellular target engagement of menin, MLL, and WDR5 in the cell environment. WDR5 is a histone methyltransferase that also engages MLL to promote its recruitment and a number of oncogenic MLL fusion proteins to target gene promoters. HepG2 cell lysates were treated with **C1** at room temperature for 60 min and then heated at different temperatures ranging from 45 °C to 70 °C for 10 min, and the fraction of soluble protein remaining was determined by Western blot assay. **C1** could significantly stabilize menin in HepG2 cell lysates while not affecting the stability of WDR5 and MLL (Fig. 1E; Fig. S9, 10). Finally, ITC revealed a nanomolar binding affinity (K_d = 78 nM) between **C1** and menin (Fig. S11). Overall, these results indicate that by engaging menin, **C1** could efficiently disrupt the interaction between menin and MLL *in vitro* and *in cellulo*.

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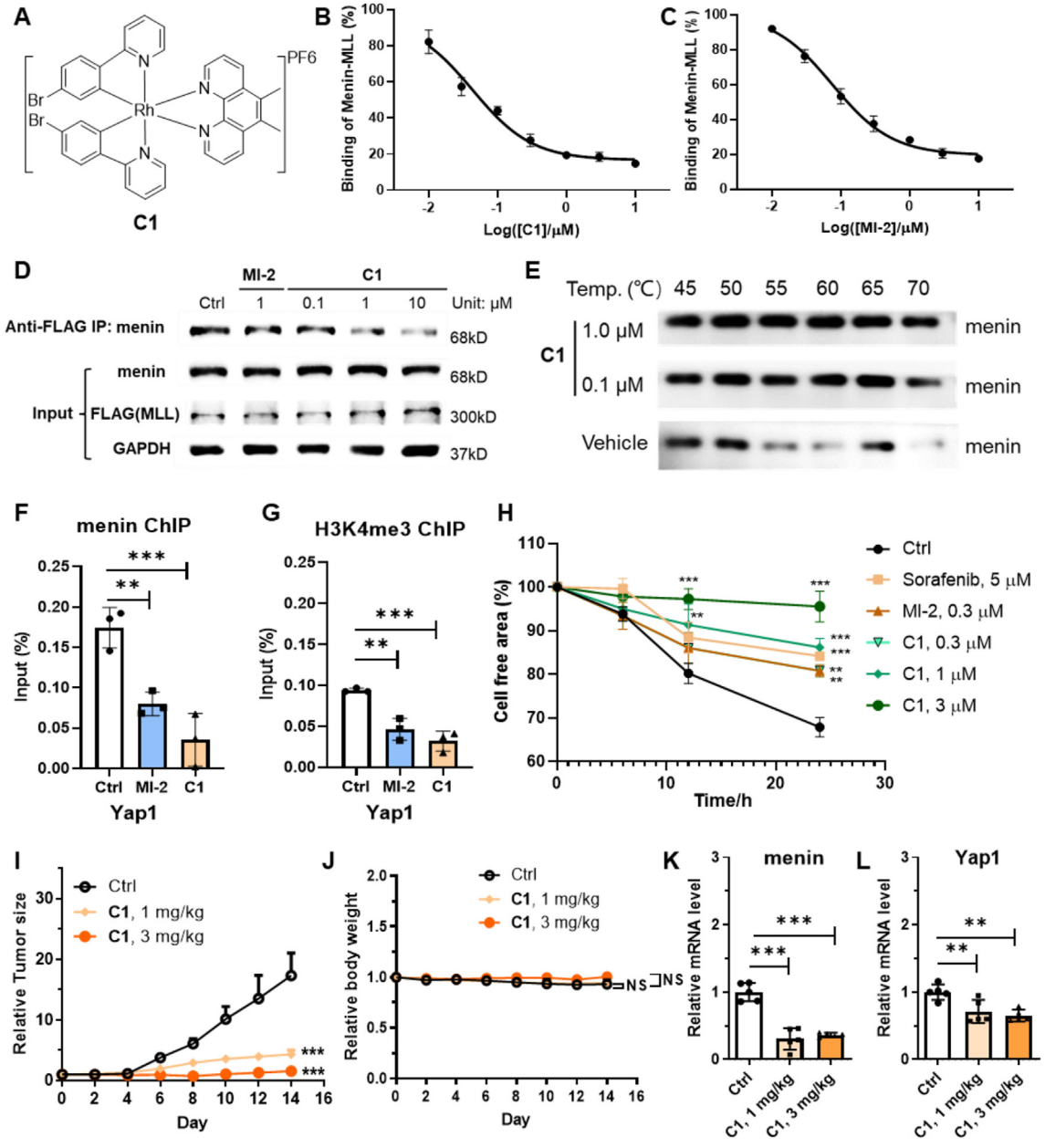

Figure 1 The menin-MLL PPI inhibitor **C1** suppressed tumor growth *in vivo*. **(A)** Chemical structure of cyclometallated rhodium(III) **C1**. **(B, C)** **C1** and MI-2 inhibit menin-MLL interaction in a dose-dependent manner in an AlphaScreen assay. **(D)** Co-IP assay of HepG2 cells treated with MI-2, **C1**, or vehicle (0.1% DMSO) for 6 h. **(E)** Thermal stabilization of menin by **C1** in cell lysates. HepG2 cell lysates were treated with **C1** at 0.1 μM or 1.0 μM. Densitometry analysis of menin content. Error bars indicate standard deviation calculated from three independent measurements. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ versus the control. **(F, G)** ChIP analysis using antibodies against menin or H3K4me3 showed that **C1** interrupted the binding of menin and reduction of H3K4me3 at the Yap1 promoter was markedly decreased. **(H)** Wound-healing kinetics in HepG2 cells was measured over time. Data presented are mean ± SEM from 3 wounds per condition. **(I)** Average tumor volume of control group versus treatment groups (**C1**: 1 mg/kg and 3 mg/kg) (mean ± SEM, $n = 5$). **(J)** Average body weight of the vehicle control group versus the treatment groups (**C1**: 1 mg/kg and 3 mg/kg). **(K)** Expression of menin and Yap1 measured by qRT-PCR of RNA extracted from tumor samples harvested at the endpoint of the vehicle control group versus the treatment groups (**C1**: 1 mg/kg and 3 mg/kg). One-way ANOVA analysis demonstrates the statistical significance of the effect observed for **C1** as compared to the control group. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Menin plays an important role in the recruitment of MLL and MLL fusion proteins to target genes, including *HOXA7*, *HOXA9*, and *HOXA13*.[3] Chromatin immunoprecipitation (ChIP) analysis demonstrated that **C1** decreased the binding of menin to the sites of the *HOXA7*, *HOXA9*, and *HOXA13* promoters, but had no effect on *CDKN1A*, *CDKN2A* or *GAPDH* promoters (Fig. S12). **C1** also decreased mRNA levels of *Yap1*, *HOXA7*, *HOXA9*, *HOXA13,* and *IL6* (Fig. S13). Menin occupancy frequently coincides with H3K4me3 at the promoter of *Yap1* to activate Yap1 transcription. Yap1 is phosphorylated and accumulates in the cytoplasm, leading to HCC cell invasion and proliferation.[5] The ChIP assays showed that **C1** reduced the binding of menin at the *Yap1* locus but not at the *GAPDH* locus (Fig. 1F; Fig. S12). Further, **C1** reduced H3K4me3 levels at the *Yap1* locus (Fig. 1F, G). qRT-PCR and immunoblotting analysis revealed that the Yap1 levels were reduced by **C1** in a dose-dependent manner (Fig. S13). p-Yap1 (Ser127) was reduced in the cytoplasm of HepG2 cells when treated with **C1** (Fig. S14). Taken together, these results suggest that **C1** is able to modulate the interaction between chromatin and menin in HepG2 cells, particularly at the Yap1 promoter.

C1 selectively inhibited HepG2 and Hep3B hepatoma cell proliferation with GI_{50} values of 0.31 $\pm$ 0.15 μM and 0.71 $\pm$ 0.02 μM respectively, while showing lower cytotoxicity to human non-small cell lung cancer A549 cells (GI_{50} = 3.57 $\pm$ 1.91 μM) and normal human HEK239T cells (GI_{50} = 8.28 $\pm$ 1.42 μM) (Table S1). The higher expression status of menin in hepatoma cells was confirmed by Western blot assay (Fig. S15). Moreover, the migration and invasion of HepG2 were markedly reduced upon treatment with **C1** as observed using a wound healing assay and a transwell assay respectively (Fig. 1H; Fig. S16, 17). Finally, menin knockdown significantly reduced **C1**-induced cytotoxicity towards HepG2 cells, suggesting that **C1** acted through menin to exert its cytotoxic effects (Fig. S18, 19).

In a mouse xenograft model of HCC, mice orally treated with **C1** showed a significant reduction of tumor volume from day 6 onwards versus the control group (Fig. 1I). No signs of gross toxicity or statistically significant organ weight loss (Fig. 1J; Fig. S20) or changes in complete blood cell count (Fig. S21) were observed between the control group and the treatment groups. Tumors in treated animals showed a significant decrease in the mRNA and protein levels of menin and Yap-1, suggesting that menin activity was dramatically inhibited *in vivo* (Fig. 1K, L). p-Yap1 (Ser127) was also slightly reduced (Fig. S22). Consistent with the *in cellulo* results, HOXA7, HOXA9, HOXA13, and IL-6 levels in tumor tissues were also decreased by **C1** (Fig. S23). Overall, the results suggest that **C1** demonstrates potent *in vivo* anti-tumor activity via its ability to inhibit menin activity and could therefore represent a potential drug candidate for HCC treatment.

In summary, this study has identified a rhodium(III)-based menin-MLL inhibitor **C1** as a promising orally bioavailable anti-tumor lead compound. **C1** could significantly inhibit the menin-MLL interaction and regulate epigenetic modulations by antagonizing the binding of menin to the Yap1 promoter. Significantly, **C1** reduces cell proliferation, migration, and invasion in *in vitro* HCC models. Moreover, **C1** reduced tumor growth *in vivo* without impairing normal hematopoiesis or inducing gross toxicity. Overall, this evidence indicates that **C1** can serve as a chemical scaffold for the development of more potent and novel menin-MLL inhibitors with druglike properties.

Conflict of interests

The authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2022.10.005.

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