Naphthol AS-E Phosphate Inhibits the Activity of the Transcription Factor Myb by Blocking the Interaction with the KIX Domain of the Coactivator p300

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Abstract

The transcription factor c-Myb is highly expressed in hematopoietic progenitor cells and controls the transcription of genes important for lineage determination, cell proliferation, and differentiation. Dere gelation of c-Myb has been implicated in the development of leukemia and certain other types of human cancer. c-Myb activity is highly dependent on the interaction of the c-Myb with the KIX domain of the coactivator p300, making the disruption of this interaction a reasonable strategy for the development of Myb inhibitors. Here, we have used bacterial Autodisplay to develop an in vitro binding assay that mimics the interaction of Myb and the KIX domain of p300. We have used this binding assay to investigate the potential of Naphthol AS-E phosphate, a compound known to bind to the KIX domain, to disrupt the interaction between Myb and p300. Our data show that Naphthol AS-E phosphate interferes with the Myb–KIX interaction in vitro and inhibits Myb activity in vivo. By using several human leukemia cell lines, we demonstrate that Naphthol AS-E phosphate sup presses the expression of Myb target genes and induces myeloid differentiation and apoptosis. Our work identifies Naphthol AS-E phosphate as the first low molecular weight compound that inhibits Myb activity by disrupting its interaction with p300, and suggests that inhibition of the Myb–KIX interaction might be a useful strategy for the treatment of leukemia and other tumors caused by deregulated c-Myb. Mol Cancer Ther; 14(6). 1276–85. ©2015 AACR. See related commentary by Liu et al., p. 1273

Introduction

Myb proteins constitute a family of highly conserved transcription factors playing important roles in lineage determination, cell proliferation, and differentiation (1, 2). The founding member of the vertebrate Myb family, c-Myb, plays a key role in the hematopoietic system. c-Myb is highly expressed in immature hematopoietic progenitor cells and its downregulation is essential for their terminal differentiation. c-Myb has also been implicated in controlling the fate of certain nonhematopoietic cells, such as the progenitor cells, in the colonic crypts (3, 4).

Recently, c-Myb has attracted attention as a potential drug target for the treatment of leukemia and other tumors (3, 6). Myb was initially discovered as the retroviral oncogene (v-myb) of avian myeloblastosis virus (7). In humans, translocations and duplications of c-myb occur in acute lymphoblastic leukemia of young children (8, 9), indicating a role of c-Myb in leukemia development. Furthermore, c-Myb also plays crucial roles in leukemias without being rearranged or mutated. Although normal hematopoietic progenitor cells also express c-Myb, leukemic cells are often more dependent on high c-Myb expression levels than their normal counterparts (2). This “addiction” to c-Myb opens up a possible therapeutic window for killing leukemic cells but sparing normal hematopoietic precursor cells. The validity of this concept was recently demonstrated in a mouse model of AML in which shRNA-mediated downregulation of c-Myb resulted in remission of the leukemia without inhibiting normal hematopoiesis (10). c-Myb has also been implicated in nonhematopoietic tumors. Around 70% of estrogen receptor–positive breast cancers show high c-Myb expression, which is required for their survival (11, 12). Furthermore, c-Myb is highly expressed in colon cancer and correlates with poor prognosis (2, 13, 14). Translocations between the c-myb and NFIB genes occur in more than 50% of adenoid cystic carcinomas, leading to the expression of c-Myb–NFIB fusion proteins (15). Finally, sequencing studies have recently shown that c-Myb is altered by translocation in a significant percentage of diffuse low-grade pediatric gliomas (16). These findings indicate that c-Myb can act as a “driver” oncogene in various tumors. Inhibiting c-Myb might therefore be a therapeutic option also for these types of cancers.

Transcriptional activation of target genes by c-Myb has been studied in detail (17–27). This work showed that the activity of c-Myb is highly dependent on its interaction with the coactivator p300 (28–31). Myb and p300 interact via a LXXLL motif located in the transactivation domain (TAD) of c-Myb and the KIX-domain of p300 (32). Mutations within the LXXLL motif disrupt the interaction and lead to an almost complete loss of Myb activity.

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(29, 30). Low-molecular weight compounds that target the Myb-p300 interaction might therefore be useful as Myb inhibitors.

Here, we describe an in vitro binding assay for the interaction of Myb with the KIX domain of p300. The assay is based on bacterial Autodisplay of the KIX domain and a soluble eGFP–Myb fusion protein containing the LXXLL motif. We show that both proteins interact with high specificity and demonstrate that Naphthol AS-E phosphate, a compound known to bind to the KIX-domain, blocks the Myb–KIX interaction in vitro, and inhibits Myb activity in vivo.

Materials and Methods

Plasmids

For Autodisplay of the KIX domain, an artificial gene was constructed, encoding the signal peptide of CTB, the KIX domain as a passenger, the linker and the β-barrel of AIDAL. The coding sequence of the KIX domain of human p300 (amino acids 556-652) was amplified by PCR with the primers 5'-CITCAGGG-GAATTCGAACAGTGGCACG-3' and 5'-GTACCTATCGTGCCTCTGTTTTCTTCAAAGC-3', digested with Xhol and KpnI and inserted into plasmid pKP10 (33). The artificial gene encoding the KIX–AIDAL-1 auto transporter was transferred to the first multiple cloning site of pCOLADuet-1 (Merck Millipore). The resulting commercial gene encoding the KIX–AIDAL-1 fusion protein under control of a T7/lac promoter. The bacterial expression vector for an eGFP–v-Myb fusion protein was generated by PCR-amplification of v-Myb coding sequences (amino acids 115 to 320) using the primers 5'-CCGGATGCACATCGCCGGGAAGGTC-3' and 5'-GGCGCGGGCCGCAAAAGGAAATCTTCAAC-3', digesting the resulting fragment with BamH I and NotI and cloning it into pGVC2 (gift from H. Moons, Institute for Biochemistry, University of Münster, Münster, Germany). The expression vector for an eGFP–hc-Myb fusion protein was constructed similarly, except that primers 5'-CCACCCGGATCCAAAAGCAATTTACCTTAC-3' and 5'-ATATCAGCGCGGCGCTTATAAAGGATCTTCAAC-3' were used. A L302A mutant derivative was generated by site-directed mutagenesis.

Expression and purification of bacterial proteins

A culture of Escherichia coli UT3600(DE3) pCO001 (encoding the KIX–AIDAL fusion protein) in lysogeny broth (LB) containing 30 µg/mL kanamycin was prepared by growing a diluted overnight culture at 37°C with vigorous shaking to an OD of 0.6. Protein expression was induced by adding IPTG (1 mmol/L final concentration). The incubation was continued at 18°C overnight under vigorous shaking. The cells were harvested by centrifugation, suspended in lysis buffer [100 mmol/L Tris, pH 7.4; 1 mmol/L EDTA; 150 mmol/L NaCl; 1% Triton-X100; 1 mmol/L dithiothreitol (DTT)]. 1 mmol/L phenylmethylsulfonylfluoride (PMSF)] and lysed by sonication. The lysate was clarified [9, 500 rpm, 60 minutes, 4°C] and loaded onto a Strept-Taggin column (IBA GmbH) in Strept equilibrium buffer [100 mmol/L Tris, pH 7.4; 150 mmol/L NaCl; 1 mmol/L EDTA). The column was washed with equilibrium buffer and the bound protein was eluted using the same buffer supplemented with 2.5 mmol/L dethiobiotin. The protein was concentrated using cen- trifugal filter tubes (Merck Millipore, exclusion size 30 kDa) and stored at ~80°C. To purify bacterial protein containing the KIX domain, a GST–p300 fusion protein (amino acids 1 to 672) was expressed using the plasmid pGex-p300K (35), as described previously (36). A construct encoding a GST-human CBP fusion protein (amino acids 1 to 692), pGex-CBP(1-692), was generated by PCR amplification of the corresponding part of human CBP and cloning into pGex-6P2. The protein were bound to glutathione sepharose and incubated for 24 hours with prescision protease (GE Healthcare) in PBS. The supernatant obtained after removal of the sepharose contains the purified p300 (1-672) or CBP (1-692). Flow cytometry, protein binding, and inhibitor testing

For Autodisplay of the KIX-AIDA-1 protein an overnight culture was diluted 1:100 and grown at 37°C under vigorous shaking to an OD of 0.6. Protein expression was induced by adding IPTG (1 mmol/L final concentration). After 60 minutes at 30°C, the cells were pelleted (5 minutes, 4°C, 3,500 × g) and suspended in phosphate-buffered saline (PBS), including the appropriate antibiotic. After overnight incubation at 30°C under vigorous shaking, the cells were harvested (5 minutes, 4°C, 3,500 × g) and suspended in PBS to an optical density of 1. Forty microliters of the suspension was then incubated with 10 µL of the desired binding partner (10 µmol/L stock concentration in PBS) for 120 minutes at 4°C. For inhibitor testing, naphthol AS-E phosphate was added to the suspension and incubated for 60 minutes at 4°C. DMSO (final concentration 1%) was used as a control. The fluorescent Myb protein was then added and further incubated at 4°C for 120 minutes. Finally, cells were washed with PBS, pelleted in a microcentrifuge (2 minutes, 12,000 × g), suspended in PBS and analyzed using a FACSaria cytometer (BD Biosciences). For each measurement, 50,000 cells were analyzed.

Molecular docking

A three-dimensional model of Naphthol AS-E phosphate was generated with MOE using the MMFF94x force field (37). A low-mode dynamics conformational search for Naphthol AS-E phosphate was performed (default settings of MOE) and the resulting conformer with lowest field energy was used for further docking, using the structure of the KIX-cMyb complex (32).

Microscale thermophoresis

To measure the dissociation constant for the Myb–KIX interaction, 50 nmol/L (final concentration) of purified eGFP–hc-Myb (241-390) and various concentrations ranging from 28 µmol/L to 0.85 nmol/L of purified bacterially expressed p300 (1-672) or CBP (1-692) were combined in PBS, incubated for 1 hour at room temperature, and filled in capillaries to perform thermophoresis measurements in a NanoTemper Monolith (NT-015) instrument. Thermophoresis was performed at 15% LED power and 40% laser power at 1,475 nm ± 15 nm. To determine the IC50 of the inhibition of the Myb–KIX interaction by napthol AS-E phosphate, purified eGFP–hc-Myb (241-390) and p300 (1-672) at constant concentrations (50 nmol/L and 28 µmol/L respectively) were combined with napthol AS-E phosphate at concentrations between 50 µmol/L and 1.52 nmol/L in PBS. Measurement of thermophoresis was performed as described above. Data from three independent experiments were normalized to either AFbound [%] = 10 × (Fbound (bound) – Fbound (unbound)) or fraction bound [AFbound [%]/amplitude] to calculate the Ki of the Myb–KIX interaction or the IC50 value for inhibition of the interaction by napthol AS-E phosphate.
In vivo assays

HD11-C3-GFP1 cells (5) were treated for 24 hours with 1 μg/mL doxycycline and Naphthol AS-E phosphate, followed by measuring their fluorescence with a microplate reader. Northern blotting of mim-1 and ribosomal protein S17 mRNAs was performed as described previously (5). Differentiation assays were performed by culturing HL60, U937, or NB4 cells for 2 days in RPMI-1640 medium containing napthol AS-E phosphate. HL60, U937, and NB4 cells were originally obtained from the ATCC. No further authentication was done by the authors. The cells were stained with phycoerythrin-labeled anti-human CD11b antibody (BD Pharmingen), and analyzed by flow cytometry or May-Grünwald staining. Apoptosis was analyzed with the Trevigen TACS annexin V kit (Gentaur GmbH). Real-time PCR was performed as described previously (5). The data were analyzed by subtracting the C_t values for c-myc (or Ada) mRNA from those for β-actin mRNA, thereby normalizing the amount of c-myc (or Ada) mRNA to that of β-actin mRNA.

Results

Development of an Autodisplay binding assay for the Myb-p300 interaction

Bacterial Autodisplay is an efficient system for the expression of recombinant proteins on the outer membrane of Gram-negative bacteria (38, 39). Autodisplay provides a powerful experimental platform for various biotechnologic applications, including the analysis of protein–protein interactions and the development of inhibitors (40, 41). To identify inhibitors for the Myb–KIX interaction, we have generated an Autodisplay screening tool, which consists of the surface-expressed KIX domain and a soluble GFP–Myb fusion protein. For Autodisplay of the KIX domain (amino acids 556 to 652), we generated an artificial E. coli AIDA-I–based autotransporter gene encoding a KIX–AIDA-I fusion protein (Fig. 1A). To confirm its expression, outer membrane proteins were prepared and analyzed by SDS-PAGE and Western blotting (Fig. 1B). Induction of the autotransporter expression by IPTG resulted in an additional protein band of approximately 60 kDa, which corresponds to the calculated size of the KIX–AIDA-I protein (Fig. 1B). The protein reacted with antibodies against the AIDA-I β-barrel, confirming its identity.

The Myb interaction partner was expressed in bacteria as a eGFP fusion construct containing the TAD of v-Myb (amino acids 115 to 320), an N-terminal eGFP-tag and a C-terminal Strep-tag for purification (Fig. 2A). The TAD contains the LXXLL motif of v-Myb that directly binds to the KIX domain (32). To demonstrate the Myb–KIX interaction, E. coli cells expressing the KIX domain were incubated with the eGFP–v-Myb protein and subsequently analyzed by flow cytometry. Incubation with the eGFP–v-Myb fusion protein increased the fluorescence of the
KIX-expressing bacteria while incubation with eGFP alone resulted only in background fluorescence (Fig. 2B, left). As additional control for the specificity of the interaction eGFP and eGFP–v-Myb were tested for binding to bacteria without induction of autotransporter expression. No binding was observed with either of the proteins (Fig. 2B, right). The interaction was also confirmed by fluorescence microscopy. Fluorescent cells were observed when KIX-expressing cells were incubated with eGFP or eGFP–v-Myb and visualized by fluorescence microscopy.

Earlier studies showed that the replacement of leucine (L302) of the LXXLL binding motif by alanine virtually completely abolished the Myb–p300 interaction (30). In the Myb–KIX complex, Leu302 occupies a deep hydrophobic cavity on the KIX domain (32), providing an explanation for the importance of this residue. To further prove the specificity of the Myb–KIX interaction in the Autodisplay binding assay, we generated wild-type and mutant fusion proteins of eGFP and the TAD of human c-Myb differing only by the L302A amino acid replacement in the mutant construct. We replaced the TAD of v-Myb by that of human c-Myb because the principal objective of developing the Myb–KIX Autodisplay binding assay was to establish an in vitro screening tool for inhibitors of human c-Myb. Figure 3 shows that the wild-type eGFP–hc-Myb was able to bind to the KIX-expressing bacteria similar to the GFP–v-Myb protein, whereas the L302A mutant did not show any binding. This demonstrated that the Myb–KIX interaction in the Autodisplay assay was highly specific and mimicked the physiologic interaction of both proteins.

Naphthol AS-E phosphate disrupts the Myb–KIX interaction

NMR-based screening of small molecules binding to the KIX domain has previously identified Naphthol AS-E phosphate (Fig. 4A) as an inhibitor of the interaction of the KIX domain with the transcription factor CREB (42). Because Myb and CREB bind to the same surface of the KIX domain, albeit in different manner (32), we investigated whether Naphthol AS-E phosphate also disrupts the Myb–KIX interaction. We incubated KIX-expressing bacteria with eGFP/hc-Myb without or with Naphthol AS-E phosphate. Figure 4B shows that the binding of Myb to the bacteria gradually decreased with increasing Naphthol AS-E phosphate concentration. We estimated the IC50 value for the inhibition to be approximately 30 μmol/L, which is lower than the IC50 value of 90 μmol/L reported for the inhibition of the CREB–KIX interaction.
This suggested that Naphthol AS-E phosphate is a slightly better inhibitor of the Myb–KIX than of the CREB–KIX interaction.

To analyze the Myb–KIX interaction and the inhibitory effect of Naphthol AS-E phosphate in a quantitative manner, we performed microscale thermophoresis (MST) experiments. MST is based on the directed movement of molecules along a temperature gradient, which is very sensitive to changes of the molecule–solvent interface caused by molecular interactions. Changes in thermophoresis can be used to detect and quantify biomolecular interactions, such as protein–protein or protein–small-molecule interactions (43). In a typical experiment, a constant amount of a fluorescent detector protein is titrated with increasing amounts of an unlabeled interaction partner. Figure 4C shows the result of a thermophoresis experiment carried out with eGFP–hc-Myb as the detector protein and serially diluted amounts of the unlabeled KIX domain (amino acids 1 to 672 of p300). The resulting curves at different concentrations of the unlabeled binding partner allowed to calculate the dissociation constant (see Materials and Methods for further details) for the Myb–KIX interaction, which turned out to be 2.5 μmol/L (Fig. 4C).

We performed additional thermophoresis experiments to determine the IC\textsubscript{50} value for the inhibition of the Myb–KIX interaction by Naphthol AS-E phosphate. In these experiments, constant amounts of eGFP–hc-Myb and the unlabeled KIX domain were incubated with increasing amounts of Naphthol AS-E phosphate. The analysis of the thermophoresis curves (Fig. 4D) confirmed that Naphthol AS-E phosphate disrupts the Myb–KIX complex with an IC\textsubscript{50} concentration of approximately 30 μmol/L.

To address how Naphthol AS-E phosphate inhibits the Myb–KIX interaction, we performed molecular docking studies (Fig. 5). As shown before (32), the LXXLL motif forms an α-helix that interacts with a hydrophobic groove on the surface of the KIX domain (Fig. 4E). Our docking studies suggest that Naphthol AS-E also binds to the hydrophobic groove by hydrophobic interactions and by interactions of the phosphate and carbonyl groups of Naphthol AS-E with an adjacent lysine residue (Fig. 4F). Part of Naphthol AS-E phosphate covers the hydrophobic pocket that is occupied by leucine 302 in the KIX-Myb complex. Naphthol AS-E phosphate therefore appears to inhibit the binding of Myb by competing for its binding site.

Previously, it was shown that p300 and its paralog CBP have nonredundant functions in hematopoietic cells and that c-Myb depends upon cooperation with p300 to a much greater extent than upon cooperation with CBP (28). It was therefore of interest to compare the binding of Myb with p300 and CBP using MST. We used a bacterially expressed CBP protein (amino acids 1 to 692), which corresponds to the p300(1-672) protein. We determined that human CBP (amino acids 1-692) interacts with a dissociation constant of 0.65 μmol/L with Myb (Supplementary Fig. S1A), Thus, Myb appears to interact more strongly with CBP than with p300. We also performed additional thermophoresis experiments to determine the effect of Naphthol AS-E phosphate on the Myb–CBP interaction (Supplementary Fig. S1B). We found that the IC\textsubscript{50} value for the inhibition of the interaction in this case was approximately 43 μmol/L. Thus, a higher concentration of Naphthol AS-E phosphate is required to disrupt the Myb-CBP compared with the Myb–p300 interaction.
Naphthol AS-E phosphate inhibits Myb activity in vivo and induces differentiation and apoptosis of leukemia cell lines

Because Myb is highly dependent on the interaction with p300, we investigated whether Naphthol AS-E phosphate inhibits Myb activity in vivo. We have recently developed a reporter cell line that allows monitoring Myb activity by the fluorescence of the cells (5, 44). This cell system is based on the chicken HD11 cell line that was stably transfected with a Myb-responsive GFP reporter gene driven by the promoter and enhancer of the Myb-inducible chicken mim-1 gene (22) and a doxycycline-inducible expression system for c-Myb (Fig. 5A). We have used these cells to evaluate the effect of Naphthol AS-E phosphate on Myb activity in vivo. Figure 5B shows that doxycycline significantly increased the fluorescence, whereas Naphthol AS-E phosphate caused a concentration-dependent inhibition of the fluorescence, indicating that it inhibits Myb activity. To demonstrate that Naphthol AS-E phosphate not simply quenches GFP fluorescence, we also examined the effect of Naphthol AS-E phosphate on the Myb-induced activation of the endogenous, Myb-inducible mim-1 gene as a more physiologic measure of Myb activity. Figure 5C shows a Northern blot demonstrating that Naphthol AS-E phosphate significantly inhibited the activation of the mim-1 gene by c-Myb. This experiment also showed that inhibition by Naphthol AS-E phosphate was not due to a decrease of the amount of c-Myb, indicating that the compound inhibits the activity of Myb and
Naphthol AS-E phosphate induces differentiation of the myeloid leukemia cell lines HL60, U937, and NB4 to investigate whether Naphthol AS-E phosphate induces the expression of endogenous c-Myb target genes by real-time PCR. As shown in Fig. 6A, treatment with 5 μmol/L Naphthol AS-E phosphate significantly inhibited the expression of the c-myc and adenosine deaminase (Ada) genes, two bona fide c-Myb target genes, in all three cell lines (45). This demonstrated that Naphthol AS-E phosphate suppresses the expression of c-Myb regulated genes in human cells. As in case of the reporter cell line (Fig. 5C) the amount of c-Myb was not decreased by Naphthol AS-E phosphate (Fig. 6D).

c-Myb is highly expressed in most hematopoietic progenitor cells and is required for maintaining their undifferentiated state (2). We therefore examined whether inhibition of c-Myb by Naphthol AS-E phosphate induces differentiation of the myeloid leukemia cell lines. Figure 6B shows that Naphthol AS-E phosphate in all cases induced differentiation (as assessed by the differentiation marker CD11b) of up to 20% of the cell population at a concentration of 5 μmol/L. Figure 6D shows microscopic images of HL60 cells treated with DMSO or Naphthol AS-E phosphate to confirm the differentiation also by morphologic criteria, such as the presence of irregularly shaped nuclei or enlarged cytoplasm.

Decreased c-Myb expression is also known to induce apoptosis (46, 47). To see whether Naphthol AS-E phosphate induces apoptosis, HL60, U937, and NB4 cells were treated for 24 hours with increasing concentrations of the compound. Apoptotic cells were quantified by annexin V staining. As shown in Fig. 6C, Naphthol AS-E phosphate induced overt apoptosis in all cases at concentrations above 25 μmol/L.

Discussion

Because of recent progress of our understanding of the role of c-Myb in leukemia and other types of cancer, c-Myb is now regarded as an attractive target for the development of small-molecule inhibitors as potential therapeutic drugs. With the exception of a recent report from our laboratory (5), which identified the sesquiterpene lactone mexicanin-I as the first low molecular weight compound that inhibits c-Myb activity, no small-molecule inhibitors of c-Myb are presently known. Mexicanin-I was identified by using the cell-based screening system described in Fig. 6A and its molecular mechanism of action is not yet known.

Here, we have adopted a novel strategy for the identification of Myb inhibitors that is based on the observation that the activity of Myb is highly dependent on its interaction with the coactivator p300. This interaction is mediated by the KIX domain of p300 and a LXXLL amino acid motif in the TAD of c-Myb. Amino acid replacements within this motif or in the KIX-domain of p300 disrupt the function of c-Myb as well as its interaction with p300, leading to dysregulation of multiple hematopoietic lineages (28, 29). Recently, it was shown that interaction of c-Myb with p300 is required for the induction of acute myeloid leukemia (AML) by human AML oncogenes (48). It therefore appears that the interaction with p300 is an "achilles heel" of c-Myb that can be exploited to inhibit its activity.

Here, we have established a protein–protein interaction assay that mimics the binding of Myb to the KIX domain of p300. We have expressed the KIX domain on the outer membrane of E. coli and demonstrated a specific interaction of the KIX-expressing
bacteria with a fluorescently labeled soluble Myb protein. The Myb–KIX interaction in this binding assay is highly specific and resembles the physiologic interaction of both proteins, as demonstrated by the L302A mutant. By using MST, we have determined that the dissociation constant of the Myb–KIX interaction is approximately 2.5 μmol/L, which is much lower than the Kd of approximately 12.5 to 15 μmol/L determined previously by using bacterially expressed KIX domain and a peptide containing the LXXLL-motif (32). This difference might be explained by the presentation of the LXXLL motif in the context of a larger part of the TAD, which might stabilize this motif for optimal binding to the KIX domain. It is also possible that the Myb–KIX interaction is supported by additional interactions between KIX and the Myb TAD that are missing when only a short peptide containing the LXXLL-motif is used. The Autodisplay binding assay described here can easily be adapted to large-scale screening, using high-speed flow cytometry or microtiter plate-based assays after immobilization of the bacteria in microtiter wells. It will therefore be interesting to exploit this system in the future to screen chemical compound libraries for inhibitory molecules.

On a side issue, we have also measured the dissociation constant for the interaction of c-Myb with the p300 paralog CBP. It was shown before that Myb is able to cooperate with both proteins but that in the hematopoietic system p300 is a more critical Myb interaction partner than CBP (28). Our data show that Myb binds somewhat stronger to CBP (Kd, 0.65 μmol/L) than to p300 (Kd, 2.5 μmol/L). Thus, the "dominant" role of p300 as a Myb cooperation partner in hematopoietic cells seems not to be due to higher affinity of p300 for binding to Myb, but must have other reasons.
Besides developing a screening tool for Myb inhibitors the second important result of our work is the identification of Naphthol AS-E phosphate as the first small-molecule inhibitor targeting the Myb–KIX interaction. Naphthol AS-E phosphate disrupts this interaction in vitro, as determined in the Autodisplay binding assay and by MST, and inhibits Myb activity in vitro, as shown by its effect on the activation of a Myb-dependent reporter gene and the endogenous Myb-inducible mim-1 gene. Molecular docking studies suggest that Naphthol AS-E phosphate interferes with the Myb–KIX interaction by binding to the same region of the KIX domain as Myb. Naphthol AS-E phosphate also inhibits the expression of known Myb target genes in several human myeloid leukemia cell lines and induces their differentiation and apoptosis, depending on the concentration of the compound. Interestingly, the IC50 value for inhibition of Myb activity in vitro is much lower (3 to 5 μmol/L) than the IC50 value for inhibition of the Myb–KIX interaction in vitro (~30 μmol/L). A similar difference was noted for the inhibition of the KIX–CREB interaction by Naphthol AS-E phosphate, which was also much more effective in vitro than in vitro (42). We have excluded that Naphthol AS-E phosphate induces degradation of Myb as an explanation for this difference. It is possible that slight reductions of the interaction with p300 might already lead to a large decrease of the Myb (or CREB) activity. Structural analysis of a trimeric complex of the KIX domain, the LXXLL motif of c-Myb and the mixed lineage leukemia (MLL) protein has previously shown that Myb and MLL bind cooperatively to different sides of the KIX domain (49). Because of cooperative binding, small changes in Myb binding might lead to larger effects on the overall composition of the Myb–KIX–MLL complex. It is also possible that posttranslational modifications or slight conformational differences between the isolated KIX domain expressed in bacteria and its eukaryotic counterpart expressed in the context of full-length p300 are responsible for the differences in the IC50 values determined in vitro and in vivo. In any case, our work has shown that Naphthol AS-E phosphate is an effective Myb inhibitor. Initial experiments using leukemia cell lines are promising in that they have shown that Naphthol AS-E phosphate induces differentiation and apoptosis and, hence, prevents their proliferation. Naphthol AS-E phosphate will therefore be interesting as a lead structure for further chemical modification to improve its performance as a Myb inhibitor. Last but not least, our work supports the idea that the disruption of the Myb–KIX interaction might be a promising strategy for the development of Myb inhibitors.

Disclosure of Potential Conflicts of Interest

J. Jose is CSO of Autodisplay Biotech GmbH. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: S. Uttarkar, K.-H. Klempnauer
Development of methodology: S. Uttarkar, S. Dukare, J. Jose, K.-H. Klempnauer
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Uttarkar, S. Dukare, M. Goblirsch, K.-H. Klempnauer
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Uttarkar, B. Ropp, M. Goblirsch, J. Jose, K.-H. Klempnauer
Writing, review, and/or revision of the manuscript: S. Uttarkar, M. Goblirsch, J. Jose, K.-H. Klempnauer
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Uttarkar, K.-H. Klempnauer
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