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Small Molecule Therapeutics

Molecular Cancer Therapeutics

Small-Molecule Disruption of the Myb/p300 Cooperation Targets Acute Myeloid Leukemia Cells

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Abstract

The transcription factor c-Myb is essential for the proliferation of hematopoietic cells and has been implicated in the development of leukemia and other human cancers. Pharmacologic inhibition of Myb is therefore emerging as a potential therapeutic strategy for these diseases. By using a Myb reporter cell line, we have identified plumbagin and several naphthoquinones as potent low-molecular weight Myb inhibitors. We demonstrate that these compounds inhibit c-Myb by binding to the c-Myb transactivation domain and disrupting the cooperation of c-Myb with the coactivator p300, a major driver of Myb activity. Naphthoquinone-induced inhibition of c-Myb suppresses Myb target gene expression and induces the differentiation of the myeloid leukemia cell line HL60. We demonstrate that murine and human primary acute myeloid leukemia cells are more sensitive to naphthoquinone-induced inhibition of clonogenic proliferation than normal hematopoietic progenitor cells. Overall, our work demonstrates for the first time the potential of naphthoquinones as small-molecule Myb inhibitors that may have therapeutic potential for the treatment of leukemia and other tumors driven by deregulated Myb. *Mol Cancer Ther; 15(12); 2905–15.* ©*2016 AACR.*

Introduction

The c-*myb* proto-oncogene was discovered more than thirty years ago as the cellular counterpart of the retroviral oncogene v-*myb* of avian myeloblastosis virus, but its relevance for human cancer has only recently been recognized (1). Rearrangements of the c-*myb* locus have been observed in acute lymphoblastic leukemia (T-ALL; refs. 2, 3). Mutations that create Myb-binding sites upstream of the *Tal1* oncogene have been found in a significant fraction of T-ALL of children (4). These changes create a "super-enhancer," leading to increased *Tal1* expression. Acute myeloid leukemia (AML) cells are often "addicted" to high levels of c-Myb expression that exceed those required for proliferation and survival of normal hematopoietic cells (1, 5, 6). Gene rearrangements and deregulation of c-*myb* expression have also been implicated in nonhematopoietic tumors, including breast cancer (7, 8), colon carcinoma (9, 10), adenoid cystic carcinoma (11), and diffuse low-grade pediatric gliomas (12). Overall, these findings have greatly fostered interest in c-Myb as a potential drug target.

c-Myb plays a key role as a transcription factor in hematopoietic cells and several other tissues (13). Among the known protein interaction partners of c-Myb, the coactivator p300 has emerged as a key driver of c-Myb activity. The interaction of Myb and p300 is mediated by the so-called KIX domain of p300 which binds to a highly conserved LXXLL-motif located in the c-Myb transactivation domain (14). Several studies have confirmed the relevance of this motif for Myb activity and its role in hematopoietic cells (15–18). Mutations leading to amino acid substitutions within the LXXLL motif (e.g., replacement of Leu-302 by Ala) virtually completely abolish Myb activity and cause hematopoietic defects (19). Targeting the c-Myb/p300 interaction therefore appears to be a valid strategy to inhibit c-Myb activity. In support of this idea, we have recently shown that Naphthol AS-E phosphate, a low molecular weight compound originally described as inhibitor of the interaction of the KIX domain with transcription factor CREB (20), disrupts the c-Myb/p300 interaction and inhibits c-Myb activity (21).

To facilitate the identification of compounds that inhibit Myb activity, we have previously established a cell-based screening system that can be used to search for Myb-inhibitory compounds (22, 23). Using this system, we have discovered that several naphthoquinones have potent Myb-inhibitory activity. Here, we have examined the mechanism and the biological consequences of Myb inhibition by these compounds.

Materials and Methods

Cells

HD11-C3-GFP1 cells were generated in our laboratory and have been described (22). HD11-C3-RL1 is a similar line based on

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Renilla luciferase instead of eGFP as reporter. HL60 cells were originally obtained from ATCC 4 years ago. No further authentication was done by the authors. Murine hematopoietic progenitor cells were isolated from the femurs of C57/BL6 wild-type mice and enriched for absence of lineage differentiation markers (lin^-) by immunomagnetic beads using the MACS Lineage Cell Depletion Kit (Miltenyi Biotec; ref. 24). Murine leukemias were generated by transplantation of oncogene (MLL-AF9 or MYC/BCL2) transduced lineage-negative cells in lethally irradiated syngeneic recipients as described previously (25). Acute myeloid leukemia (AML) blast cells were obtained from the bone marrow of patients diagnosed with AML at the University of Münster Hospital (Münster, Germany). Blasts were enriched (usually >90%) by density centrifugation. $CD34^+$ hematopoietic progenitor cells were isolated by magnetic cell sorting from leukapheresis samples of healthy donors undergoing harvest for allogeneic stem cell transplantation. All patients and donors provided written consent, and all studies were approved by the local ethical board. Plumbagin and related naphthoquinones were obtained from Sigma-Aldrich. Cell viability was analyzed by an MTS assay (23). Trolox and vitamin C were added together with plumbagin. N-acetylcysteine treatment (NAC) was performed by preincubating the cells for 1 hour with NAC followed by transferring them to fresh medium. If not indicated otherwise, cells were treated with compounds for 12 to 16 hours.

Transfections

QT6 fibroblasts were transfected by calcium-phosphate coprecipitation, and reporter gene activities were analyzed as described previously (26). The luciferase reporter genes pGL4-5xMRE(GG)-Myc-Luc and pG5E4-38Luc contain 5 tandem copies of Myb- or Gal4-binding sites, respectively (27, 28). Cotransfections were performed with the β-galactosidase reporter gene pCMVβ (Clontech) and luciferase values were normalized against the β-galactosidase activity to compensate different transfection efficiencies. Reporter studies were performed in at least three independent experiments, with replicate transfections in each experiment. Expression vectors for v-Myb (pCDE26v-Myb) and c-Myb (pCDNA3-chc-Myb) have been described previously (29). A mutant lacking all cysteine residues (pCDE26v-Myb/CallA) was generated by oligonucleotide-directed mutagenesis, converting all cysteine codons to alanine codons. pGal4/c-Myb encodes a Gal4/chicken c-Myb fusion protein containing c-Myb amino acid sequences 244 to 500. pKIX/VP16 and Gal4/VP16 encode fusion proteins of the VP16 transactivation domain and the KIX domain of p300 (amino acids 556-652) or the Gal4 DNA-binding domain. Expression of endogenous *mim-1* and ribosomal protein S17 mRNAs was analyzed as described previously (22).

Lentiviral infections

A lentivirus encoding a c-terminally truncated c-Myb (c-MybΔ3) was generated by replacing the RFP coding region of pLVX-DsRed-Monomer-C1 (Clontech) by the coding sequence of human c-Myb, truncated after amino acid 390. Viral particles were generated by cotransfecting the resulting plasmid (pLVX-c-MybΔ3) with lentiviral packaging plasmids, using Hek293T cells. Infected HL60 cells were selected with 2 μg/mL puromycin.

Real-time PCR

Quantitative real-time PCR was performed as described previously (22) with the following primers: *ACTB* mRNA: 5′-CGTCCACCGCAAATGCTT-3′ and 5′-GTTTTCTGCGCAAGTTAGGT-3′; c-*MYC* mRNA: 5′-TGCGTGACCAAGATCCC-3′ and 5′-CGCACAAGAGTTCCGTA-3′; *ADA* mRNA: 5′-ACCTGGCTGGAGATGAGACC-3′ and 5′-TTTTTGAGCCGAATGACTGC-3′; *CDC2* mRNA: 5′-CTGGAGTTGAGTAACGAGCTGA-3′ and 5′-TTGGATTCTATCCCTCCTGG-3′; *c-KIT* mRNA 5′-TGATTTTCCTGGATGGATGG-3′ and 5′-TGGGATTTTCTCTGCGTTCT-3′. Data were analyzed by subtracting the C_t values for Myb target genes from those for *ACTB*, thereby normalizing the corresponding mRNA amounts to β-actin mRNA.

Microscale thermophoresis

To analyze the interaction of naphthoquinones with the Myb transactivation domain extracts of QT6 cells transfected with GFP/Myb (202–442), GFP/Myb (1–201), GFP/Myb (278–328), or GFP were prepared in 50 mmol/L HEPES, pH 7.4, 120 mmol/L sodium chloride, 1 mmol/L EDTA, 6 mmol/L EGTA, and 0.5% Nonidet P-40. Naphthoquinone concentrations ranging from 1.4 nmol/L to 50 μmol/L were combined with constant amounts of cell extract, incubated for 1 to 2 hours at room temperature and filled in capillaries to perform thermophoresis measurements in a NanoTemper Monolith (NT.015) instrument. Thermophoresis was performed at 1,475 ± 15 nm. Data from several independent experiments were normalized to ΔFnorm [‰] (10^*(Fnorm (bound) − Fnorm(unbound)) or fraction bound (ΔFnorm [‰]/amplitude) to calculate K_d values.

Differentiation and apoptosis assays

HL60 cells were cultured for 2 days in the presence of plumbagin and/or all-trans-retinoic acid (ATRA). The cells were analyzed by May-Grünwald staining or by flow cytometry using phycoerythrin-labeled anti-human CD11b antibody (BD Pharmingen).

Colony formation assays

Viable cells (500–1,000 as determined by Trypan blue staining) were seeded per dish in growth factor–supplemented methylcellulose medium. All experiments were performed in triplicates and repeated several times. Colonies with more than 50 cells were counted on day 7.

Results

5-hydroxy-2-methyl-1,4-naphthoquinone inhibits c-Myb activity

We have previously established a cell-based screening system to identify compounds that inhibit Myb activity (22, 23). This system is based on a chicken macrophage cell line that expresses c-Myb in a doxycyline-inducible manner and carries a GFP reporter gene driven by the *cis*-regulatory elements of the highly Myb-inducible chicken *mim-1* gene (ref. 30; Fig. 1A). We have used this cell line to screen a number of plant-derived sesquiterpene lactones as well as several compounds, which were selected for the presence of so-called α,β–unsaturated carbonyl groups, for Myb-inhibitory activity. Such reactive chemical groups are responsible for the biological activities of many sesquiterpene lactones and other compounds. One of the molecules that showed a strong inhibitory activity in this screening system was 5-hydroxy-2-methyl-1,4-naphthoquinone, a natural compound also known as plumbagin (Fig. 1B). This compound inhibited the Myb-dependent reporter activity at an EC_{50} concentration of approximately 0.5 μmol/L. The inhibition was not due to unspecific

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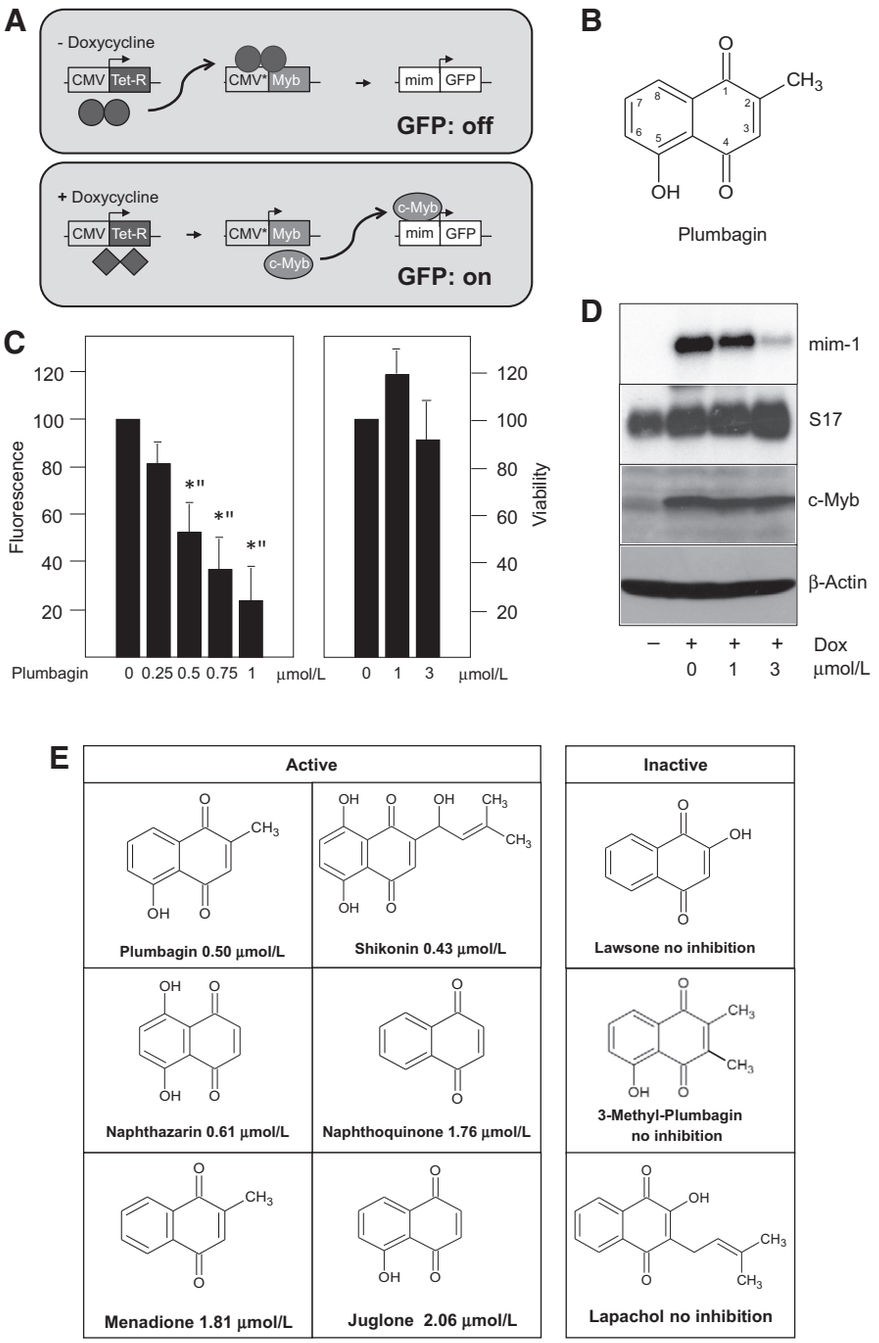

Figure 1.

Plumbagin suppresses Myb activity. **A,** Schematic illustration of the reporter cell line HD11-C3-GFP1. The cells carry a stably transfected eGFP reporter gene, an expression vector for the Tet-repressor (Tet-R), and a c-Myb expression vector, which harbors Tet operator sites in the CMV promoter at the transcriptional start site (CMV*). **B,** Structure of plumbagin. **C,** HD11-C3-GFP1 cells were grown in the presence of doxycycline and plumbagin, as indicated. Columns on the left and right show fluorescence and cell viability, respectively. Asterisks indicate statistical significance (*, $P < 0.05$, Student t test). **D,** HD11-C3-GFP1 cells grown in the presence or absence of doxycycline (Dox) and plumbagin were analyzed by Northern blotting for expression of *mim*-1 and ribosomal protein S17 mRNAs (top) and by Western blotting for c-Myb and β-actin (bottom). **E,** The inhibitory activities of different naphthoquinones were determined as in **C,** Using HD11-C3-RL1 cells. The numbers indicate the EC_{50} concentrations for Myb inhibition.

effects on cell viability, as determined by an MTT assay (Fig. 1C) and was confirmed by northern blot analysis of the expression of the endogenous Myb-inducible *mim-1* mRNA (Fig. 1D).

To compare the inhibitory activities of different naphthoquinones, we employed a cell system similar to the one illustrated in Fig. 1A except that *Renilla* luciferase was used instead of GFP as reporter. This allowed us to measure the inhibitory activities of the naphthoquinones independently of their intrinsic fluorescence. Fig. 1E illustrates the structures of the different naphthoquinones and the EC_{50} concentrations for inhibition of Myb activity. The most active compounds, plumbagin, shikonin, and naphthazarin, showed EC_{50} values below 1 µmol/L juglone, menadione, and unsubstituted 1,4-Naphthoquinone being slightly less active, while lawsone, 3-Methyl-Plumbagin, and lapachol were inactive. We noted that all active compounds possess at least one unsubstituted electrophilic carbon atom in the quinone ring while the inactive ones have either both positions substituted, or (in case of lawsone) an electron-donating OH-group neighboring the unsubstituted carbon which lowers the electrophilicity at this position. This relationship indicates that Myb-inhibitory

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activity is associated with potential electrophilic reactivity of the molecules as previously also observed with sesquiterpene lactones (23).

Plumbagin targets the transactivation domain of Myb

We performed reporter assays to address whether 5-hydroxy-2-methyl-1,4-naphthoquinone inhibits the DNA binding or the transactivation function of Myb. A fusion protein consisting of the Myb DNA-binding domain and the VP16 activation domain, was not inhibited, whereas the stimulation of the same reporter gene by v-Myb was clearly inhibited (Fig. 2A and B). Thus, plumbagin does not inhibit the DNA-binding activity of Myb. To assess its effect on the activity of the Myb transactivation domain, we employed a fusion protein of the Gal4 DNA-binding domain and the Myb transactivation domain. Fig. 2B shows that plumbagin inhibited the activity of the Gal4/Myb fusion protein. As control, the activity of the Gal4/VP16 protein was not affected. Overall, these experiments showed that plumbagin inhibits the activity of the Myb transactivation domain.

Plumbagin binds to the Myb transactivation domain

Naphthoquinones can exert inhibitory activities by generation of reactive oxygen species (ROS) or by alkylation of nucleophilic groups in proteins, mainly cysteine residues, due to the presence of reactive α,β–unsaturated carbonyl groups (31). To elucidate which of these mechanisms is responsible for Myb inhibition, we asked whether antioxidants are able to rescue Myb activity in the presence of plumbagin. We treated the cells described in Fig. 1A with Trolox or vitamin C as scavengers of ROS in addition to plumbagin. Figure 2C shows that these compounds were unable to rescue Myb activity. However, pretreating the cells with NAC restored Myb activity (Fig. 2D). We therefore concluded that naphthoquinones do not inhibit Myb activity by generation of ROS. That NAC was able to rescue Myb activity suggested that the inhibition might be due to alkylation of cysteine residues of Myb. Indeed, we found that all tested naphthoquinones showing Myb-inhibitory activity reacted readily with NAC under formation of covalent adducts detected by UHPLC/QqTOF MS analyses, whereas inactive compounds did not react with NAC (data not shown).

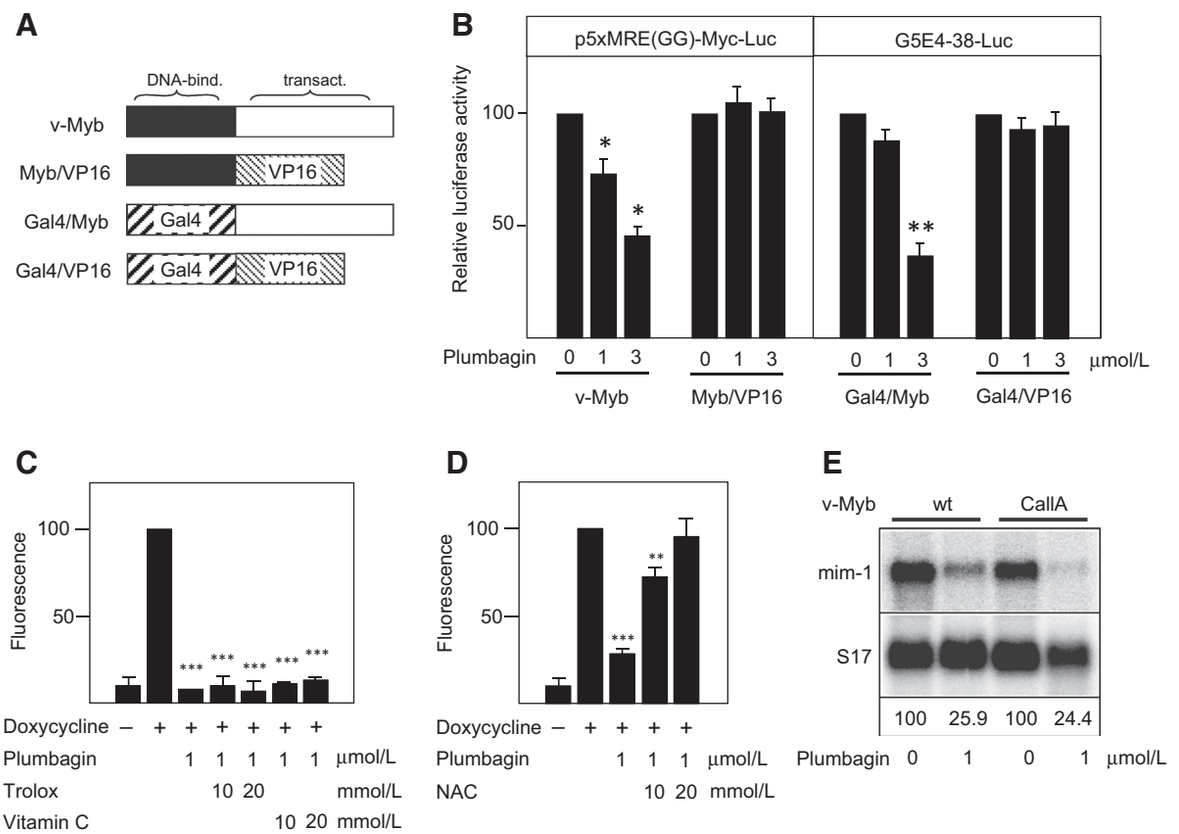

Figure 2.

Characterization of the inhibitory effect of plumbagin on Myb activity. **A,** Schematic illustration of Myb- and Gal4-fusion proteins. **B,** QT6 fibroblasts were transfected with the Myb-inducible luciferase reporter gene p5xMRE(GG)-Myc-Luc or the Gal4-dependent reporter gene pG5E4-38-Luc and the expression vectors indicated at the bottom. The columns show the average luciferase activity of cells treated with plumbagin. Asterisks indicate statistical significance (*, $P < 0.05$; **, $P < 0.01$, Student t test). **C** and **D,** Fluorescence of HD11-C3-GFP1 cells incubated for 12 hours with doxycycline and treated with plumbagin, trolox, and vitamin C or pretreated with NAC. Fluorescence was normalized to cells treated only with doxycycline. **E,** Inhibition of wild-type and a cysteine-free mutant of v-Myb by plumbagin. HD11 cells transfected with expression vectors for wild-type or the cysteine-free (CallA) mutant of v-Myb were treated with or without 1 µmol/L plumbagin. Endogenous *mim-1* and S17 mRNA expression was analyzed by Northern blotting. The numbers below the lanes indicate the relative amounts of *mim-1* RNA. Asterisks indicate statistical significance (**, $P < 0.01$; ***, $P < 0.001$, Student t test).

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To investigate whether the observed inhibition was due to the direct alkylation of cysteines in the Myb protein, we generated a cysteine-free version of v-Myb by replacing all cysteine residues with alanine. We used v-Myb instead of c-Myb to reduce the number of residues to be mutated. We then expressed the wild-type and cysteine-free version of v-Myb in HD11 cells and analyzed the expression of the endogenous *mim-1* gene to monitor Myb activity. Figure 2E shows that the cysteine-free v-Myb was able to induce *mim-1* expression, indicating that the cysteines are not essential for Myb activity. Importantly, the ability of plumbagin to inhibit Myb was indistinguishable between wild-type and cysteine-free v-Myb, demonstrating that plumbagin does not inhibit Myb by alkylating cysteine residues of this protein.

Next, we considered the possibility that plumbagin binds to the Myb transactivation domain in a noncovalent manner. Such a mechanism would also be consistent with the rescuing effect of NAC because the formation of a covalent adduct of plumbagin and NAC would lead to a bulky substituent at the C3 atom of plumbagin, which might hinder its interaction with Myb. We employed microscale thermophoresis (MST), a biophysical method based on the directed movement of molecules along a temperature gradient, to investigate whether plumbagin binds to the Myb transactivation domain. MST is very sensitive to changes of the molecule/solvent interface caused by molecular interactions and is used to detect and quantify biomolecular interactions, such as protein–small-molecule interactions (32). Typically, a constant amount of a fluorescent detector protein is titrated with increasing amounts of an unlabeled interaction partner. Figure 3A shows results of MST experiments with a fusion protein of eGFP and the c-Myb transactivation domain as the detector protein and serially diluted amounts of plumbagin. The resulting curve at different concentrations of plumbagin indicated binding of the compound to eGFP/Myb and allowed to calculate the dissociation constant for this interaction. Several independent replicate MST experiments resulted in a K_d value of approximately 0.87 μmol/L for the binding of plumbagin to eGFP/Myb. Plumbagin

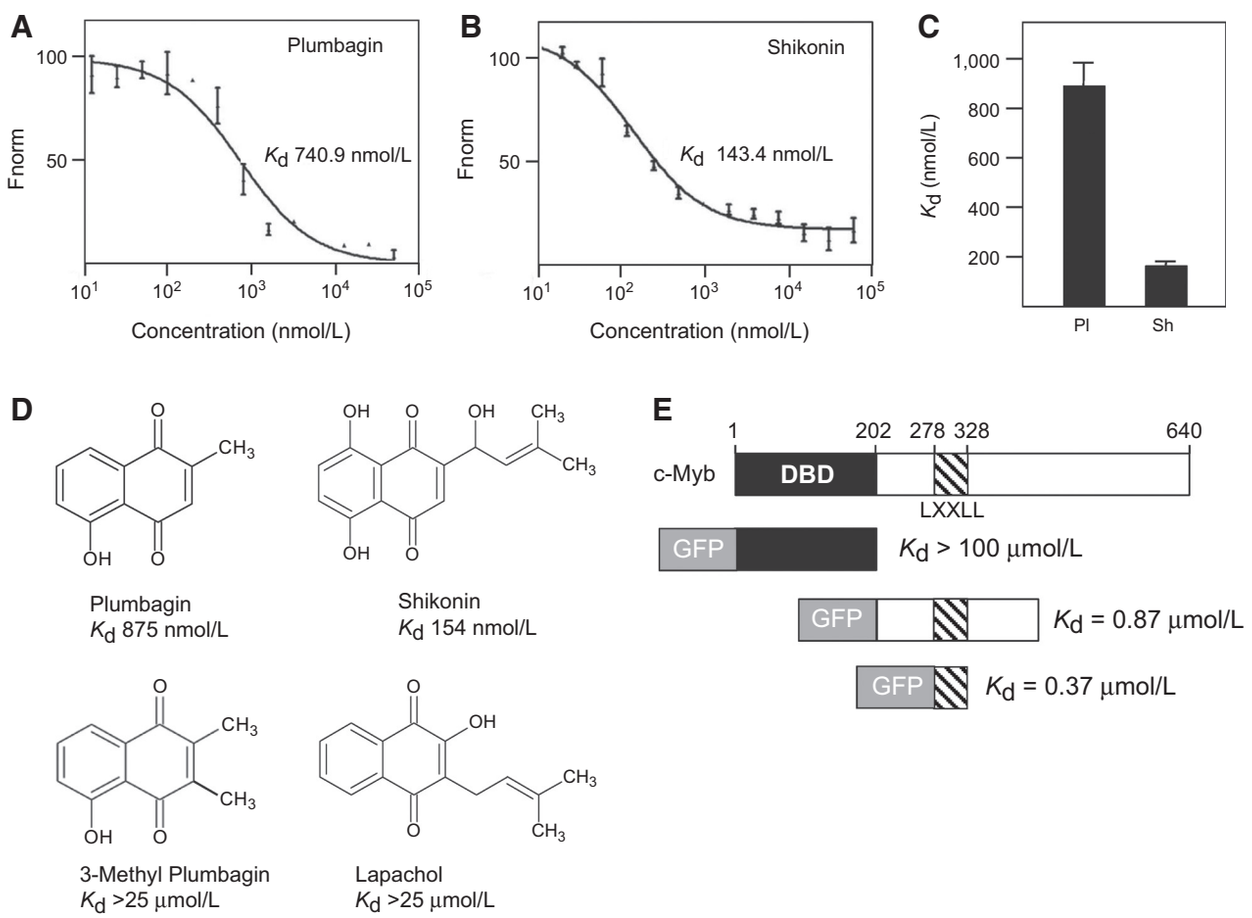

Figure 3.

Microscale thermophoresis reveals binding of plumbagin to the transactivation domain of Myb. Constant amounts of extract from QT6 cells transfected with GFP/Myb (202–442) were titrated with plumbagin (**A**) or shikonin (**B**) from 1.4 nmol/L to 50 μmol/L. The normalized fluorescence (Fnorm 1/1,000) was plotted against the concentration of the compounds. **A** and **B** Show the results of single MST experiments. The columns (**C**) show the estimated dissociation constants (and their standard deviations) for binding of plumbagin and shikonin, determined from independent experiments. The structures of plumbagin, shikonin, 3-methyl-plumbagin, and lapachol are shown with the corresponding K_d values (**D**). **E** Summarizes MST-experiments with different GFP-Myb fusion proteins and the corresponding K_d values for binding of plumbagin. The DNA-binding (DBD) and transactivation domain containing the LXXLL motif are highlighted.

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showed only very weak binding to eGFP expressed on its own (K_d ca. 40 μmol/L), which indicated that the high-affinity binding to eGFP/Myb is mediated by the Myb part of the fusion protein. We also performed MST experiments with shikonin, which inhibits Myb more potently, and with lapachol and 3-methyl-plumbagin, which do not inhibit Myb. Figure 3B and C shows that shikonin has slightly higher affinity for the Myb transactivation domain than plumbagin (K_d ca. 0.15 μmol/L), whereas lapachol and 3-methyl-plumbagin interact very poorly (K_d >25 μmol/L; Fig. 3D). Thus, the affinities of these compounds for binding to the Myb transactivation domain parallel their inhibitory activities, supporting the idea that plumbagin and shikonin inhibit Myb activity by direct binding.

To narrow down the binding region for plumbagin, we used an eGFP fusion protein that contains amino acids 278–328 of c-Myb and harbors the LXXLL motif in its center. MST experiments with extracts of cells expressing this protein showed strong binding of plumbagin (K_d ca. 0.37 μmol/L), indicating that plumbagin binds close to the KIX-binding site of c-Myb (Fig. 3E). We also performed MST experiments with a fusion protein of eGFP and the DNA-binding domain of c-Myb, which showed very weak binding (K_d > 100 μmol/L).

Plumbagin disrupts the cooperation of Myb with the coactivator p300

To understand how the binding of plumbagin to the Myb transactivation domain inhibits Myb activity, we considered the possibility that plumbagin disturbs the cooperation of Myb and p300, the main driver of the transcriptional activity of Myb. Figure 4A shows that increased expression of p300 counteracts the inhibitory effect of plumbagin, consistent with the notion that plumbagin interferes with the cooperation of Myb and p300.

Previous work has shown that p300 acetylates lysine residues in the C-terminal part of c-Myb (33, 34). Furthermore, plumbagin has been reported to inhibit the HAT activity of p300 (35), raising the question whether plumbagin acts by inhibiting the acetylation

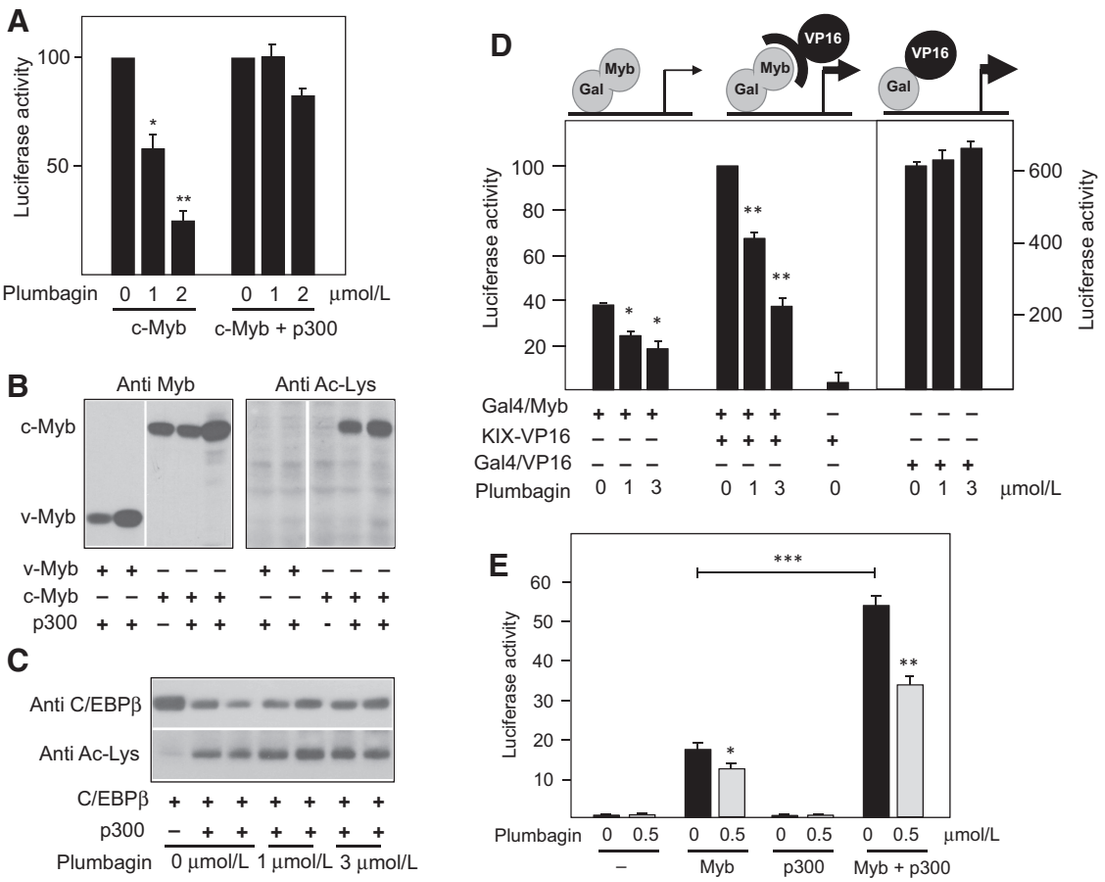

Figure 4.

Plumbagin disrupts the Myb/p300 interaction. **A,** QT6 fibroblasts were transfected with the Myb-responsive luciferase reporter gene p5xMRE(GG)-Myc-Luc and expression vectors for human c-Myb (3 μg) and p300 (5 μg). The cells were incubated with or without plumbagin and analyzed for luciferase activity. **B,** QT6 fibroblasts were transfected with the indicated expression vectors for v-Myb, c-Myb, and p300. Total cell extracts were analyzed by Western blotting with antibodies against Myb and acetyl-lysine. **C,** QT6 fibroblasts were transfected with expression vectors for C/EBPβ and p300. Total cell extracts were analyzed as in **B**. **D,** QT6 fibroblasts were transfected with the Gal4-responsive luciferase reporter gene pG5E4-38-Luc and the expression vectors indicated below the columns. The cells were analyzed as in **A**. **E,** QT6 fibroblasts were transfected with the Myb-responsive luciferase reporter gene p5xMRE(GG)-Myc-Luc and expression vectors for v-Myb (0.5 μg) and full-length p300 (2 μg), as indicated below the columns. The cells were incubated with or without plumbagin and analyzed for luciferase activity. Asterisks indicate statistical significance (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, Student t test).

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of c-Myb. However, such a mechanism appears unlikely to be responsible for the inhibition of Myb activity for several reasons. First, as shown in Fig. 2, plumbagin also inhibits the activity of v-Myb, which lacks the C-terminal domain of c-Myb and is not acetylated by p300 (Fig. 4B). Although this does not exclude that inhibition of acetylation of other proteins cooperating with Myb is involved, the acetylation of Myb itself does not appear to play a role in the inhibitory mechanism. Second, the estimated IC_{50} concentration for inhibition p300 by plumbagin in a HAT assay is 25 μmol/L (35). Hence, significantly higher concentrations of plumbagin than used here are required to inhibit the HAT activity of p300. Accordingly, using C/EBPβ as an unrelated protein that is acetylated by p300, no inhibition of HAT activity was observed at the concentrations of plumbagin that are effective in inhibiting Myb activity (Fig. 4C). As an alternative explanation of the inhibitory effect of plumbagin, we therefore investigated whether plumbagin disrupts the interaction of Myb and p300. We performed two-hybrid experiments using expression vectors for Gal4/Myb and p300/VP16, which contains the KIX domain of p300 that is responsible for the Myb/p300 interaction. As expected, expression of p300/VP16 increased the activity of the Gal4/Myb protein, reflecting the interaction of Myb with the KIX domain of p300. Plumbagin inhibited the activity of the Gal4/Myb protein and, importantly, also the increased activity in the presence of p300/VP16, suggesting that plumbagin disrupts the Myb–KIX interaction (Fig. 4D). Figure 4E demonstrates that plumbagin causes a similar inhibitory effect when full-length p300 instead of the KIX-VP16 protein was coexpressed with v-Myb. Taken together, these results suggest the disruption of the Myb/p300 interaction as the likely mechanism of action of plumbagin. It might appear as an amazing coincidence that the same compound inhibits both Myb/p300 interaction and the HAT activity of p300; nevertheless, our data clearly argue against inhibition of acetylation of Myb by plumbagin as the relevant inhibitory mechanism.

Plumbagin inhibits the expression of Myb target genes and induces myeloid differentiation

To explore the influence of plumbagin on hematopoietic cells, we examined the expression of Myb-target genes in the promyelocytic leukemia line HL60. Figure 5A shows that several Myb target genes were significantly downregulated in cells treated for 48 hours with 0.5 μmol/L plumbagin. To demonstrate that downregulation of Myb target genes is due to a direct effect of plumbagin on Myb activity, we focused on the c-*MYC* gene, whose mRNA has a very short half-life (36). Figure 5C shows that c-*MYC* expression was inhibited significantly already after 2 hours.

We also found that plumbagin induced the differentiation of HL60 cells in a concentration-dependent manner in up to 20 % of the cell population, as demonstrated by the differentiation marker CD11b (Fig. 5D and F). ATRA significantly enhanced this effect of plumbagin. Differentiation was also visualized microscopically by the appearance of irregularly shaped nuclei and an increase of the cytoplasm of the cells (Fig. 5E). Analysis of the fraction of Annexin V–positive cells showed that plumbagin concentrations below 1 μmol/L induced apoptosis only weakly (data not shown).

Plumbagin affects a variety of biological processes via different targets (31), which raised the question of whether the induction of differentiation by plumbagin was due to the inhibition of Myb or to a Myb-independent mechanism. We infected HL60 cells with a lentivirus expressing a C-terminally truncated Myb (c-MybΔ3) or with the parental lentivirus encoding RFP. Truncation of the C terminus of c-Myb removes a negative regulatory domain, resulting in a protein with enhanced transactivation and transforming potential (37). Figure 5E shows that the cells expressing ectopic Myb differentiated less efficiently in response to plumbagin compared with control cells. Treatment with shikonin resulted in a higher percentage of differentiated cells, consistent with its higher Myb inhibitory activity. As with plumbagin, the effect of shikonin was significantly decreased due to ectopic expression of Myb. Together, these results indicate that the effects of both naphthoquinones on differentiation of HL60 cells are indeed due, at least in part, to the inhibition of Myb.

Plumbagin suppresses the proliferation of primary AML cells but not that of normal hematopoietic progenitor cells

To investigate the effect of plumbagin on primary leukemia cells, we used two mouse models of AML that are based on retrovirally induced expression of an MLL-AF9 fusion protein (38) or c-Myc/Bcl2 (39) in hematopoietic progenitor cells. Leukemias were induced in C57BL/6 mice by transplantation of lineage-negative progenitor cells infected with MLL-AF9 or c-Myc/Bcl2 retrovirus. c-Kit–positive leukemic cells, which represent leukemia stem cells (38), and c-Kit–negative bulk leukemic cells were then subjected to colony formation assays in the absence or presence of 0.5 μmol/L plumbagin. As control for normal hematopoietic cells, we used lineage-negative bone marrow cells from healthy mice. Figure 6A shows that colony formation of leukemic cells was suppressed by plumbagin, whereas normal cells were not significantly affected. To explore the effect of plumbagin on primary human cells, we performed colony formation assays with $CD34^+$ cells isolated from healthy donors and with leukemic blasts from patients with AML (Fig. 6B). Colony assays performed with cells from AML patients showed that plumbagin inhibited proliferation in most cases while independent preparations of $CD34^+$ cells from healthy donors showed no reduction of colony formation. Overall, these data showed that AML cells are significantly more sensitive to plumbagin than normal hematopoietic progenitors.

Discussion

Recent insight into the role of c-Myb in leukemia and other tumors has made c-Myb an attractive target for the development of small-molecule inhibitors (1). A key observation supporting the idea to develop Myb inhibitors as potential drugs for treatment of leukemia is that acute myeloid and lymphoid leukemia cells often require (or are "addicted" to) higher levels of c-Myb for proliferation and survival than normal hematopoietic progenitor cells (1, 5, 6). Transcriptome-wide expression studies have revealed that the self-renewal program of leukemic stem cells is distinct from that of normal hematopoietic stem cells and requires high levels of Myb activity (6, 40), explaining the "addiction" of leukemic cells to c-Myb. Thus, partial inhibition of Myb activity might suffice to eradicate leukemic cells without impairing normal hematopoiesis. Experimental support for this concept has come from studies in a mouse model of AML in which shRNA-induced downregulation of Myb eliminated the leukemic cells but maintained normal hematopoiesis (40).

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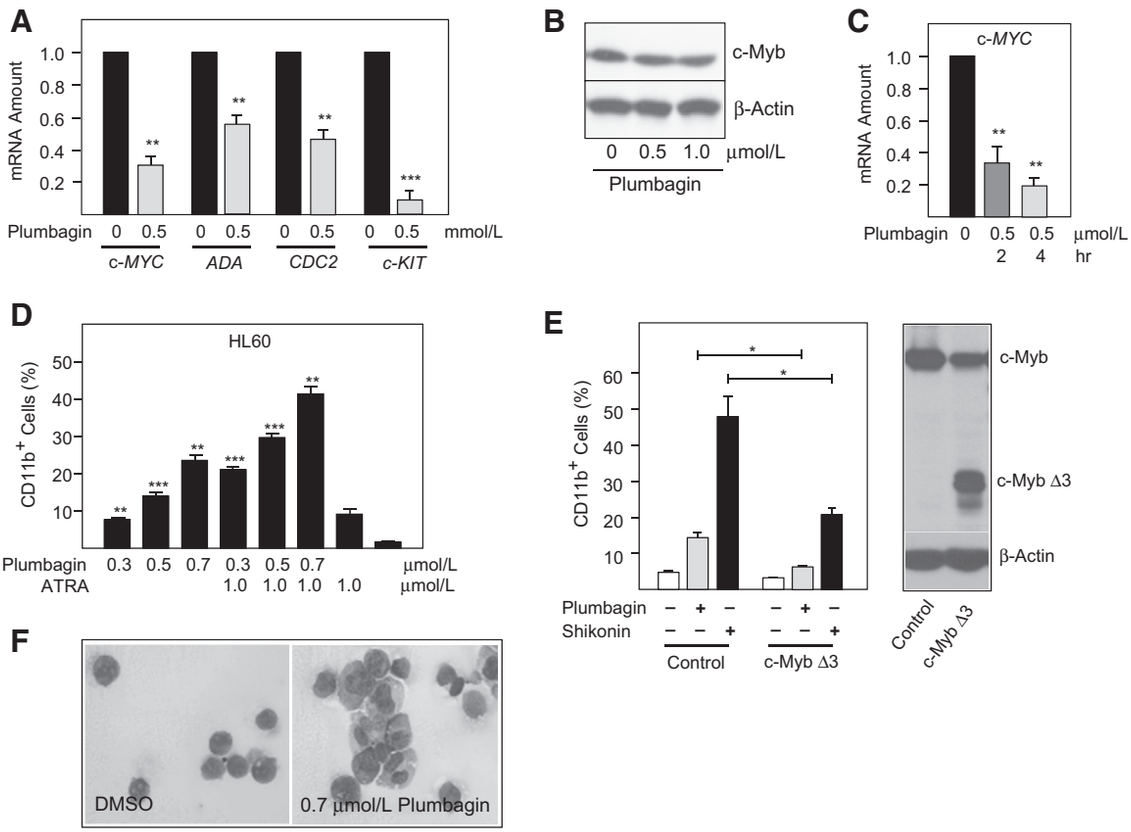

Figure 5.

Plumbagin suppresses the expression of Myb target genes and induces differentiation of HL60 cells. **A,** Real-time PCR analysis of mRNA expression in HL60 cells treated for 48 hours with or without plumbagin. Columns show the amount of the indicated mRNAs normalized to β-actin mRNA. **B,** Western blot analysis of c-Myb and β-actin expression in HL60 cells treated for 48 hours with plumbagin. **C,** Real-time PCR analysis of c-*MYC* mRNA after 2 or 4 hours of plumbagin treatment. **D,** Differentiation of HL60 cells treated for 2 days with the indicated concentrations of plumbagin or/and ATRA. Columns indicate the percentage of CD11b-positive cells, as determined by flow cytometry. **E,** HL60 cells infected with control lentivirus or lentivirus encoding c-MybΔ3 were treated for 2 days with plumbagin or shikonin. Columns indicate the percentage of CD11b-positive cells. Asterisks indicate statistical significance (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, Student *t* test). Extracts of the infected cells were analyzed by Western blotting for Myb and β-actin expression (right). **F,** May-Grünwald staining of HL60 cells treated for 2 days with DMSO or plumbagin.

We have previously identified two low-molecular weight compounds, naphthol AS-E phosphate and celastrol that inhibit Myb activity by disrupting the interaction of Myb with the coactivator p300. This has provided proof-of-principle that c-Myb can be targeted by small-molecule inhibitors (21, 41). Here, we have characterized naphthoquinones as potent Myb inhibitors, the most active of which inhibit Myb at submicromolar concentrations. Importantly, these compounds bind directly to the Myb transactivation domain and thereby disrupt the cooperation of Myb with the KIX-domain of p300. With the exception of the α-helical LXXLL motif (14) the overall structure of the Myb transactivation domain is not known, precluding the identification of possible naphthoquinone-binding sites by molecular docking studies. The shortest protein construct showing high-affinity binding of plumbagin encompassed amino acids 278–328 of c-Myb with the LXXLL motif located in the center. Thus, plumbagin must bind in the immediate vicinity of the LXXLL motif.

Remarkably, all naphthoquinones that are active as Myb inhibitors possess unsubstituted reactive carbon atoms in conjugation with the quinone carbonyl groups (so-called α,β-unsaturated carbonyl groups) and react covalently with NAC, whereas the inactive derivatives do not, suggesting that the ability to undergo covalent interactions might be required for their inhibitory activity. However, we have clearly excluded alkylation of cysteine residues of Myb as the inhibitory mechanism. Interactions with other nucleophilic amino acids such as lysine amino groups cannot be ruled out, but are less likely to occur at physiologic pH. Nevertheless, it is possible and remains to be investigated that the reactivity of these compounds plays a role in the inhibitory mechanism. One might envision that the naphthoquinones, when bound to Myb, alkylate p300, or another protein to which they are presented via the Myb transactivation domain. p300 has several cysteine-rich domains that are important for its function and might be targets for such alkylations.

We have shown that plumbagin inhibits the expression of Myb-regulated genes and induces myeloid differentiation, as expected if Myb is inhibited. Naphthoquinones have a broad spectrum of biological activities (31). Numerous studies have analyzed the cellular pathways affected by these compounds and identified

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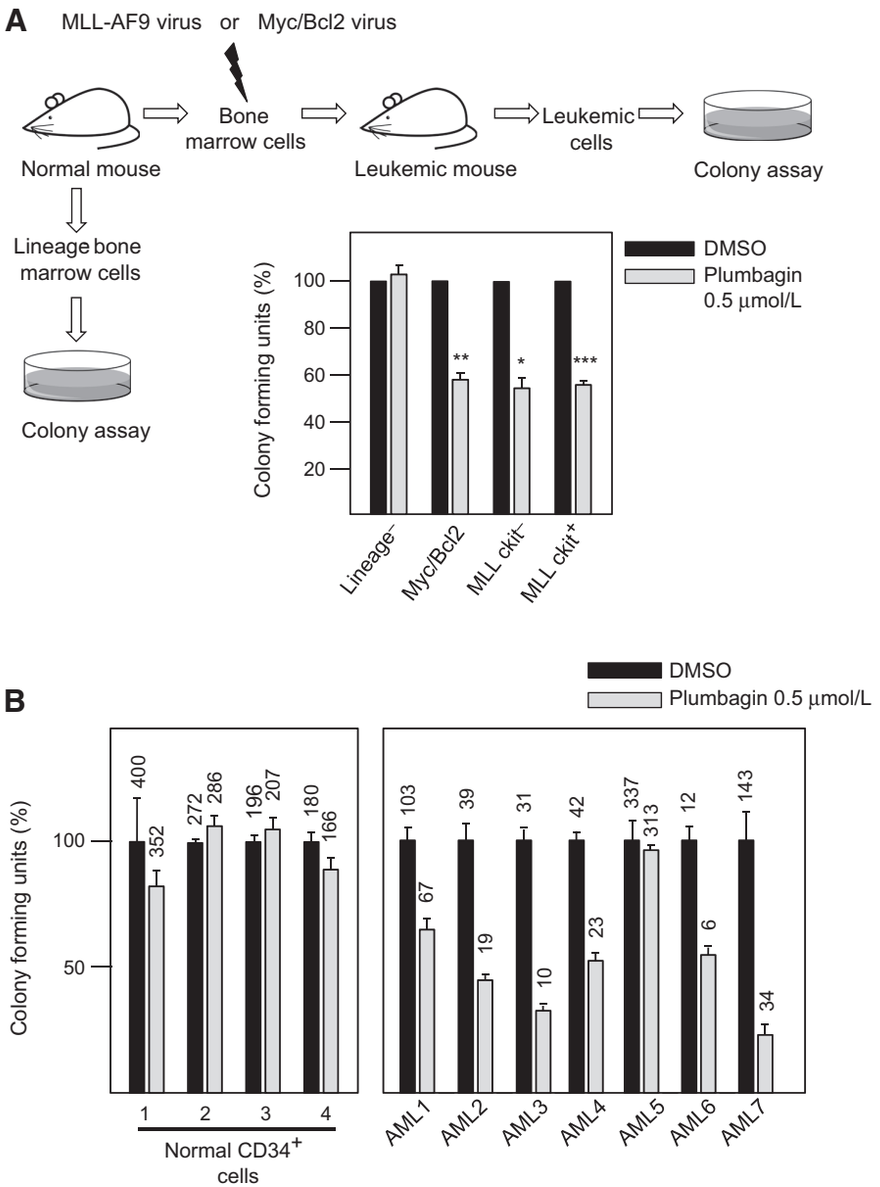

Figure 6.

Impact of plumbagin on the proliferation of primary AML cells and normal hematopoietic progenitors. **A,** Effect of plumbagin in mouse models of AML. As illustrated schematically, lineage-negative bone marrow cells from healthy mice and leukemic blasts from mice were subjected to colony assays in the absence or presence of 0.5 μmol/L plumbagin. Leukemias were generated by transduction of progenitor cells with Myc/Bcl2 or MLL/AF9 retroviruses. MLL-AF9–derived leukemic cells were sorted into c-Kit–negative (bulk blast cells) and c-Kit–positive cells (leukemic stem cells). Equal numbers of cells were plated without (blue bars) or with (red bars) 0.5 μmol/L plumbagin in each case. Columns show colony numbers as percent of DMSO-treated controls. Asterisks indicate statistical significance (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, Student t test). **B,** Influence of plumbagin on primary human cells. Colony assays were performed with $CD34^+$ hematopoietic progenitor cells from healthy donors and leukemic blasts from different AML patients.

potential target proteins, although in many cases significantly higher concentrations than the ones used here were required to elicit effects. Several studies have implicated plumbagin also as a potential antitumor drug for the treatment of prostate and ovarian cancer and glioblastoma (42–44). It is therefore clear that naphthoquinones affect other targets besides Myb. Nevertheless, the finding that ectopic expression of Myb diminished the naphthoquinone-induced differentiation of HL60 cells indicates that the induction of differentiation by these compounds to is due, at least in part, to inhibition of c-Myb. One of our most interesting observations is the finding that leukemic cells derived from mouse models of AML or from patients with AML are more sensitive to plumbagin than normal murine or human hematopoietic progenitor cells. This accords with the increased requirement of leukemic versus normal hematopoietic cells for c-Myb activity and suggests that a "therapeutic window" may exist that would allow targeting of leukemic cells by a Myb inhibitor while maintaining normal hematopoiesis.

Naphthol AS-E and celastrol inhibit Myb by blocking the Myb–KIX interaction through binding to the KIX-domain (21, 41), whereas plumbagin and related naphthoquinones, as shown here, inhibit the Myb–KIX interaction by binding to Myb itself. It will be interesting to combine these inhibitors to investigate whether this enhances their activities. Together with our recent work our data underscore the importance of the Myb–KIX interaction for Myb activity and demonstrate that this interaction can be targeted by small molecules, suggesting that a Myb inhibitor–based therapeutic approach for the treatment of acute myeloid leukemia might in principle be feasible. As Myb is also involved in other human cancers, such as adenoid cystic carcinoma, the potential use of Myb inhibitors might not be restricted to leukemia.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Uttarkar, T. Piontek, S. Dukare, W.E. Berdel, T.J. Schmidt, K.-H. Klempnauer

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Uttarkar, T. Piontek, S. Dukare, C. Schomburg, W.E. Berdel, T.J. Schmidt, K.-H. Klempnauer

Writing, review, and/or revision of the manuscript: S. Uttarkar, S. Dukare, W.E. Berdel, C. Muller-Tidow, T.J. Schmidt, K.-H. Klempnauer

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Uttarkar, P. Schlenke, C. Muller-Tidow, K.-H. Klempnauer

Study supervision: S. Uttarkar, K.-H. Klempnauer

Other (provided substances for testing and contributed to previous results based on which tests were performed): T.J. Schmidt

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Molecular Cancer Therapeutics

Small-Molecule Disruption of the Myb/p300 Cooperation Targets Acute Myeloid Leukemia Cells

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