Identification of small molecules that induce apoptosis in a Myc-dependent manner and inhibit Myc-driven transformation

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The Myc transcription factor plays a central role in the regulation of cell cycle progression, apoptosis, angiogenesis, and cellular transformation. Myc is a potent oncoprotein that is deregulated in a wide variety of human tumors and is therefore an attractive target for novel cancer therapies. Using a cellular screening approach, we have identified low-molecular-weight compounds, Myc pathway response agents (MYRAs), that induce apoptosis in a c-Myc-dependent manner and inhibit Myc-driven cellular transformation. MYRA-A inhibits Myc transactivation and interferes with the DNA-binding activity of Myc family proteins but has no effect on the E-box-binding protein USF. In contrast, MYRA-B induces Myc-dependent apoptosis without affecting Myc transactivation or Myc/Max DNA binding. Our data show that cellular screening assays can be a powerful strategy for the identification of candidate substances that modulate the Myc pathway. These compounds can be useful tools for studying Myc function and may also be of therapeutic potential as leads for drug development.

cellular screen | chemical library | proliferation | Myc pathway | DNA-binding

The c-Myc transcription factor is a key regulator of proliferation, cell growth, differentiation, apoptosis, angiogenesis, and transformation (1, 2). It is a basic helix-loop-helix leucine zipper (bHLHZip) protein that carries out its functions through dimerization with the bHLHZip protein Max. The heterodimer binds to and activates promoters containing a specific DNA sequence CACGTG, called E-box (3, 4). Transactivation by Myc involves recruitment of histone acetyltransferases (HATs) to target genes through adaptor proteins, such as TRRAP (5–7). Myc also binds to the Miz-1 protein at initiator elements and thereby represses the transcription of Miz-1 target genes such as the cyclin-dependent kinase (cdk) inhibitor p15INK4b (8–11). Another Myc family protein, Mnt, is expressed in both proliferating and differentiating cells in all tissues analyzed and has been suggested to function as a Myc antagonist (12–14).

c-myc was identified as an oncogene, and its role in tumorigenesis *in vivo* was confirmed in studies in transgenic mice with constitutive Myc expression (15). Myc tumorigenesis was enhanced by introduction of antiapoptotic proteins such as Bcl-2 or Bcl-X_L (16, 17), or by an impaired p53 pathway (18–21). Using transgenic mice with tetracycline-inducible Myc expression, Felsher and colleagues (22, 23) induced malignant T cell lymphomas, acute myeloid leukemia, and liver tumors under sustained Myc expression. Inactivation of c-Myc caused tumor regression accompanied with rapid proliferation arrest, apoptosis, and differentiation in hematopoietic malignancies and osteosarcoma but tumor dormancy in liver malignancies. Reactivation of c-Myc resulted in enhanced apoptosis of hematopoietic tumors and osteosarcoma but restoration of malignancy in the liver cancer model (22–24). Similarly, Evan and colleagues (25, 26) have used a conditional transgenic expression system (Myc-ER^{TAM}) to investigate the effect of c-Myc activation in distinct tissues. Sustained activation of Myc-ER^{TAM} in the skin induced a complex neoplastic lesion involving marked hyperplasia of undifferentiated suprabasal cells, angiogenesis, and overt papil-

lomatosis. Subsequent deactivation of Myc triggered complete papilloma regression (25). Coexpression of c-Myc and Bcl-X_L in pancreatic β cells induced angiogenic and invasive tumors in the transgenic mice whereas deactivation of c-Myc induced rapid apoptosis and vascular degeneration in established pancreatic tumors (26). These findings demonstrate that inactivation of Myc in proliferating cells induces apoptosis and differentiation and that even brief inactivation can result in sustained loss of malignant properties. However, brief inactivation of c-Myc was not sufficient for sustained regression of c-Myc-induced mammary adenocarcinomas or in tumors of pancreatic islets and skin epidermis (27, 28). Even though tumor regrowth rather than enhanced apoptosis was observed upon Myc reactivation in mammary adenocarcinomas, still approximately 50% of the tumors regressed completely after the first inactivation of Myc and around 17% of the tumors maintained the dependency on Myc even after three rounds of Myc induction and inactivation (27). Recently, Nilsson *et al.* (29) showed that disabling the Myc target gene ornithine decarboxylase (Odc) markedly delayed lymphoma development both in E μ -Myc;Odc^{+/-} transgenic mice and in E μ -Myc mice treated with an Odc inhibitor. Taken together, these studies suggest that, whereas the outcome of Myc activation and reactivation is tissue-specific, targeting of the Myc pathway could have significant effects in cancer treatment.

Several criteria make Myc an attractive target for tailored cancer therapy. The Myc protein is expressed only in cycling cells, activates both proliferation and apoptosis (2, 30–33), and is deregulated in a wide spectrum of human tumors often associated with poor differentiation and aggressiveness (34, 35). Numerous efforts using different approaches have been made to interfere with c-Myc to revert or eliminate malignant cells (reviewed in refs. 36–38). Two groups have performed screening assays using libraries of low-molecular-weight compounds. Based on an *in vitro* FRET-assay, Berg *et al.* (39) reported the identification of four compounds that interfered with Myc/Max dimerization. One of these substances inhibited transformation of chicken embryo fibroblasts driven by Myc and another blocked the transformation driven by Jun or Myc oncoproteins, whereas the remaining two had no effect in the focus formation assay. None of these compounds inhibited transformation driven by Src (39). In another study, Yin *et al.* (40) used the yeast two-hybrid system for identification of low-molecular-weight compounds that blocked Myc/Max interaction. These substances resulted in different effects on cell proliferation in parental and myc-null cells, but no difference was observed between parental and Myc-overexpressing cells. In contrast to the above-mentioned screens that were designed to specifically target Myc/Max dimer-

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Abbreviations: MYRA, Myc pathway response agent; BL, Burkitt lymphoma; LCL, lymphoblastoid cell line; CMV, cytomegalovirus; USF, upstream stimulatory factor.

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ization, we have used a cellular screening approach to enable identification of small molecules with different mechanisms of action. Using this strategy, we identified low-molecular-weight compounds that induce apoptosis in a Myc-dependent manner and inhibit transformation driven by c-Myc.

Results

Identification of Small Molecules by a Cellular Screening Assay. To identify substances that interfere with the Myc pathway, we have used cells with inducible Myc expression and screened for low-molecular-weight compounds that selectively affected cell viability of Myc-overexpressing cells compared with cells with WT Myc levels. To optimize the conditions for Myc induction, Tet-Myc mouse fibroblasts were treated with different doxycycline concentrations in a time-course experiment. As expected, c-Myc expression was induced in a dose-dependent manner, and, although the induced expression of Myc was slightly decreased at 66 h, Myc was still maintained at high levels during 3 days after treatment with 2 μ g/ml doxycycline (Fig. 1A). We performed the screen by treating

Tet-Myc cells with each of the 1,990 compounds of the NCI diversity set library 18 h after doxycycline addition and analyzed cell viability after 48 h by both the WST-1 assay and crystal violet staining. Doxycycline alone did not have any affect on proliferation (data not shown). Several compounds showed predominant growth suppression of c-Myc-induced cells compared with uninduced cells. Two of these compounds, NSC339585 and NSC45641, were selected for further characterization, because the relative viability of uninduced cells was >2-fold higher than that of induced cells after treatment. These substances are referred to as Myc pathway response agent (MYRA)-A and MYRA-B, respectively, (Fig. 1B). As shown in Fig. 1C, the cell viability of Myc-induced cells was only 10% after treatment with MYRA-A but 37% for treated WT cells. In response to MYRA-B, the viability of Myc-induced cells was around 33% whereas the corresponding viability of uninduced cells was $\approx$ 76%. To examine whether incubation with MYRAs influenced Myc levels, we performed Western blot analysis. As shown in the *Inset* in Fig. 1C, MYRA treatment did not affect Myc expression levels. Although the pattern of growth and death

differed between the untreated and treated uninduced cells, the remaining attached cells appeared morphologically intact. In contrast, the majority of MYRA-treated, Myc-induced cells displayed features of cell death such as a round shape and detachment (Fig. 1D).

To analyze the effects of MYRA treatment on human cells with different Myc levels, we used p493-6 B cells with tetracycline-regulated Myc expression (ref. 41 and Fig. 1E). Both MYRA-A and MYRA-B affected the viability of p493-6 cells with high Myc expression to a larger extent compared with the cells with lower Myc levels. The IC_{50} values for both MYRA-A and MYRA-B were more than twice as high for p493-6 + Tet (Myc-off) compared with p493-6 + Tet (Myc-on), $>40 \mu\text{M}$ versus $18\text{--}20 \mu\text{M}$, respectively (Fig. 1E). Notably, MYRA-B had a more pronounced effect than MYRA-A and showed almost no effect on p493-6 + Tet (Myc-off) cells at concentrations up to $40 \mu\text{M}$. Similarly, the MYRAs predominantly affected human Burkitt lymphoma (BL) cells with Myc translocations compared with lymphoblastoid cell lines (LCLs) lacking Myc translocations, as illustrated by the lower IC_{50} values for the BL cells (Fig. 1F). Taken together, these results support the data obtained from the mouse cells indicating that MYRA-A and MYRA-B are more efficient in cells with high compared to those with low Myc levels.

We next verified the effects of MYRA-A and MYRA-B on TGR-1 (c-myc WT), HO15.19 (c-myc-null), and HOMyc3 (c-myc-overexpressing) Rat-1 cells (42–44). MYRA treatment resulted in different effects depending on the c-Myc status of the cells, with the most predominant inhibition in HOMyc3 cells. The IC_{50} values for HOMyc3 cells were ≈ 3 and $10 \mu\text{M}$ for MYRA-A and MYRA-B, respectively, whereas the corresponding IC_{50} values for TGR-1 cells were 5 and $50 \mu\text{M}$ (Fig. 2A). Because the HO15.19 myc-null cells have a longer doubling time than the TGR-1 cells (42), we treated these cells with compounds from 48 h up to 96 h. After 96 h of treatment, the IC_{50} of MYRA-A and MYRA-B for HO15.19 cells were around 10 and $140 \mu\text{M}$ (Fig. 2A), respectively, whereas no effects were observed after 48 h (data not shown). As shown in Fig. 2B, MYRA-treated HOMyc3 cells were sparsely distributed compared with the corresponding TGR-1 cells. In contrast, MYRA treatment had virtually no effect on HO15.19 cells (Fig. 2B). Collectively, these results indicated that the effects of MYRAs on cell viability is c-Myc-dependent.

Candidate Substances Induce Myc-Dependent Apoptosis. Because we observed a predominant decrease in cell viability of Myc-overexpressing cells after MYRA treatment, we analyzed whether this effect was due to apoptosis. Hoechst staining revealed many condensed and fragmented nuclei in MYRA-treated HOMyc3 cells and, although to a somewhat lesser extent, some distorted and condensed nuclei in treated TGR-1 cells, indicating that these compounds induced apoptotic cell death (Fig. 2C). In contrast, drug-treated HO15.19 cells displayed similar nuclear morphology as untreated cells. To confirm these data, we used an apoptosis ELISA and found that both MYRA-A and MYRA-B induced significant apoptosis in HOMyc3 cells after 48 h of treatment (Fig. 2D). Importantly, these compounds induced an enhanced apoptotic response in cells with Myc overexpression compared with cells with WT Myc levels. MYRA-A induced a 4-fold increase in apoptosis in HOMyc3 cells compared with TGR-1 cells, whereas the corresponding apoptosis induction was 7-fold for MYRA-B. In contrast, no significant apoptosis was observed in HO15.19 cells even after 96 h of treatment (Fig. 2D). Taken together, these data support the notion that both compounds induced apoptotic cell death in a c-Myc-dependent manner.

Effects of MYRAs on the DNA-Binding Activity of the Myc/Max Complex. Because binding to target sequences is essential for c-Myc function, we analyzed whether the identified compounds interfered with the DNA binding of the Myc/Max complex by EMSA in HL60

Fig. 2. Induction of c-Myc-dependent apoptosis by MYRAs in Rat-1 cell lines. (A) Rat-1 cells with different c-Myc expression were treated with 0–7 μM MYRA-A or 0–80 μM MYRA-B, and cell viability was analyzed by crystal violet staining. (B) Phase contrast images of Rat-1 cells treated with 3 μM MYRA-A, 40 μM MYRA-B, or with DMSO (control). (C) Hoechst staining of Rat-1 cells treated with MYRA-A, MYRA-B, or with DMSO. Apoptotic nuclei are indicated by arrows. (D) Apoptosis was analyzed by using the Cell Death Detection ELISA^{PLUS} kit after treatment with 3 μM MYRA-A, 40 μM MYRA-B, or with DMSO. Data represent the mean and SD of three independent experiments.

cells (45, 46). The identity of specific bands corresponding to the DNA binding of Myc/Max, Mnt/Max, and upstream stimulatory factor (USF) was confirmed by antibody supershift experiments (Fig. 3A). The effects of MYRAs on DNA binding were assessed by a titration from 12.5 to 100 μM . We found that MYRA-A interfered with the DNA binding of Myc/Max and Mnt/Max complexes in a dose-dependent manner but had no effect on the DNA binding of the E-box-binding transcription factor USF (Fig. 3A). In contrast, MYRA-B had no effect in EMSA even at

Fig. 4. Effects on luciferase activity by the treatment with MYRAs. CV1 cells were transiently transfected with pSP-Myc or pSP-vector together with minM4Luc (A), or were transfected either with CMV-p53 or CMV-vector and the p53-reporter (B), or with CMV-reporter alone (C). pCMV- β -galactosidase was included in all transfections. Twenty-four hours posttransfection, the cells were treated with the indicated concentrations of MYRA-A and MYRA-B. The luciferase activities were normalized for β -galactosidase as a control of transfection efficiencies, and the mean values with SD of at least three independent experiments are shown. (D) Myc immunoprecipitation was performed by using cell extracts from Tet-Myc cells treated with MYRA-A *in vivo* (Left), or *in vitro* (Center) and from Myc/Max transfected COS cells (Right) that had been incubated with MYRA-A *in vitro*. The inhibitor of Myc/Max dimerization 10058-F4 was used as control for the *in vitro* experiment. Precipitated protein complexes were separated by SDS/PAGE and analyzed by Western blotting for Myc and Max.

were further verified in Rat-1 cells with different Myc levels. Notably, the effect of the candidate compounds on cell viability was more significant in cells with c-Myc overexpression (HOMyc3) compared with cells with WT c-myc (TGR-1), whereas they did not show significant effects in c-myc-null cells (HO15.19).

Morphological changes of cells and nuclei showed that treatment with MYRAs induced apoptotic cell death in a Myc-dependent manner, which is supported by the quantitative data from the apoptosis ELISA assay. To our knowledge, there have been no previous reports of the identification of small molecules that induce apoptosis in a Myc-dependent manner. Berg *et al.* (39) did not indicate that their compounds induced apoptosis. Although Yin *et al.* (40) observed apoptosis after treatment with their substances for >1 week, no difference on cell viability was observed between Myc-overexpressing and control cells.

We observed that MYRA-A interfered with the DNA binding not only of Myc/Max but also of Mnt/Max and Max/Max complexes. These results are not surprising because Mnt and Myc share DNA-binding site and regulate an overlapping set of target genes *in vivo* (12). In addition, loss of Mnt has been shown

Fig. 5. Inhibition of soft agar colony formation by MYRAs. Rat-1a-myc (A) and MR (B) cells were seeded in soft agar and treated with different concentrations of MYRA-A and MYRA-B. For each compound, two of the concentrations used (in μ M) are shown. Representative plates from two independent experiments are presented.

to induce similar effects as Myc overexpression, such as induction of Myc target genes, accelerated proliferation and apoptosis, and transformation of primary fibroblasts in conjunction with Ras (47, 48). It has been proposed that Myc functions by relief of Mnt repression (48, 49). Therefore, it will be interesting to analyze whether the Myc-dependent effects of these compounds occur through interference with both Myc and Mnt function. However, MYRA-A did not show any effect on the DNA-binding activity of USF, which binds to the same E-box (50). Thus, MYRA-A could possibly discriminate Myc network members from other E-box-binding proteins. The effective concentrations of MYRA-A in the EMSA were higher than those used in the cell viability assays, which is similar to the results obtained for other compounds (39). One possibility is that the compound accumulates in cells to reach effective intracellular concentrations. We did not obtain evidence that interference with Myc/Max dimerization by MYRA-A was the underlying mechanism. However, based on the results obtained in the EMSA and luciferase assays, we hypothesize that MYRA-A might distinguish Myc family proteins from other E-box-binding transcription factors as a result of direct or indirect interference with DNA-binding. MYRA-B, on the other hand, might act through effects on variant E-box promoters, by interaction with other transcriptional factors that bind Myc such as Miz-1 (9), by interference with adaptor proteins, or by an indirect mechanism. It is also interesting to note that MYRA-B displayed differential inhibition of soft agar growth having only weak inhibition on MR cells compared with its effect on Rat-1a-myc cells. This finding might reflect different conditions for transformation and/or might be due to different cell type-specific changes in these two cell lines. It will be important to analyze the effects of MYRAs on the Myc pathway in more detail to find key players and possible new therapeutic candidates in the Myc-mediated apoptosis pathway.

Ideally, a Myc-targeting therapy should intervene only with cells with deregulated Myc and not affect the majority of normal cells *in vivo*. The observation that MYRAs predominantly induced apoptosis in cells with Myc overexpression is in line with this ambition. Our study shows that cellular screening assays can be efficient tools to identify low-molecular-weight compounds that target the Myc pathway by different mechanisms of action. These compounds could be useful tools for gaining further

insights into Myc-regulated pathways and may also provide a starting point for medical chemistry and drug design.

Materials and Methods

Cell Culture and Media. Tet-Myc cells containing a tetracycline-inducible c-myc gene (51) were cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% tetracycline-free FCS (Clontech). The human B cell line p493-6 (41) was a kind gift from G. Bornkamm (National Research Center for Environment and Health, Munich). All B cell lines were cultured in RPMI medium 1640. TGR-1 and HO15.19 Rat-1 cells (42) were kindly provided by J. Sedivy (Brown University, Providence, RI). HOmyc3 Rat-1 cells with 2- to 4-fold c-Myc overexpression (43, 44) were a kind gift from M. Cole (Dartmouth Medical School, Hanover, NH). Rat-1 and Rat-1a-myc cells (52) were grown in DMEM whereas MR (53), COS, and CV1 cells were grown in IMDM.

Cellular Library Screen. Three thousand Tet-Myc cells were seeded per well in 96-well plates and were cultured for 18 h in the presence or absence of 2 $\mu\text{g/ml}$ doxycycline before treatment with compounds from the diversity set library provided by the National Cancer Institute (Bethesda) (<http://dtp.nci.nih.gov>).

Cell viability was assayed using the WST-1 Cell Proliferation Reagent (Roche Diagnostics) according to the manufacturer's instructions, followed by crystal violet staining.

EMSA. Whole-cell extracts from either transfected COS cells or logarithmically growing HL60 cells were prepared, incubated with candidate compounds, and analyzed by EMSA as described (45, 51).

Supporting Information. Further details are available in *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site.

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