Pharmacologic Inhibition of the Menin–MLL Interaction Leads to Transcriptional Repression of PEG10 and Blocks Hepatocellular Carcinoma

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Abstract

Hepatocellular carcinoma (HCC) accounts for approximately 85% of primary malignant liver tumors and is the fifth most common cancer in the world, leading to over 600,000 deaths each year, emphasizing the need for new therapies. Upregulation of menin was reported in HCC patients and high levels of menin correlate with poor patient prognosis. The protein–protein interaction between menin and histone methyltransferase mixed lineage leukemia 1 (MLL1) plays an important role in the development of HCC, implying that pharmacologic inhibition of this interaction could lead to new therapeutic strategy for the HCC patients. Here, we demonstrate that the menin–MLL inhibitor MI-503 shows antitumor activity in in vitro and in vivo models of HCC and reveals the potential mechanism of menin contribution to HCC. Treatment with MI-503 selectively kills various HCC cell lines and this effect is significantly enhanced by a combination of MI-503 with sorafenib, the standard-of-care therapy for HCC. Furthermore, MI-503 reduces sphere formation and cell migration in in vitro HCC models. When applied in vivo, MI-503 gives a strong antitumor effect both as a single agent and in combination with sorafenib in mice xenograft models of HCC. Mechanistically, treatment with MI-503 downregulates expression of several genes known to play a critical role in proliferation and migration of HCC cells, including PEG10, and displaces the menin–MLL1 complex from the PEG10 promoter, resulting in reduced H3K4 methylation and transcriptional repression. Overall, our studies reveal a mechanistic link between menin and genes involved in HCC and demonstrate that pharmacologic inhibition of the menin–MLL interaction might represent a promising therapeutic approach for HCC.

Introduction

Hepatocellular carcinoma (HCC) accounts for approximately 85% of malignant liver tumors and is the fifth most common cancer in the world, leading to over 600,000 deaths each year (1, 2). The majority of HCC patients are diagnosed at a late stage of the disease, when curative therapies are no longer effective (3), and most patients die about 6 months after diagnosis (1, 4, 5). Furthermore, patients who initially respond to curative therapies frequently experience disease recurrence, resulting in an overall 5-year survival of only 7% (6), while patients with surgically resectable tumors have survival rates still below 40% (7). All these findings strongly support the need for new and more effective therapies for HCC. One recent new treatment strategy for advanced HCC patients is the use of the multitargeted kinase inhibitor sorafenib (3), which blocks the activity of Raf serine/threonine kinases and various receptor tyrosine kinases (8). Sorafenib inhibits the proliferation of HCC cells and blocks tumor angiogenesis, resulting in an increase of about three months in the median survival of HCC patients (8). However, sorafenib has only limited clinical efficacy with undesired side effects (9), strongly implying the need for more efficacious therapies for HCC.

Recently it has been demonstrated that menin, a scaffold protein encoded by the multiple endocrine neoplasia type 1 (MEN1) gene, is involved in promoting hepatocellular carcinoma (10). Menin is a highly specific binding partner of mixed lineage leukemia 1 (MLL1, also known as KMT2A; refs. 11, 12), a histone methyltransferase that catalyzes the trimethylation of H3K4 (H3K4me3) (13), and is required for the recruitment of the MLL1 complex to the target genes (14). Menin upregulation was reported in HCC patients, and high levels of menin correlate with poor patient prognosis (10). Indeed, menin knockdown substantially represses HCC cell proliferation, blocks colony formation, and inhibits tumor growth in the xenograft model of HCC (10). Likewise, heterozygous loss of Men1 reduces development of HCC in vivo (10).

The function of menin in liver tumorigenesis has been linked to the transcriptional regulation of the Yes-associated protein (Yap1), an important oncogene in HCC (15) and a downstream target of the Hippo pathway (16, 17). Menin occupancy at the promoter region of Yap1 has been noted to coincide with H3K4me3, a histone mark regulated by MLL1 (10), implying a potential involvement of the menin–MLL1 interaction in HCC development. We, and others, have previously shown a critical role of the menin interaction with MLL1 and/or MLL fusion complex from the PEG10 promoter, resulting in reduced H3K4 methylation and transcriptional repression.
proteins in acute leukemias with translocations of the MLL gene (12, 18), as well as in solid tumors, including metastatic prostate cancer (19). All these studies imply that the menin–MLL1 interaction might play a more general role in cancer, including liver tumorigenesis; therefore inhibition of this interaction with small molecules might represent a novel therapeutic approach for HCC treatment. On the other hand very limited effect observed in HCC cells following treatment with a weak menin–MLL1 inhibitor MI-1 (no significant effect on cell viability in liquid culture and <25% inhibition of colony formation and cell migration when applied as a single agent at 4 μmol/L; ref. 20) raises questions regarding the potential therapeutic value of blocking the menin–MLL1 interaction as a treatment for liver cancer.

We have recently reported very potent small-molecule inhibitors of the menin–MLL1 interaction with optimized drug-like properties, including MI-503 (IC50 = 14 nmol/L, Kd = 9 nmol/L), which demonstrated very potent activity in both in vitro and in vivo models of MLL leukemia (18, 21–23) as well as in castration-resistant prostate cancer (19). As the in vitro potency of MI-503 is >140-fold better than MI-1 and it demonstrates strong and selective activity in cancer cells, we selected MI-503 to study the effect of pharmacologic inhibition of the menin–MLL1 interaction in liver cancer. Here, we performed a systematic evaluation of the MI-503 in HCC models to assess whether pharmacologic inhibition of the menin–MLL1 interaction might represent a new therapeutic strategy for liver cancer. Treatment with MI-503 had a very pronounced effect in various models of HCC, both in vitro and in vivo, and this effect was enhanced when MI-503 was combined with sorafenib, the current standard-of-care therapy.

In patients with advanced HCC, Pharmacologic inhibition of menin-MLL1 by MI-503 strongly downregulated the expression level of genes previously linked to HCC, including PEG10 (24, 25), thereby providing a new mechanistic insight into the role of menin–MLL1 and H3K4me3 in HCC. Overall, our findings demonstrate that pharmacologic inhibition of the menin–MLL1 interaction can block progression of HCC, thus validating this protein–protein interaction as an attractive target for therapeutic intervention in liver cancer.

Materials and Methods
Chemistry
Chemical synthesis and chemical characterization of MI-503 and MI-372 compounds have been described previously (18).

Cell culture
The HepG2 (low metastatic; ref. 26) and Hep3B (low metastatic; ref. 27) human HCC cell lines were obtained from ATCC in 2014 while the remaining cell lines (MHCC97, PLC/PRF/5, SNU449, and SNU423) were received from Dr. Ilona Kryczek (University of Michigan, Ann Arbor, MI). HepG2, Hep3B, and PLC/PRF/5 cells were maintained in Eagle’s minimum essential medium (EMEM; ATCC) with 10% FBS and 1% penicillin/streptomycin (Gibco). ASC52 cell line was received from Dr. Elizabeth Lawlor (University of Michigan, Ann Arbor, MI) and cultured in mesenchymal stem cell (MSC) basal medium with Mesenchymal Stem Cell Growth Kit for Adipose and Umbilical-derived MSC Low Serum Components and G418 (ATCC). All cell lines were used in the described experiments before reaching the 10th passage after thawing the cells out and were negative for mycoplasma prior performing these studies as assessed by the TOOK-E PCR Mycoplasma Detection Kit.

Cell viability assay
Cell viability assays are described in the Supplementary Information.

Cellular thermal shift assay
Cellular thermal shift assay (CETSA) was performed as described previously (19, 28). Experimental details are provided in the Supplementary Information.

Real-time qPCR
Cells were plated at a concentration of 0.25 × 10^5 cells/mL in the 12-well plate in duplicates and treated with MI-503 or DMSO for 6 and/or 13 days followed by RNA isolation according to the manufacturer’s protocol (RNaseasy kit, Qiagen), RNA reverse transcription to cDNA, and qPCR (see Supplementary Information for details).

RNA Sequencing studies
HepG2 cells were plated in the 12-well plates at the initial concentration of 0.4 × 10^6 cells/mL and treated with 3 μmol/L MI-503 or DMSO (0.25%) in triplicates. After 3 days of treatment, cells were trypsinized, counted, and viable cell number was adjusted to the original concentration. Media were changed and compound or DMSO was resupplied at that time. Cells were harvested after 3 more days of incubation. Total RNA was isolated from cells using RNaseasy kit (Qiagen), amplified, and quality was assessed using the TapeStation (Agilent). Sequencing was performed at the University of Michigan DNA Sequencing Core. Samples with RIN (RNA Integrity Numbers) of 8 or greater were prepped using the Illumina TrueSeq mRNA kit (Illumina). RNA was converted to mRNA using a polyA purification. cDNA library was created using reverse transcriptase, barcoded, and sequenced using 4 samples per lane on a HiSeq 2000 (Illumina) in High Output mode. Sequenced reads were aligned to human reference genome using Bowtie and Tophat (version 2.0.3). Differential gene expression analysis was done using program Cuffdiff. Genes with P < 0.05 and a fold change greater than 2-fold were considered significant. Gene Set Enrichment Analysis (http://www.broadinstitute.org/gsea/index.jsp) was performed on gene expression of MI-503–treated versus DMSO-treated cells.

Sphere formation and cell migration assays
Sphere formation and cell migration assays are described in Supplementary Information.

In vitro combination studies
HepG2 and Hep3B cells were plated at a concentration of 0.45 × 10^5 cells/mL in a 24-well plate and treated with 0.25% DMSO, sorafenib, MI-503, or both compounds. After 4 days (for HepG2) or 5 days (for Hep3B) of incubation cells were transferred to the 96-well plate in quadruplicates. The MTT cell proliferation assay kit (Roche) was then applied. Plates were read for absorbance at 570 nm using a PHERAstar BMG microplate reader. Drug interactions were analyzed by CalcuSyn program (Biosoft) based on the analytical method of Chou and Talalay (29).
Chromatin immunoprecipitation

A total of 1.5 × 10⁶ HepG2 cells plated in 10-cm dish were treated with DMSO (0.25%) or MI-503 at 4 μmol/L, 2 μmol/L, and 1 μmol/L concentrations for 6 days. After 3 days of incubation, cell number was adjusted to the starting concentration, media were changed, and fresh compound was added. Following 6 days of incubation, cells were fixed with 1% paraformaldehyde for 10 minutes at room temperature. Cells were then quenched with glycine for 5 minutes at room temperature. ChIP was performed using Magna ChIP A/G kit (Millipore) according to the manufacturer's protocol. The cell lysate was prepared and equal amounts of DNA isolated from either DMSO or MI-503–treated samples was immunoprecipitated using antibodies against Menin (Bethyl Laboratories), MLL1 (Millipore), H3 (Abcam), H3K4me3 (Abcam), and IgG (Millipore) overnight at 4°C. The DNA was eluted and subjected to quantitative PCR using SYBR Green primers for PEG10 (see Supplementary Information for primer sequences and corresponding genomic reference coordinates).

Animal studies

All animal studies were performed in compliance with the guidelines of the University of Michigan Committee for Use and Care of Animals and Unit for Laboratory Animal Medicine (ULAM) under the protocol PRO00005043. Freshly thawed HepG2 or Hep3B cells (ATCC) were cultured in EMEM with 10% FBS and 1% penicillin/streptomycin antibiotics. On the day of injection, cells were reseeded in serum-free medium at 1 × 10⁶ cells/mL and mixed 1:1 with Matrigel. A total of 0.5 × 10⁶ HepG2 or Hep3B cells (ATCC) were cultured in EMEM with 107 cells/mL and mixed 1:1 with Matrigel. A total of 0.5 × 10⁶ cells/mL were injected to the flank of the 6-week-old female athymic nude mice. When tumor size reached approximately 100 mm³, mice were randomized into four groups with each group containing 8–9 mice to provide over 80% power to detect 50% effect. Mice were then treated with vehicle (25% DMSO, 25% PEG400, 50% PBS), MI-503 (dissolved in vehicle), sorafenib (dissolved in 12.5% Cremophor, 12.5% Ethanol and 75% water), or both compounds once daily at the designated doses using intraperitoneal (vehicle and MI-503) or oral, (sorafenib) administration. Body weight and tumor sizes were monitored 3 times per week. The tumor volume was calculated according to the formula: tumor volume = a × b²/2 (a, long diameter; b, short diameter). Treatment continued until the mean tumor size in the vehicle group reached approximately 1,200 mm³. The experimenters were not blind to group assignment and outcome assessment.

Statistical analysis

Student t test (unpaired, two-tailed) was used to calculate significance level between treatment groups to calculate P values. P values of less than 0.05 were considered significant. Graph generation and statistical analysis were performed using GraphPad Prism version 6.02 software (GraphPad).

Accession numbers

RNA-seq data were deposited at the NCBI Gene Expression Omnibus under the accession number GSE103327.

Results

Menin is overexpressed in liver cancer cells

To understand the tumorigenic function of menin in hepatocellular carcinoma, we compared the expression level of MEN1, which encodes menin, in normal liver and liver tumor samples by analyzing the Oncomine datasets (30). Comparison of the MEN1 expression level between the two sets of normal liver and HCC patient samples (31) revealed a significant upregulation of MEN1 in the liver tumor samples (Fig. 1A, consistent with the previous studies; ref. 10). We further investigated the role of menin in liver cancer by assessing the level of menin in several human HCC cell lines: HepG2, Hep3B, SNU449, SNU423, MHCC97, PLC/PRF/5. Western blot analysis confirmed that menin is expressed and detectable in each of these HCC cell lines, although some variability in the menin expression was observed (Supplementary Fig. S1A).

Studies by Xu and colleagues (10) support the importance of the menin interaction with MLL in hepatocellular carcinoma. Therefore, we used the panel of HCC cell lines to evaluate the expression level of two MLL family members, MLL1 (KMT2A) and MLL2 (KMT2B), which are both capable of strong interaction with menin (11). Interestingly, we found much higher expression level of MLL1 than MLL2 in all HCC cell lines tested (Supplementary Fig. S1B) which suggests that MLL1, rather than MLL2, plays an important role in liver cancer through a direct interaction with menin.

Menin–MLL inhibitor blocks proliferation of HCC cells

Knock down of menin substantially represses proliferation and reduces colony formation in HCC cells (10). To determine whether pharmacologic inhibition of the menin–MLL interaction affects growth of liver cancer cells, we used our recently reported and very potent menin–MLL inhibitor MI-503 (Supplementary Fig. S1C), which has demonstrated strong activity in MLL leukemia (18) and prostate cancer (19) models. Treatment with MI-503 inhibited the proliferation of HepG2 liver cancer cells in a dose- and time-dependent manner, with a more pronounced effect observed upon prolonged treatment (over 7 days; Fig. 1B). The need for a prolonged treatment in the HCC cells is consistent with the effects we observed in MLL leukemia (18) and prostate cancer cells (19). Further examination of MI-503 in a panel of HCC cell lines revealed a strongly decreased viability of these cells after 7 and 12 days of treatment, with a half-maximal growth inhibitory (GI₅₀) concentration ranging from 0.5 to 3.2 μmol/L after 12 days of treatment (Fig. 1C; Supplementary Table S1). Importantly, treatment with a structurally similar but much weaker menin–MLL inhibitor, MI-372 (Supplementary Fig. S1C), did not show an effect on HCC cell proliferation (Supplementary Fig. S1D; Supplementary Table S1). These results validate that targeted inhibition of the menin–MLL1 by MI-503 inhibits the growth of HCC cells. We have also tested the effects of MI-503 treatment in normal adipose-derived ASC52 mesenchymal stem cells, which served as a negative control cell line, and observed no substantial effect on cell growth up to 6 μmol/L of MI-503 (Fig. 1D; Supplementary Table S1). These data are consistent with no or limited effect of MI-503 in other negative control cell lines as we reported previously (18, 19) and demonstrate specificity of MI-503 on proliferation of liver cancer or other menin–MLL–dependent cells (18). To assess whether cell growth inhibition correlates with menin engagement by MI-503, we performed the CETSA. MI-503 treatment of HepG2 cells at 45°C induced a thermal stabilization of menin when compared with the DMSO-treated cells (Fig. 1E; Supplementary Fig. S1E), confirming that MI-503 binds to menin in HCC cells.
MI-503 blocks cell migration and sphere formation in HCC cells

The menin–MLL1 interaction was shown to contribute to the aggressive nature of HCC (10). As cell migration is a property of cancer cells associated with cancer metastasis, we assess the effect of menin inhibition on HCC cell migration using the cell culture wound closure assay (32) by measuring the rate of wound closure after treatment with MI-503. The migration of both HepG2 and...
Hep3B cells was markedly reduced upon treatment with MI-503 when compared with the DMSO-treated cells (Fig. 2A–D; Supplementary Fig. S2A and S2B). Furthermore, we have also evaluated the effect of MI-503 on sphere formation in HepG2 and Hep3B cell lines, both of which were previously shown to retain the capacity of sphere formation (33, 34), a propensity of cancer stem cells (35). Treatment with MI-503 strongly inhibited sphere formation in both cell lines as indicated by markedly reduced sphere numbers (Fig. 2E and G) and substantially smaller sphere sizes when compared with DMSO-treated cells (Fig. 2F and H). Overall, all these data confirm strong effect of the MI-503 menin–MLL inhibitor in HCC cell lines, supporting a potential therapeutically active value of menin–MLL inhibitors in HCC.

**MI-503 affects expression of genes linked to HCC**

Previous studies have suggested that menin promotes liver tumorigenesis by regulating the expression and function of YAP1, an important “driver gene” in HCC, through an epigenetic mechanism involving the H3K4me3 histone mark (10). To investigate whether pharmacologic inhibition of the menin–MLL1 interaction affects YAP1 expression, we treated HepG2 cells with MI-503 and assessed YAP1 expression by qRT-PCR. We found a dose-dependent reduction in YAP1 expression after 6 days of treatment with MI-503, but this effect was relatively modest, with only approximately 40% downregulation of YAP1 observed following treatment with 2 μmol/L MI-503 (Fig. 3A). Furthermore, downregulation of YAP1 expression did not become more pronounced after prolonged (13 days) treatment of HepG2 cells with MI-503 (Fig. 3A).

To further investigate the effect of menin–MLL1 inhibition on gene expression changes in HCC cells, we performed the RNA sequencing (RNA-seq) studies in HepG2 cells following treatment with MI-503. We found 291 and 552 genes down- and upregulated, respectively, which showed at least 2-fold change in the expression level following treatment with MI-503 (Supplementary Table S2). We then have performed the GSEA analysis and found significant enrichment for genes up and downregulated in a subclass of HCC with activated CTNNB1 gene encoding β-catenin (36). A set of genes up- and downregulated upon treatment with MI-503 is, respectively, down and upregulated in this subtype of HCC (Fig. 3B and C). The CTNNB1 gene encodes β-catenin and its mutations lead to the activation of Wnt signaling, which plays an essential role in development of HCC by controlling cell proliferation, apoptosis, cell cycle, and motility (37–39). Mutations in CTNNB1 are highly prevalent in HCC, representing the second most frequently mutated gene in HCC (40). Indeed, the HepG2 cell line represents the liver cancer cell line characterized by activated Wnt signaling and expresses a truncated β-catenin lacking the Ser/Thr domain that regulates its degradation (ref. 41; Supplementary Table S3). The observation that MI-503 reverses gene expression signature in HCC with activating CTNNB1 mutations supports the potential application of menin–MLL1 inhibitors in HCC with activated Wnt signaling pathway.

The RNA-seq data for HepG2 cells treated with MI-503 showed only a minor reduction (~20%) in the YAP1 expression, which did not reach statistical significance (Supplementary Table S2); this was likely due to a lower sensitivity of this method compared with qRT-PCR (see above). Interestingly, within the top genes strongly downregulated following treatment with MI-503, we found several genes known to promote proliferation or migration of HCC cells, including PEG10, SLC38A4, and SEMA3C (Supplementary Table S2). These results were further validated by the qRT-PCR studies in both HepG2 and Hep3B cells, demonstrating a strong reduction in the expression level of these genes following treatment with MI-503 (Fig. 3D and E).

The PEG10 (Paternally Expressed Gene 10) gene is not expressed in normal adult liver (24) but is highly overexpressed in HCC (24, 25). Indeed, the Oncomine (30) analysis has confirmed the high overexpression of PEG10 in HCC in different sets of patient samples when compared with normal liver samples (Fig. 3F). Furthermore, significant correlation of PEG10 and MEN1 expression was found in the HCC patient samples but not in normal liver samples (Fig. 3G and H), suggesting a positive regulation of PEG10 by menin. Importantly, PEG10 promotes cancer cell growth in HCC and is associated with poor patient survival and with tumor recurrence (24, 25). Therefore, inhibition of HCC cell growth induced by MI-503 (Fig. 1C) could, at least in part, result from the downregulation of PEG10. As mentioned above, treatment of HCC cells with MI-503 also leads to downregulation of both SLC38A4, a gene activated in HCC that confers growth and survival advantages in the premalignant and malignant lesions (42), and SEMA3C (semaphorin 3C), which plays an important role in migration of HCC cells (ref. 43; Fig. 3D and E; Supplementary Table S2). These results suggest that pharmacologic inhibition of the menin–MLL interaction can block hepatocellular carcinoma by its effects on pathways associated with the proliferation and migration of HCC cells.

Interestingly, among genes markedly upregulated upon treatment of HepG2 cells with MI-503 we found GDF15 (Growth Differentiation Factor 15; Supplementary Table S2). The qRT-PCR studies performed in HepG2 and Hep3B cells demonstrated approximately 3-fold increase in GDF15 expression following treatment with MI-503, and this effect was dose dependent (Fig. 3D and E). Downregulation of GDF15 was previously found to be associated with HCC development, while restoration of GDF15 level attenuated the progression of HCC (44); however, the role of this gene in HCC seems to be quite complex (45). Nevertheless, we selected this gene as a positive control in the gene expression studies as its level increases following menin–MLL1 inhibition, but the functional consequences of GDF15 changes due to MI-503 treatment of HCC cells still need further investigation.

**PEG10 is a direct target of menin–MLL1 complex**

The finding that treatment of HCC cells with MI-503 resulted in downregulation of PEG10 prompted us to perform chromatin immunoprecipitation (ChIP) experiments in HepG2 cells to determine whether the menin–MLL1 complex regulates PEG10 expression through a direct binding to this gene. We first assessed menin binding to PEG10 and found strong enrichment of menin at two sites (P-B and P-C; Fig. 4A and B). We then assessed the effect of MI-503 on the binding of the menin–MLL1 complex to PEG10 at the sites with highest menin occupancy (Fig. 4A and B). We observed a strong, dose-dependent reduction in both menin and MLL1 binding to the PEG10 promoter after 6 days of treatment with MI-503 (Fig. 4C and D). MLL1 is a histone methyltransferase that catalyzes H3K4 tri-methylation, representing a chromatin activation mark. Therefore, we assessed whether reduced binding of the menin–MLL1 complex to PEG10 induced by MI-503 affects the H3K4 tri-methylation mark at those sites. We found that MI-503 treatment resulted in marked reduction in H3K4me3 at both sites on PEG10 (Fig. 4C and D) consistent with the reduced expression of PEG10 (Fig. 3D). These results support...
an epigenetic regulation of PEG10 by the menin–MLL1 complex and demonstrate that pharmacologic inhibition of the menin–MLL1 interaction releases this complex from PEG10 and leads to a reduction in H3K4 trimethylation and downregulation of PEG10 expression.

Sorafenib sensitizes HCC cells to MI-503 treatment
Sorafenib, a multikinase inhibitor of tyrosine protein kinases with a validated therapeutic activity against HCC, has been approved for clinical use in patients with unresectable hepatocellular carcinoma (1). We explored whether sorafenib would sensitize HCC cells to pharmacologic inhibition of the menin–MLL1 interaction by testing a combination of sorafenib and MI-503. Both sorafenib and MI-503 were used at concentrations required for effective growth inhibition in HCC cells (ref. 4; Fig. 5A and C).

The inhibitory effect of MI-503 on HepG2 cell growth was strongly enhanced by a combination with sorafenib (Fig. 5A). The combination indices (CIs) calculated for the simultaneous treatment of HepG2 cells with MI-503 and sorafenib ranged from 0.45 to 0.66, suggesting strong synergistic effect (Fig. 5A and B). Similarly, the combination of MI-503 and sorafenib resulted in a more pronounced inhibition of cell growth in Hep3B cells when compared...
Figure 3.
Menin–MLL inhibitor inhibits genes involved in proliferation and migration of HCC cells. A, Quantitative RT-PCR analysis of YAP1 expression performed in HepG2 cells after 6 and 13 days of treatment with MI-503 or DMSO. Data represent mean of duplicates ± SD. B and C, Gene Set Enrichment Analysis (GSEA) of genes downregulated (B) or upregulated (C) upon treatment with MI-503 in HepG2 cells as compared with genes from the Wnt signaling overexpressed in patient samples. The heatmaps show genes comprising the leading edge of the GSEA plots. Red, high expression; blue, low expression. Triplicate samples were used for global gene expression studies. D and E, Quantitative RT-PCR analysis of PEG10, SEMA3C, SLC38A4, and GDF15 expression performed in HepG2 (D) or Hep3B (E) cells after 6 days of treatment with MI-503 or DMSO. Data represent mean of triplicates ± SD. Expression of genes in A, D, and E was normalized to GAPDH and referenced to DMSO-treated cells. F, Expression level of PEG10 in normal liver samples compared with hepatocellular carcinoma as obtained from the analysis of two datasets (Roessler liver, Roessler liver 2) in the Oncomine database. G and H, Correlation of PEG10 with MEN1 expression in HCC (G) or normal liver samples (H). Gene expression is shown as log2 median-centered intensity. NES, normalized enrichment score; FDR, false discovery rate.
Figure 4.
Epigenetic regulation of PEG10 by menin-MLL1 in HCC. A, Diagram showing the location of primers at the promoter and downstream regions of PEG10. Genomic coordinates are provided in Supplementary Methods. B, Menin binding to different regions (P-A – P-D) of PEG10 obtained from the ChIP experiment performed in HepG2 cells. C and D, ChIP experiments performed in HepG2 cells upon 6 days of treatment with various concentrations of MI-503 or DMSO to detect menin and MLL1 binding as well as H3K4me3 on PEG10. Real-time PCR was performed on the precipitated DNAs with P-B (C) and P-C (D) primers for PEG10 genomic regions. IgG was used as a control. Data represent mean of triplicates ± SD.
with treatment with either compound individually (Fig. 5C). The calculated CI values \(<0.66\) support synergistic effect of MI-503 and sorafenib (Fig. 5C and D). Overall, these results demonstrate that sorafenib sensitizes HCC cells to the pharmacologic inhibition of the menin–MLL1 interaction.

**MI-503 inhibits tumor growth in vivo in HCC xenograft models**

The pronounced effects of MI-503 observed in HCC cell lines prompted us to assess the therapeutic potential of the menin–MLL inhibitor in in vivo models of HCC. We have recently shown that MI-503 has a favorable pharmacokinetic profile in mice and has demonstrated strong in vivo efficacy in mice models of MLL leukemia (18) and in castration-resistant prostate cancer (19), which emphasizes its suitability as a candidate for in vivo studies. Here, we used MI-503 both as a single agent and in combination with sorafenib to assess the in vivo efficacy of the menin–MLL1 inhibition in two mouse models of HCC: HepG2 and Hep3B xenograft models. The HepG2 or Hep3B cells were implanted into the flank of athymic nude mice. Treatment with MI-503 and/or sorafenib was then initiated when the tumors reached approximately 100 mm³ volume and was continued until the volume of the tumor in the vehicle-treated mice reached approximately 1,200 mm³ (2–3 weeks). Once-daily intraperitoneal administration of MI-503 at a 35 mg/kg dose caused a strong reduction (>50%) in the tumor growth in both HepG2 and Hep3B xenograft models (Fig. 6A and B). Furthermore, an 85% inhibition of the tumor growth was observed in both xenograft models when MI-503 was combined with sorafenib (Fig. 6A and B). Importantly, no substantial signs of toxicity (e.g., no significant changes in mouse body weight or liver enzymes level), were observed in the mice during the treatment (Supplementary Fig. S3). These results support a potential therapeutic value of menin–MLL inhibitors in

**Figure 5.**
Sorafenib sensitized HCC cells to the treatment with MI-503. A, MTT cell viability assay performed after 4 days of treatment of HepG2 cells with MI-503, sorafenib, and both agents tested simultaneously. Data represent mean of quadruplicates ± SD. B, Isobologram obtained from analysis of the combination of MI-503 and sorafenib in HepG2 cells after 4 days of treatment. Combinatorial indices (CI < 1) indicate synergistic effect between these two agents in HepG2 cells. C, MTT cell viability assay performed after 5 days of treatment of Hep3B cells with MI-503, sorafenib, or both agents tested simultaneously. Data represent mean of quadruplicates ± SD. D, Isobologram obtained from analysis of the combination of MI-503 and sorafenib in Hep3B cells after 5 days of treatment. Combinatorial indices (CI < 1) indicate synergistic effect between these two agents in Hep3B cells.
HCC. As sorafenib alone leads to >50% tumor growth inhibition in both xenograft models (Fig. 6A and B) it remains to be explored whether lower doses of both compounds could result in even stronger combinatorial effect in vivo.

We next examined gene expression in the tumor samples collected from the HepG2 xenograft mice treated with MI-503, sorafenib, combination of both agents or vehicle. The tumor samples collected from mice treated with MI-503 as a single agent showed a marked reduction in the expression level of PEG10 and SLC38A4, which are both growth-promoting genes in HCC cells (Fig. 6C). Upregulation of GDF15 was also observed in MI-503–treated mice when compared with vehicle-treated control mice (Fig. 6C). These results were consistent with the on target activity of MI-503 and gene expression studies performed in vitro in the HepG2 and Hep3B cells (Fig. 3D and E). In contrast, we did not observe statistically significant effect on the expression level of...
PEG10, SLC38A4, or GDF15 in the tumor samples isolated from mice treated with sorafenib alone (Fig. 6C). These results indicate a different mechanism of action for sorafenib and MI-503 in HCC cells. Interestingly, combinatorial treatment of MI-503 and sorafenib in mice resulted in a similar trend in the gene expression changes as observed for MI-503 alone (especially for the downregulated genes), but the effects are less pronounced and did not reach statistical significance (Fig. 6C). Taken together, the reduction in the tumor growth in the in vivo models of HCC induced by MI-503 correlated with the decrease in the expression level of genes linked to HCC, thereby validating the on-target mechanism of action of MI-503. These results demonstrate that pharmacologic inhibition of the menin–MLL interaction and/or its combination with sorafenib could represent an effective approach for HCC treatment.

Discussion

HCC represents an aggressive and incurable cancer that requires new therapies for effective patient treatment. Menin is upregulated in HCC (Fig. 1A) and a high level of menin expression correlates with poor patient survival, emphasizing an important role of menin in HCC (10). As menin interaction with MLL1 was indicated to play an important role in the development of HCC, we hypothesized that pharmacologic inhibition of this interaction could represent a new therapeutic approach in HCC. Here, we established that MI-503, a potent small-molecule inhibitor of the menin–MLL1 interaction, has a pronounced antitumor effect in both in vitro and in vivo models of HCC. Treatment with MI-503 results in effective killing of HCC cells and reduces sphere formation and cell migration. The combination of MI-503 with sorafenib, multikinase inhibitor currently used in the clinic for HCC treatment, synergistically enhanced the effects of MI-503 on proliferation of HCC cells. Furthermore, in vivo application of MI-503 resulted in marked reduction in tumor growth in HepG2 and Hep3B xenograft models, and these effects were enhanced by combination of MI-503 with sorafenib. Overall, our studies demonstrate that pharmacologic inhibition of the menin–MLL interaction has a pronounced effect in HCC models, both in vitro and in vivo, and this approach might be promising as therapy for HCC patients.

Previous studies have suggested that menin function in liver tumorigenesis is associated with transcriptional activation of YAP1, an important oncogene in HCC (10, 15). However, here we found that pharmacologic inhibition of the menin–MLL1 interaction had only a modest effect on the YAP1 expression in HepG2 cells. Instead, we found strong downregulation of other genes involved in HCC cell growth or migration, including PEG10, SLC38A4, and SEMA3C, following treatment with the MI-503 menin–MLL1 inhibitor. We further validated that MI-503 reduces the binding of menin and MLL1 to PEG10 and decreases the H3K4me3 level at PEG10, providing an important mechanistic link between PEG10 and the menin–MLL1 axis in HCC. Fig. 6D. Furthermore, global gene expression studies have identified that MI-503 affects expression of genes associated with a subclass of HCC with mutations in CTNNB1, which encodes β-catenin, thereby providing a link to the Wnt signaling pathway that is known to play a crucial role in a subset of HCC (37–39). Overall, our studies demonstrate that the role of menin in HCC is much more complex than the transcriptional regulation of YAP1 and Hippo pathway, as suggested previously (10). The menin–MLL1 complex participates in epigenetic activation of several genes involved in HCC pathogenesis, rather than regulating a specific pathway in HCC.

We have previously demonstrated strong antitumor effects of the MI-503 menin–MLL1 inhibitor in in vitro and in vivo models of MLL leukemias (18), prostate cancer (19), and Ewing sarcoma (46). In addition, our earlier menin–MLL1 inhibitor, MI-2, also strongly inhibited tumor cell growth in pediatric gliomas with H3.3K27M mutations (47) and downregulated genes involved in the estrogen receptor–positive breast cancer (48). In the study presented here, we demonstrate that pharmacologic inhibition of the menin–MLL1 interaction by MI-503 leads to the effective killing of HCC cells and pronounced in vivo tumor growth inhibition, providing an another example of the antitumor effect resulting from blocking the menin–MLL1 interaction. The results of these studies might suggest a novel and promising treatment for HCC patients by using the menin–MLL1 inhibitors either as single agents or in combination with known drugs, including sorafenib. While menin–MLL1 inhibitors may represent valuable anticancer agents, further studies are needed to assess inhibition of the menin–MLL1 interaction in the context of tumor suppressor function of menin in endocrine tissues (49). Although loss of menin results in MEN1 tumors, many MEN1 missense mutations are outside of the MLL-binding site on menin (50), suggesting that other menin interactions may be required for the tumor suppressor activity of menin. Clinical studies are needed to address the utility of menin–MLL1 inhibitors in HCC and other cancers.

Disclosure of Potential Conflicts of Interest

T. Cierpicki reports receiving a commercial research grant, has ownership interest (including patents), and is a consultant/advisory board member for Kura Oncology. J. Grembecka reports receiving a commercial research grant, has ownership interest (including patents), and is a consultant/advisory board member for Kura Oncology, Inc. No potential conflicts of interest were disclosed by the other authors.

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Conception and design: B. Malik, T. Cierpicki, J. Grembecka
Development of methodology: B. Malik, T. Cierpicki
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Kempinska, B. Malik, S. Klossowski, S. Shukla, H. Miao
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K. Kempinska, B. Malik, J. Wang, J. Grembecka
Writing, review, and/or revision of the manuscript: B. Malik, S. Klossowski, S. Shukla, J. Wang, T. Cierpicki, J. Grembecka
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): B. Malik, D. Borkin, J. Wang
Study supervision: T. Cierpicki, J. Grembecka

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References

1. Llovet JM, Bruix J. Molecular targeted therapies in hepatocellular carcinoma. Hepatology 2008;48:1312–27.
2. Llovet JM, Burroughs A, Bruix J. Hepatocellular carcinoma. Lancet 2003; 362:1907–17.
3. Balogh J, Victor D III, Asham EH, Burroughs SG, Boktour M, Saharai A, et al. Hepatocellular carcinoma: a review. J Hepatocellular Carcinoma 2016; 3:41–53.
4. Liu L, Cao Y, Chen C, Zhang X, McNabola A, Wilkie D, et al. Sorafenib blocks the RAF/MEK/ERK pathway, inhibits tumor angiogenesis, and induces tumor cell apoptosis in hepatocellular carcinoma model PLC/PRF/5. Cancer Res 2006;66:11851–8.
5. Feo F, Pascale RM, Simile MM, De Miglio MR, Muroni MR, Calvisi D. Genetic alterations in liver carcinogenesis: implications for new preventive and therapeutic strategies. Crit Rev Oncog 2000;10:11–62.
6. Bosch FX, Ribes J, Diaz M, Clases R. Primary liver cancer: worldwide incidence and trends. Gastroenterology 2004;127:S5–S16.
7. Takenaka K, Kawahara N, Yamamoto K, Kajiyama K, Maeda T, Itasaka H, et al. Results of 280 liver resections for hepatocellular carcinoma. Arch Surg 1996;131:71–6.
8. Cheng AL, Kang YK, Chen Z, Tsao CJ, Qin S, Kim JS, et al. Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomised, double-blind, placebo-controlled trial. Lancet Oncol 2009;10:25–34.
9. Bertot LC, Sato M, Tatsiresi R, Yoshida H, Koike K. Mortalit[y and complication rates of percutaneous ablative techniques for the treatment of liver tumors: a systematic review. Eur Radiol 2011;21:2584–96.
10. Xu B, Li SH, Zheng R, Gao SB, Ding LH, Yin ZY, et al. Menin promotes hepatocellular carcinogenesis and epigenetically up-regulates Vap1 transcription. Proc Natl Acad Sci U S A 2013;110:17480–5.
11. Grebecka J, Belcher AM, Hartley T, Cierpicki T. Molecular basis of the mixed lineage leukemia-menin interaction: implications for targeting mixed lineage leukemias. J Biol Chem 2010;285:4090–8.
12. Yokoyama A, Somervaille TC, Smith KS, Rosenblatt-Rossen O, Meyerson M, Chng VW. The menin tumor suppressor protein is an essential oncogenic cofactor for MLL-associated leukemogenesis. Cell Growth 2007;123:207–18.
13. Schuettengruber B, Chourrout D, Vervoort M, Leblanc B, Cavalli G. Identiﬁcation of HCC is related to gene alterations and experimental proof. Nat Cancer Inst 2012;34:1877–82.
14. Milne TA, Hughes CM, Lloyd R, Yang Z, Rozenblatt-Rosen O, Dou Y, et al. Menin and MLL cooperatively regulate expression of cyclin-dependent kinase inhibitors. Proc Natl Acad Sci U S A 2005;102:749–54.
15. Zender L, Spector MS, Xue W, Flemming P, Gordon Cardo C, Silke J, et al. Identiﬁcation and validation of oncogenes in liver cancer using an integrative oncogenic approach. Cell 2006;125:1253–67.
16. Zhou D, Conrad C, Xia F, Park JS, Payer B, Yin Y, et al. Mst1 and Mst2 maintain hepatocyte quiescence and suppress hepatocellular carcinoma development through inactivation of the Vap1 oncogene. Cancer Cell 2009;16:425–38.
17. Zhao B, Li B, Lei Q, Guan KL. The Hippo-YAP pathway in organ size control and tumorigenesis: an updated version. Genes Dev 2010;24:862–74.
18. Borkin D, He S, Miao H, Kemipinska K, Pollock J, Chase J, et al. Pharmacologic inhibition of the Menin-MLL interaction blocks progression of MLL leukemia in vivo. Cancer Cell 2015;27:589–602.
19. Malik R, Khan AP, Asangani IA, Cieslik M, Prendergast FR, Wang X, et al. Targeting the MLL complex in castration-resistant prostate cancer. Nat Med 2015;21:344–52.
20. Gao SB, Xu B, Ding LH, Zheng QL, Zhang L, Zheng QF, et al. The functional and mechanistic relatedness of EZH2 and menin in hepatocellular carcinoma. J Hepatol 2014;61:832–9.
21. He S, Malik B, Borkin D, Miao H, Shukla S, Kempinska K, et al. Menin-MLL inhibitors block oncogenic transformation by MLL-fusion proteins in a fusion partner-independent manner. Leukemia 2016;30:508–13.
22. Borkin D, Pollock J, Kemipinska K, Puzohiti T, Li X, Wen B, et al. Property focused structure-based optimization of small molecule inhibitors of the protein-protein interaction between menin and mixed lineage leukemia (MLL). J Med Chem 2016;59:892–913.
23. Grebecka J, He S, Shi A, Purohit T, Muntean AG, Sorenson RJ, et al. Menin-MLL inhibitors reverse oncogenic activity of MLL fusion proteins in leukemia. Nat Chem Biol 2012;8:277–84.
24. San H, Ha SY, Hwang SH. PABP2 expression of PEG10 is associated with poor survival and tumor recurrence in hepatocellular carcinoma. Cancer Treat Res 2015;47:844–52.
25. Ip WK, Lai PW, Wong NL, Sy SM, Beleshki B, Squier JA, et al. Identiﬁcation of PEG10 as a progression related biomarker for hepatocellular carcinoma. Cancer Lett 2007;250:284–91.
26. Liu L, Ren ZG, Shen Y, Zhu XD, Zhang W, Xiong W, et al. Identiﬁcation of the SOX4 target genes and clinical value of miRNA-167 in hepatocellular carcinoma. J Hepatol 2014;61:832–9.
27. Lu M, Cleary ML. The menin tumor suppressor protein is an essential oncogenic cofactor for MLL-associated leukemogenesis. Genes Dev 2010;24:862.
28. Liang CC, Park AY, Guan JL. In vivo scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. Nat Protoc 2007;2:329–33.
29. Cao L, Zhou Y, Zhai B, Liao J, Xu W, Zhang R, et al. Sphere-forming cell subpopulations with cancer stem cell properties in human hepatoma cell lines. BMC Gastroenterol 2011;11:71.
30. Uchida Y, Tanaka S, Aihara A, Adikrisna R, Yoshitake K, Matsumura S, et al. Analogy between sphere forming ability and stemness of human hepatoma cells. Oncol Rep 2010;24:1147–51.
31. Weiswald LB, Bellet D, Dangles-Marie V. Spherical cancer models in tumor biology. Neoplasia 2015;17:1–15.
32. Zhang Y, Villanueva A, Hoshida Y, Peix J, Newell P, Minguez B, et al. FOIL gains of VEGFA and molecular classiﬁcation of hepatocellular carcinoma. Cancer Res 2008;68:6779–88.
33. Laurent-Puig P, Legoix P, Bleuteau O, Belghiti J, Franco D, Binot F, et al. Genetic alterations associated with hepatocellular carcinomas deﬁne distinct pathways of hepatocarcinogenesis. Gastroenterology 2001;120:1763–73.
34. Liu L, Xue SX, Chen YT, Xue JL, Zhang CJ, Zou F. Aberrant regulation of Wnt signaling in hepatocellular carcinoma. World J Gastroenterol 2016;22:7486–99.
35. Waisberg J, Saba GT. Wnt-/-beta-catenin pathway signaling in human hepatocellular carcinoma. World J Hepatol 2015;7:2631–5.
36. Royaud S, Rickman DS, de Reyniers A, Balbaud C, Rebuissin S, Jeannot E, et al. Transcriptome classiﬁcation of HCC is related to gene alterations and to new therapeutic targets. Hepatology 2007;45:42–52.
37. Lachenmayer A, Alsinet C, Savic R, Cabello I, Tollanin S, Hoshida Y, et al. Wnt-pathway activation in two molecular classes of hepatocellular carcinoma and experimental modulation by sorafenib. Clin Cancer Res 2012;18:4997–5007.
38. Kondoh N, Inamaki N, Arii M, Hada A, Hatasue K, Matsuoe I, et al. Activation of a system A amino acid transporter, ATAI/NLC38A1, in human hepatocellular carcinoma and perineoplastic liver tissues. Int J Oncol 2007;31:81–7.
39. Liao YL, Sun YM, Chau GY, Chau YP, Lai TC, Wang JL, et al. Identiﬁcation of SOX4 target genes using phylogenetic footprinting-based prediction from expression microarrays suggests that overexpression of SOX4 potentiates metastasis in hepatocellular carcinoma. Oncogene 2008;27:5578–89.

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44. Yu J, Shen B, Chu ES, Teoh N, Cheung KF, Wu CW, et al. Inhibitory role of peroxisome proliferator-activated receptor gamma in hepatocarcinogenesis in mice and in vitro. Hepatology 2010;51:2008–19.
45. Zimmers TA, Jin X, Gutierrez JC, Acosta C, McKillop IH, Pierce RH, et al. Effect of in vivo loss of GDF-15 on hepatocellular carcinogenesis. J Cancer Res Clin Oncol 2008;134:753–9.
46. Svoboda LK, Bailey N, Van Noord RA, Krook MA, Harris A, Cramer C, et al. Tumorigenicity of Ewing sarcoma is critically dependent on the trithorax proteins MLL1 and menin. Oncotarget 2017;8:458–71.
47. Funato K, Major T, Lewis PW, Allis CD, Tabar V. Use of human embryonic stem cells to model pediatric gliomas with H3.3K27M histone mutation. Science 2014;346:1529–33.
48. Dreijerink KM, Groner AC, Vos ES, Font-Tello A, Gu L, Chi D, et al. Enhancer-mediated oncogenic function of the menin tumor suppressor in breast cancer. Cell Rep 2017;18:2359–72.
49. Chandrasekharappa SC, Teh B. Functional studies of the MEN1 gene. J Intern Med 2003;253:606–15.
50. Murai MJ, Chruszcz M, Reddy G, Grembecka J, Gierpicki T. Crystal structure of menin reveals binding site for mixed lineage leukemia (MLL) protein. J Biol Chem 2011;286:31742–8.
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