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Selective Inhibition of c-Myc/Max Dimerization and DNA Binding by Small Molecules

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Summary

bZip and bHLHZip protein family members comprise a large fraction of eukaryotic transcription factors and need to bind DNA in order to exert most of their fundamental biological roles. Their binding to DNA requires homo- or heterodimerization via α-helical domains, which generally do not contain obvious binding sites for small molecules. We have identified two small molecules, dubbed Mycro1 and Mycro2, which inhibit the protein-protein interactions between the bHLHZip proteins c-Myc and Max. Mycros are the first inhibitors of c-Myc/Max dimerization, which have been demonstrated to inhibit DNA binding of c-Myc with preference over other dimeric transcription factors in vitro. Mycros inhibit c-Myc-dependent proliferation, gene transcription, and oncogenic transformation in the low micromolar concentration range. Our data support the idea that dimeric transcription factors can be druggable even in the absence of obvious small-molecule binding pockets.

Introduction

Aberrantly high levels of the transcription factor c-Myc are found in most human cancers and have been linked to one out of seven cancer deaths in the United States. [1]. The transcription factor c-Myc is involved in fundamental cellular processes including cell cycle progression, growth, oncogenic transformation, and apoptosis [2–5]. Its expression and posttranscriptional stabilization are tightly regulated upon induction by growth stimulatory signals. Conditional overexpression of c-Myc in genetic model systems leads to tumorigenesis, which can be reverted by inactivation of the c-Myc transgene [6–9]. This suggests that inactivation of c-Myc may be a novel approach toward the treatment of human cancers, which display increased activities of c-Myc, e.g., lung, colon, and breast carcinomas, or Burkitt's lymphoma.

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c-Myc is a member of the basic helix-loop-helix leucine zipper protein family. All known biological activities of c-Myc require heterodimerization with its activation partner Max [10] from the same protein family. c-Myc regulates up to 15% of all genes in an organism [11] by two mechanisms: first, binding of c-Myc/Max heterodimers to specific recognition sites (E-box elements) within promoter regions activates gene transcription, and second, indirect recruitment of c-Myc/Max dimers to DNA via the zinc-finger protein Miz-1 leads to repression of c-Myc-regulated genes [12, 13]. Therefore, the most direct and thorough approach toward the inhibition of c-Myc functions involves the inhibition of the protein-protein interactions required for association of c-Myc with its binding partner Max, both in the presence and absence of the DNA sequence to which c-Myc/Max binds directly (Figure 1). However, the discovery of inhibitors of c-Myc/Max heterodimers is hampered by the large protein-protein interface between the two basic helix-loop-helix leucine zipper (bHLHZip) proteins, consisting of the second helix of the helix-loop-helix domain and the leucine zippers (3206 $Å^2$ of buried interface) [14]. Additionally, and perhaps even more importantly, the crystal structure does not reveal any obvious binding pockets for small molecules, prohibiting any rational predictions about the nature of substances that might inhibit c-Myc/Max dimer formation. In the light of the dramatic stabilization of c-Myc/Max interactions by nine orders of magnitude upon addition of DNA [15], it is conceivable that the presence of DNA significantly impairs a compound's effectiveness toward inhibition of c-Myc/Max association, perhaps dependent on the location of the compound's binding epitope. Therefore, in order to effectively inhibit the functions of c-Myc, it is of particular importance for an inhibitor of c-Myc/Max dimerization to maintain its inhibitory properties in the presence of the c-Myc/Max DNA binding motif while showing preference over the inhibition of DNA binding of other dimeric transcription factors. However, no small molecule has been reported to fulfill this criterion in in vitro assays (i.e., not cell-based assays) to date.

Of the first two reported nonpeptidic inhibitors of c-Myc/Max dimerization, referred to as IIA6B17 and IIA4B20, only IIA6B17 maintained its inhibitory properties in the presence of DNA in vitro [16]. Unfortunately, the activity of IIA6B17 also extended to the related basic zipper (bZip) family protein Jun, which limits its potential to serve as a molecular research tool. Recently, four compounds consisting of planar and hydrophobic chemical building blocks were reported to inhibit Myc/Max association in the presence of DNA [17]. Two of these compounds, referred to as NY2267 and NY2280, inhibited c-Myc-dependent oncogenic transformation with preference over transformation dependent on Jun but affected gene transcription dependent on both c-Myc and c-Jun to the same extent. Interestingly, fatty acids have been described to inhibit DNA binding of c-Myc [18], although contradictory statements have been made regarding the effectiveness of inhibition by saturated fatty acids [19]. In addition, four compounds

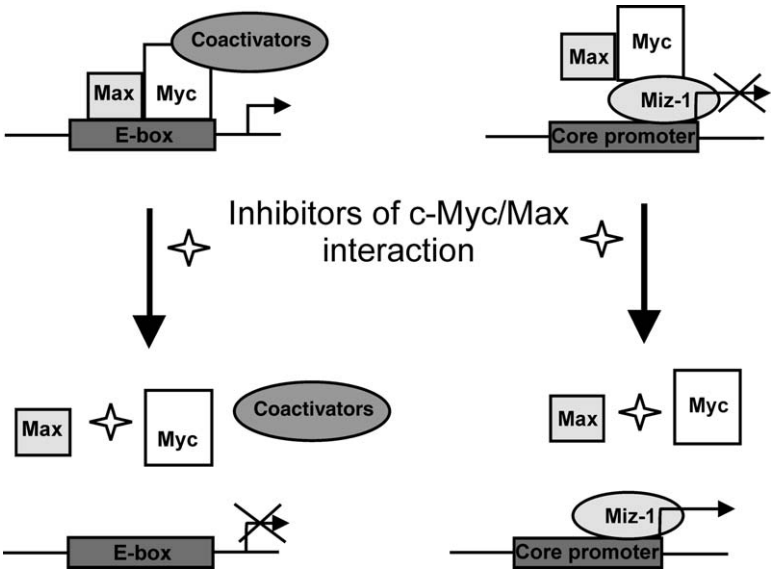

Figure 1. Inhibition of c-Myc by Inhibitors of the c-Myc/Max Interaction

c-Myc can activate gene transcription by direct binding of c-Myc/Max heterodimers to specific DNA binding elements referred to as E-box elements and can repress gene transcription by indirect DNA binding of c-Myc/Max dimers via the zinc-finger protein Miz-1. Since all known biological functions of c-Myc require binding to its activating partner Max, inhibition of the interaction between c-Myc and Max by a small organic molecule (indicated by the star) would inhibit all known biological functions of c-Myc.

structurally not related to one another were reported to inhibit dimerization of c-Myc and Max, but data about the compounds' abilities to inhibit DNA binding of c-Myc in vitro were not presented. The substances were shown to suppress growth of c-Myc-transformed cells in nude mice; however, no information was provided about the compounds' effect on anchorage-independent growth of cell lines transformed by oncogenes other than Myc [20]. Finally, a small molecule called "MYRA-A" was reported to inhibit DNA binding of Myc family proteins without interfering with c-Myc/Max dimerization [21]. In this manuscript, we detail the identification of Mycro1 and Mycro2, the first small molecules demonstrated to show a preference for the inhibition of the c-Myc/Max association over the inhibition of related transcription factors in the presence of their DNA binding motif in vitro.

Results and Discussion

Small-Molecule Inhibitors of c-Myc/Max Dimerization and DNA Binding Identified by High-Throughput Screening of Chemical Libraries

We have developed assays based on fluorescence polarization, which analyzed binding of Myc/Max, Max/Max, Jun/Jun, and C/EBPα/C/EBPα dimers to their respective fluorophore-labeled DNA target sequences (Figure 2A) [22]. Screening of a diverse selection of 17,298 small molecules for their abilities to disrupt binding of c-Myc/Max to a fluorescein-labeled oligonucleotide containing the binding motif CACGTG (Z' value [23] of the assay: 0.65 ± 0.07) resulted in the identification of a small molecule dubbed Mycro1 (pronounced "mick-ro," for *Myc* activity-*r*educing *o*rganic substance and to highlight its comparably small size in relation to the inhibited protein-protein interface) (Figure 2B). Analysis of structure-activity relationships with commercially available derivatives revealed a derivative (**2**), (referred to as Mycro2), which displayed similar activity in this assay (Figures 2B–2D). Both Mycros inhibited Myc/Max DNA binding with good activities (IC_{50} values: Mycro1, 30 ± 5 μM; Mycro2, 23 ± 4 μM) and show preference over the inhibition of binding of Max/Max homodimers (59% similar) to the same DNA probe (IC_{50} values: Mycro1, 72 ± 13 μM; Mycro2, 54 ± 9 μM). DNA binding of the related bZip proteins CEBPα and Jun, which also requires the formation of the respective homodimers, was inhibited to an even smaller extent in the fluorescence polarization assays (Figures 2C and 2D). Like c-Myc/Max, dimerization of Max/Max, CEBPα/CEBPα, and Jun/Jun is mediated by leucine zippers, which contain four to five heptad repeats consisting of hydrophobic amino acids (e.g., leucine) and polar amino acids and mediate protein-protein interactions by hydrophobic and electrostatic interactions. Mycros had virtually no effect on interactions between two SH2 domains and their binding partners (see Figures S1A and S1B in the Supplemental Data online), suggesting that the effect of Mycros on DNA binding of c-Myc/Max and the related transcription factors results from specific interactions with the α-helical dimerization motifs. Unfortunately, the binding site of Mycros cannot be assessed by mutation or deletion experiments, as mutations or deletions in the dimerization domains of c-Myc and Max result in loss of their abilities to dimerize. A closely related regioisomer of Mycro2 (**3**) (Figure 2B) had lost virtually all activity (4% ± 13% inhibition of c-Myc/Max DNA binding at 100 μM (**3**)) (see Figure S1C). To verify the results of the fluorescence polarization assay in a fluorescence-independent assay format, we analyzed the molecules' abilities to interfere with DNA binding of c-Myc in electrophoretic mobility shift assays (EMSA). Consistent with the results obtained in the fluorescence polarization assay, both Mycros inhibited DNA binding of c-Myc and displayed selectivity for inhibition of DNA binding of c-Myc/Max over Jun/Fos heterodimers (Figures 2E and 2F). The control compound **3**, which was inactive in the FP assay, was also inactive in EMSA (see Figure S1D). To investigate whether the observed reduction in DNA binding of c-Myc originates from inhibition of the DNA-protein interaction between c-Myc/Max dimers and DNA or from inhibition of protein-protein interactions between c-Myc and Max, we analyzed the effect of Mycros on the interaction between CFP-tagged c-Myc and GST-tagged Max in

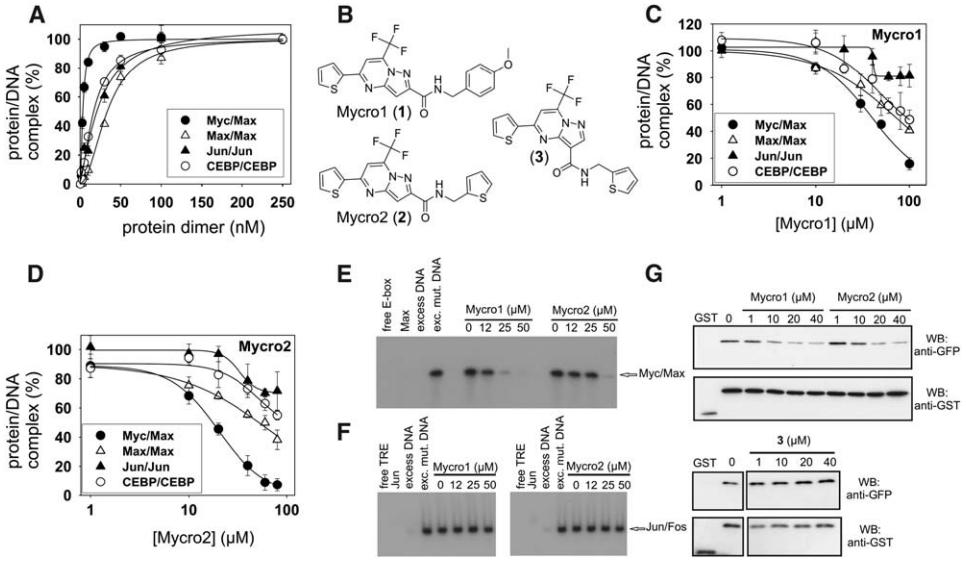

Figure 2. Mycros Inhibit DNA Binding of c-Myc by Blocking the Interaction between c-Myc and Max

(A) Binding of the transcription factor dimers c-Myc/Max, Max/Max, Jun/Jun, und C/EBPα-C/EBPα to fluorophore-labeled oligonucleotides comprising their DNA target sequences as assessed by fluorescence polarization.

(B) Chemical structures of Mycro1 (**1**), Mycro2 (**2**), and the inactive derivative **3**.

(C) Activity profile of Mycro1 against DNA binding of c-Myc/Max, Max/Max, Jun/Jun, and C/EBPα-C/EBPα as assessed by fluorescence polarization. Error bars represent standard errors of the mean (SEM).

(D) Activity profile of Mycro2 against DNA binding of c-Myc/Max, Max/Max, Jun/Jun, and C/EBPα/C/EBPα as assessed by fluorescence polarization. Error bars represent SEM.

(E) Activity profile of Mycro1 and Mycro2 against DNA binding of c-Myc/Max heterodimers in EMSA. All assigned lanes contain c-Myc, Max, and labeled E box, except the first lane (labeled E Box only, no proteins) and the second lane (Max and labeled E Box, no c-Myc).

(F) Activity profile of Mycro1 and Mycro2 against DNA binding of Jun/Fos heterodimers in EMSA. All assigned lanes contain Jun, Fos, and labeled TRE, with the exception of the first lane on each gel (labeled TRE only, no proteins) and the second lane (Jun and labeled TRE, no Fos).

(G) Mycro1 and Mycro2 inhibit the protein-protein interactions between CFP-tagged c-Myc and GST-tagged Max in a GST pull-down assay. The anti-GFP antibody used does not discriminate between the various GFP mutants.

a GST pull-down assay (Figure 2G). Both Mycros already inhibited binding between c-Myc and Max at 10 μM, while the negative control compound **3** had no effect. Considering the stabilization of c-Myc/Max interactions upon addition of DNA, the relatively low activity differences in these two assay types could be explained by a model by which Mycros interact with the proteins, preferentially c-Myc, and thereby lock monomeric c-Myc in a conformation that is unable to bind to Max and therefore also unable to bind DNA. Hence, DNA binding would no longer represent a stabilizing factor. The low activity of Mycros on DNA binding of Jun/Jun (Figures 2C and 2D) and Jun/Fos (Figure 2F), and especially, the virtual absence of inhibition of the SH2-domain proteins STAT3 and Lck (see Figures S1A and S1B) strongly argues against an alternative model of explanation, that of unspecific protein aggregation and precipitation. Compounds **1–3** fully comply with Lipinski's "rule of five" [24] and thereby fulfill an important empirical criterion for bioavailable substances.

Mycros Inhibit Cell Proliferation and Cell Cycle Progression in a c-Myc-Dependent Manner

Because c-Myc regulates a wide range of target genes essential for cell function, even a small decrease in c-Myc activity conferred by the inhibitors can be expected to have a noticeable biological effect in cellular assays. c-Myc is required for entry into S phase and cell cycle progression [3, 25] of almost all cell types. Consistent with their in vitro activities, Mycros inhibited proliferation of the c-Myc-dependent tumor cell lines Raji (Burkitt's lymphoma), MCF-7 (breast carcinoma), and U-2OS (osteosarcoma) at 10 and 20 μM (Figures 3A–3C). Similarly, proliferation of c-Myc-dependent NIH/3T3 cells was also inhibited by both Mycros at 10 and 20 μM (Figure 3D). We have not observed an increase in the number of floating cells in the tissue culture medium upon addition of Mycros, indicating the absence of gross toxicity. Since complete inhibition of c-Myc in c-Myc-dependent cells can be expected to cause cell death, the absence of toxicity indicates that at the concentrations used in tissue culture (10 μM and 20 μM), Mycros only partially inhibited c-Myc, consistent with the incomplete inhibition of c-Myc at these concentrations observed in vitro (Figure 2). Time-dependent analysis of S-phase entry in a BrdU incorporation assay revealed that the effect on proliferation of NIH/3T3 cells was at least in part due to a significant delay in S-phase entry caused by the inhibitors, consistent with scientific evidence linking c-Myc activity with S-phase entry [3, 25] (Figure 3E). In contrast to the inhibition of these four c-Myc-dependent cell lines, proliferation of the pheochromocytoma cell line PC-12, in which the Myc binding protein Max is rendered dysfunctional by homozygous mutation and which therefore proliferate independent of c-Myc [26], was not inhibited by either Mycro, indicating that the inhibition of cancer cell proliferation by Mycros occurs via a c-Myc-dependent mechanism (Figure 3F).

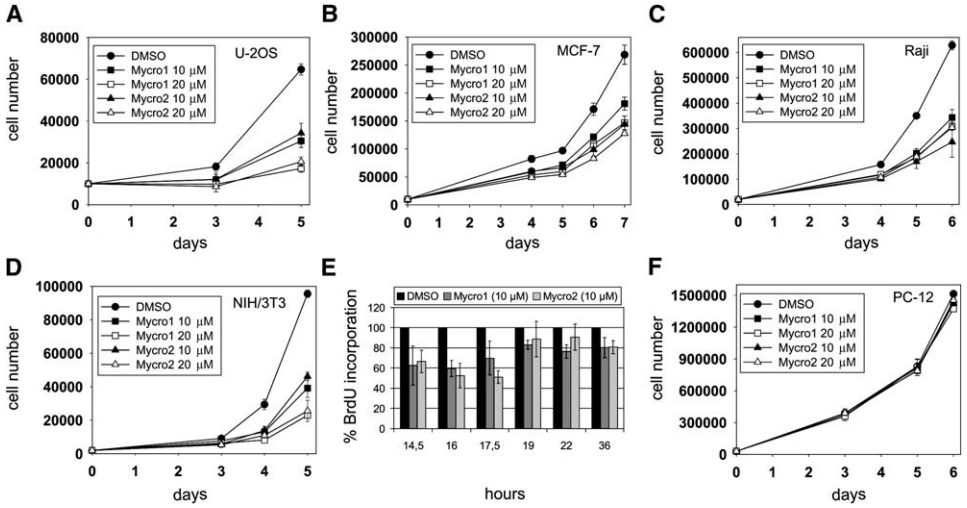

Figure 3. Mycro1 and Mycro2 Inhibit c-Myc-Dependent Cell Proliferation and Delay S-Phase Entry

Mycro1 and Mycro2 inhibit proliferation of the c-Myc-dependent cell lines U-2OS (A), MCF-7 (B), Raji (C), and NIH/3T3 (D). (E) Mycro1 and Mycro2 delay S-phase entry as analyzed by a BrdU incorporation assay in NIH/3T3 cells. (F) Mycro1 and Mycro2 do not inhibit proliferation of the c-Myc-independent cancer cell line PC-12. Error bars represent standard deviation (SD).

Mycros Inhibit c-Myc-Dependent Gene Transcription

Genome-wide analysis of gene transcription has revealed that c-Myc activates or represses a wide range of target genes, but usually by not more than a factor of three [11], which renders the detection of changes in the transcription levels of target genes a difficult task. To assess the effect of Mycros on c-Myc-induced gene transcription, we therefore set up a reporter assay that analyzes the effect of Mycros on the transcriptional activity of c-Myc and other dimeric transcription factors (see Figure S2). Both Mycro1 and Mycro2 strongly inhibited c-Myc-induced gene transcription at 10 μM (Figure 4A) in HEK293 T cells cotransfected with an expression vector for c-Myc and a luciferase reporter bearing four Myc/Max binding sites. The activities of Mycros in this assay appear to be slightly higher than in the DNA binding assays (Figures 2C–2E); however, it should be kept in mind that the potency of any small-molecule inhibitor is likely to depend on the precise assay conditions, especially with respect to the concentrations of proteins, salts, or other macromolecules. Since no in vitro assay will precisely reflect intracellular conditions, it would be unrealistic to expect exactly the same potencies of a compound in vitro as in cells. The activity of a reporter construct containing multiple binding sites for a subgroup of dimeric bZip transcription factors, refered to as AP-1, was reduced to a smaller extent (Figure 4B). AP-1 is a term for dimeric complexes that comprise members of the Jun, Fos, ATF, and Maf protein subfamilies, all of which are dimeric bZip proteins [27]. The main AP-1 proteins in mammalian cells are Jun and Fos [28], and the specificity of Mycro2 for Myc over AP-1 in the luciferase assays reflects the selectivity profile for Myc versus Jun observed in the DNA binding assays (Figures 2C–2F). Transcription dependent on dimerization of the transcription factor serum-response factor (SRF) was not or only slightly inhibited by Mycro1 or Mycro2, respectively (Figure 4C). Consistent with its inactivity in vitro, derivative 3 did not interfere with c-Myc dependent transcription (Figure 4A).

Mycros Inhibit c-Myc-Dependent Anchorage-Independent Growth

Rat1a fibroblasts can be transformed by stable overexpression of c-Myc (Rat1a/c-*myc*) or v-Src (Rat1a/v-src), which enables them to grow anchorage independently as colonies in soft agar. Transformation by v-Src may be partially dependent on c-Myc [29]; however, v-Src has also been shown to signal independently of c-Myc [30]. Therefore, incomplete inhibition of c-Myc can be

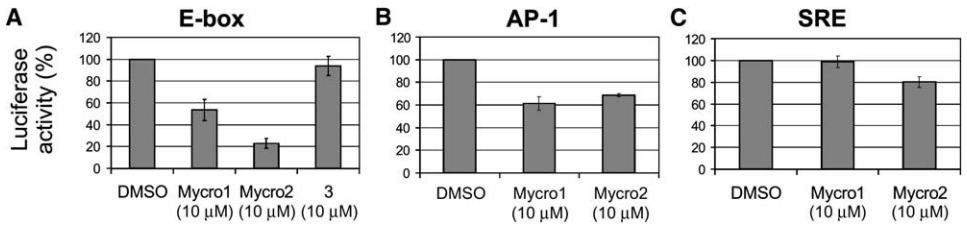

Figure 4. Mycro1 and Mycro2 Selectively Inhibit Transcription in a c-Myc-Dependent Reporter Gene Assay

(A) Mycro1 and Mycro2 inhibit c-Myc-dependent luciferase gene activation.

(B) Effect of Mycro1 and Mycro2 on transcriptional activity of the AP-1 transcription factors induced by addition of 12-O-tetradecanoylphorbol 13-acetate.

(C) Effect of Mycro1 and Mycro2 on serum response factor-dependent luciferase gene activation.

Error bars represent standard error of the mean (SEM).

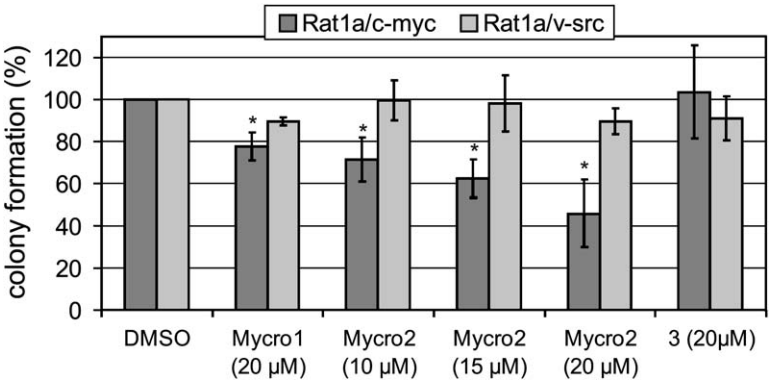

Figure 5. Mycro1 and Mycro2 Selectively Reduce Anchorage-Independent Growth of c-Myc-Transformed Rat1a Cells

Rat1a cells transformed with c-Myc (Rat1/c-*myc*) or v-Src (Rat1a/v-src) were seeded in soft agar and treated with the indicated concentration of test compounds. Colonies larger than 0.13 mm in diameter were counted. *$p < 0.015$ as compared to DMSO. Error bars represent standard deviation (SD).

expected to have only a minor effect on anchorage-independent growth of Rat1a/v-src cells, as has previously been observed with other c-Myc inhibitors [16, 17]. Neither Rat1a cells overexpressing c-Jun nor the c-Myc target HMG-I/Y [31] formed a significant number of colonies in soft agar in the time frame of the assay, which prohibited their use as specificity controls. Twenty micromolar Mycro1, and especially 20 μM Mycro2, inhibited anchorage-independent growth of Rat1a/c-*myc* cells, as observed by the significantly reduced number of large soft agar colonies (diameter > 0.13 mm) (Figure 5 and see Figure S3). In contrast, only a minor reduction of the number of large soft agar colonies formed by Rat1a/v-src cells was observed. This indicates that Mycros inhibited c-Myc functions only partially at concentrations up to 20 μM, as was observed in vitro (Figure 2), and thereby selectively interfere with the ability of Rat1a/c-*myc* cells to proliferate anchorage independently. Derivative **3** had no effect on c-Myc-dependent growth in soft agar, consistent with its lack of activity in vitro and on gene transcription.

The selective effect of Mycros on c-Myc activity both in vitro and in cellular systems could make them useful research tools for the analysis of hitherto unresolved biological questions. Inhibition of c-Myc in tumor cell lines by Mycros can be expected to downregulate expression levels of c-Myc-regulated genes and could therefore be used to complement the existing approaches toward the identification of c-Myc target genes [1, 11]. Selective inhibition of c-Myc by a cell-permeable agent could also used to achieve a better understanding of the conditions under which c-Myc promotes either proliferation or apoptosis [32]. In a related application, Mycros could be used to study the conditions under which tumors induced by tissue-specific overexpression of c-Myc in animal models [33] or by injection of c-Myc-transformed cells in immunosuppressed mice can be reverted by a pharmacological agent. Considering that Mycro1 and Mycro2 completely fulfill Lipinski's "rule of five" [24] and that they display selective activity against c-Myc in the low micromolar concentration range, it is well conceivable that Mycros could serve as starting points for drug discovery programs.

Significance

Transcription factors of the bZip and bHLHLZip protein families need to dimerize and bind to DNA in order to exert most of their diverse biological functions. Their dimerization occurs via α-helical domains, which generally do not contain obvious binding sites for small molecules. One member of the bHLHZip protein family, the oncoprotein c-Myc, is overexpressed in many human tumors and is considered to be a potential therapeutic target for the treatment of certain human tumors. Since all biological activities of c-Myc known to date require binding to its activation partner Max, c-Myc can be inactivated by agents that block the protein-protein interactions between c-Myc and Max. We have identified two low-molecular weight compounds, dubbed Mycro1 and Mycro2, which inhibit c-Myc/Max dimerization in the low micromolar concentration range. Mycro1 and Mycro2 maintain their inhibitory activities in the presence of the c-Myc/Max DNA binding motif and are the first inhibitors of Myc/Max dimerization that were demonstrated to inhibit DNA binding of c-Myc/Max in vitro with preference for c-Myc/Max over related dimeric transcription factors. Both Mycro1 and Mycro2 exert c-Myc-dependent effects in cellular assays. This selectivity represents a milestone in the development of agents that specifically target the large protein-protein interfaces between transcription factor subunits and should make Mycros useful research tools to address unresolved questions of c-Myc biology. The data presented here demonstrate that the dimerization of transcription factors can be potently and specifically inhibited, even in the absence of obvious small-molecule binding pockets, by drug-like molecules in the low micromolar concentration range.

Experimental Procedures

Fluorescence Polarization Assays and High-Throughput Screening

The sequence of the oligonucleotides used was as follows. c-Myc/Max and Max/Max: 5′-fluorophore-CACGTGGTCTGGG-3′ and 5′-CCCAGACCACGTG-3′; 5-carboxyfluorescein was used as fluorophore during screening, and Texas red was used as fluorophore during validation and characterization. Jun/Jun: 5′-Texas red-ATGACTCATATCGGTCC-3′ and 5′-GGACCGATATGAGTCAT-3′; C/EBPα-C/EBPα: 5′-Texas red-TTGCGCAATATCGGTC-3′ and 5′-GACCGATATTGCGCAA-3′. Oligodeoxynucleotides were used at a final concentration of 3 nM. Fluorescence polarization assays were performed at final buffer concentrations of 60 mM Tris/HCl (pH 7.5), 150 mM NaCl, 9 mM $MgCl_2$, 0.6 mM dithiothreitol, 3 mM EDTA, 0.1% Nonidet P-40, and 10% DMSO. Proteins only contained the respective DNA binding and dimerization domains and were used at a concentration close to their K_d-values (final concentrations: 5 nM Myc/Max, 30 nM Max/Max, 30 nM Jun/Jun, 30 nM C/EBPα/C/EBPα). Proteins were incubated with test compounds in Eppendorf

tubes at room temperature for 1 hr prior addition of the respective labeled oligodeoxynucleotides. For the determination of Z′ [23], 5 nM Myc/Max dimer was incubated for 1 hr with one equivalent of 9E10 antibody, which inhibits Myc/Max dimerization by binding to the leucine zipper of c-Myc, or the corresponding buffer without antibody. Three independent experiments, in which the fluorescence polarization of both the protein bound fluorescein-labeled oligodeoxynucleotide (binding experiments without antibody) and the free fluorescein-labeled oligodeoxynucleotide (binding reaction containing 9E10) were analyzed in 48 duplicate wells, were performed. 8,298 compounds from Chemical Diversity and 9,000 compounds from Maybridge were tested for their ability to interfere with c-Myc/Max dimerization and DNA binding. Binding curves and inhibition curves were fitted with SigmaPlot (SPSS Science Software GmbH). All competition curves were repeated three times in independent experiments.

Chemical Compounds

Mycro1 (N-(4-methoxybenzyl)-5-(thiophen-2-yl)-7-(trifluoromethyl)-pyrazolo[1,5-a]pyrimidine-2-carboxamide) and Mycro2 ((5-(thiophen-2-yl)-N-(thiophen-2-ylmethyl)-7-(trifluoromethyl)pyrazolo[1,5-a]pyrimidine-2-carboxamide) were purchased from ChemDiv (Mycro1, compound code 1762-0456; Mycro2, compound code 1762-0445). Compound 3 (5-(thiophen-2-yl)-N-(thiophen-2-ylmethyl)-7-(trifluoromethyl)pyrazolo[1,5-a]pyrimidine-3-carboxamide) was purchased from Asinex (compound code BAS 00923999).

Electrophoretic mobility shift assays (EMSA) were performed essentially as described [16]. MycCFP (333 nM) and Max (333 nM) were mixed in dimerization buffer (200 mM HEPES [pH 7.5], 500 mM KCl, 30 mM $MgCl_2$, 2 mM DTT, 10 mM EDTA). To 18 μl of protein mixture in an Eppendorf tube, 2 μl of 10× stock solutions of the test compounds were added and incubated for 1 hr at room temperature (final DMSO concentration: 10%). Subsequently, 10 μl of binding buffer (33 mM HEPES [pH 7.5], 83 mM KCl, 5 mM $MgCl_2$, 0.3 mM DTT, 1.6 mM EDTA, 15 ng/μl poly dI/dC [Amersham], and 200,000 cpm of a freshly labeled, double-stranded DNA oligonucleotide [5′-AGTTGACCACGTGGTCTGGG-3′]) were added. The sequence of the mutated double-stranded oligonucleotide was 5′-AGTTGAC TACGTAGTCTGGG-3′. Similarly, MBP-tagged v-Jun and v-Fos (333 nM each) were incubated under otherwise identical conditions with a double-stranded oligonucleotide comprising the TRE motif (5′-AGTCAGAATGACTCATATCGGTC-3′). The sequence of the mutated TRE oligonucleotide was 5′-AGTCAGAAAGACTCTTATCG GTC-3′. Twenty minutes after addition of the binding buffer, protein-DNA complexes were resolved on a 4% acrylamide gel (45 mM Tris-borate, 1 mM EDTA), and gels were dried before autoradiography. The intensities of the protein-DNA complexes were compared to the intensities obtained from a standard curve of protein concentrations with SigmaPlot (SPSS Science Software GmbH).

GST Pull-Down Assays

For GST pull-down assays, 2 μg of recombinant, affinity-purified GST-Max or GST alone were incubated with 30 μl of washed glutathione-agarose beads in a total volume of 500 μl GST binding buffer (5% glycerine, 5 mM Tris/HCl [pH 7.5], 50 mM NaCl, 0.5 mM DTT, 0.5 mM EDTA, 2.5% DMSO, 1% BSA) overnight at 4°C with gentle rotation. After washing the beads, recombinant, affinity-purified Myc-CFP and 25 μl of a 20× stock of test compound or DMSO were added in a total volume of 500 μl GST binding buffer. Samples were again incubated overnight at 4°C and washed. Beads were boiled for 10 min in 40 μl of 3× Lämmli buffer, and samples were resolved by SDS-PAGE. Proteins were transferred to nitrocellulose and probed with an anti-GFP antibody, followed by incubation with horseradish peroxidase (HRP)-coupled secondary antibody.

Proliferation Assays

Growth curves were performed in 24-well tissue culture plates. Cells were seeded at the following densities: NIH/3T3 cells, 2,000 cells/well; U-2OS cells, 10,000 cells/well; MCF-7 cells, 10,000 cells/well; Raji cells, 20,000 cells/well; PC-12 cells, 30,000 cells/well. Eight hours later, each compound or DMSO (final concentration, 0.2%) was added. Media containing the test compounds were replaced on day three. Cells were trypsinized and manually counted at the indicated times.

BrdU Incorporation Assay

2,500 NIH/3T3 cells were seeded in 96-well microtiterplates. Eight to 10 hr later, the culture media was replaced with media containing 0.1% FCS and 10 μM of test compound or DMSO (final DMSO concentration, 0.3%). After 24 hr, cells were stimulated for 16 hr by media containing 10% FCS in the presence of the test compounds. Subsequently, cells were labeled with 100 μg/ml BrdU in media containing 10% FCS for 4 hr. BrdU incorporation was monitored with the 5-Bromo-2′-deoxy-uridine-Labeling and Detection Kit III (Roche Applied Science) according to the manufacturer's instructions.

Luciferase Reporter Assays

108,000 HEK293 T cells were seeded in 12-well microtiter plates and transfected with 500 ng reporter vector together with 375 ng full-length c-Myc under the control of a CMV promoter and 12.8 ng of pRL-CMV vector (Promega) with the calcium phosphate precipitation technique. The reporter vectors carried either four E-box elements cloned into the pGL3 promoter vector (pGL3-E-Box) or four mutated E boxes (pGL3-E-boxMut). To monitor AP-1-dependent gene transcription, HEK293 T cells were transfected with either 10 ng of pAP-1-Luc (BD Biosciences, Clontech) or 10 ng of pAP1-Mut-Luc, containing four mutated copies of the AP1 enhancer and 6.4 ng of pRL-CMV. Five hours after transfection, the media was replaced with fresh media containing the test compounds. For AP-1 luciferase assays, the fresh media contained an additional 10 ng/ml of TPA (12-O-tetradecanoyl phorbol-13-acetate). For the SRF-dependent luciferase assay, HEK293 T cells were cotransfected with 40 ng of the reporter vector 3D.ALuc [34] containing three SRF binding sites in the pGL3 framework, 50 ng of pEF.SRF.VP16 containing SRF codons 1–412 fused to VP16 codons 410–490, or empty vector, and 100 ng of pRL-TK vector (Promega). Luciferase assays were performed with a Dual-Luciferase Reporter System (Promega) according to the manufacturer's instructions. Luciferase activities were assessed 24 hr later.

Soft Agar Colony Assay

4,000 Rat1a/c-*myc*, Rat1a/v-src or Rat1a/pLXSN cells were suspended in 200 μl of 0.2% agar containing MEM supplemented with 10% fetal bovine serum and 100 μg/ml penicillin/streptomycin, the test compounds, or DMSO (final DMSO concentration, 1%) and seeded on top of a 300 μl layer consisting of the same media containing 0.7% agar in 24-well plates. Cells were fed every 3–4 days by adding 100 μl 0.2% agar in the same medium containing the appropriate compound concentration. Cells were stained after 10–14 days with 1 mg/ml iodonitrotetrazoliumchloride. Plates were scanned, and colonies found to be larger than 0.13 mm by comparison with a circular mask (Corel PhotoPaint) were counted.

Supplemental Data

Supplemental Data including the construction of the plasmids and three experimental figures are available at http://www.chembiol.com/cgi/content/full/13/7/745/DC1/.

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