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Selective Inhibition of c-Myc/Max Dimerization by a Pyrazolo[1,5-*a*]-pyrimidine

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The c-Myc proto-oncogene is involved in many human tumors, and needs to bind to its activation partner Max for all of its known biological activities.[1–4] Inhibition of the protein–protein interactions between c-Myc and Max by cell-permeable molecules is therefore an attractive goal.[5–8] Dimerization between c-Myc and Max occurs via α-helical domains comprising leucine zipper motifs, which display no obvious binding sites for inhibitory ligands.[9] We recently identified two pyrazolo[1,5-*a*]pyrimidines which inhibit c-Myc/Max dimerization from a diverse collection of chemicals (Figure 1a).[10] These compounds, dubbed Mycro1 and Mycro2, inhibited c-Myc/Max dimerization and DNA binding with preference over other structurally related transcription factors in vitro, and exhibited c-Myc dependent effects in cellular assays. To explore the chemical space around the pyrazolo[1,5-*a*]pyrimidine core structure for substitution patterns which are associated with activity against c-Myc/Max dimerization, and to possibly identify a Myc/Max dimerization inhibitor with improved properties, we screened a 1438-membered pyrazolo[1,5-*a*]pyrimidine library based on the structures of Mycro1 and Mycro2 (Figure 1b and Figure S1 in the Supporting Information).

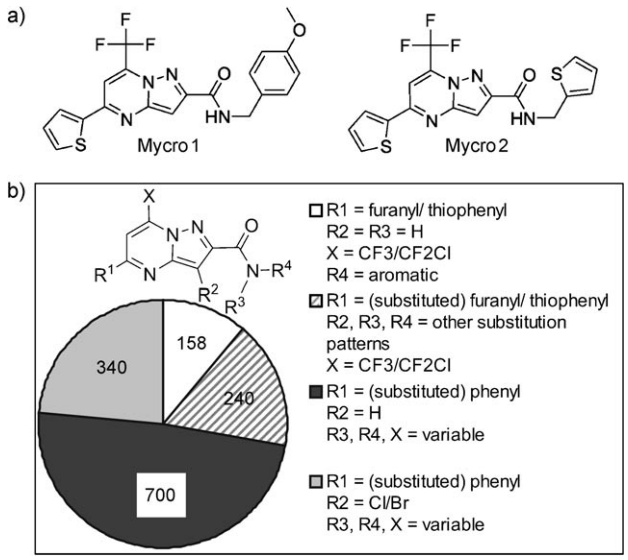

Figure 1. a) Structures of the c-Myc/Max dimerization inhibitors Mycro1 and Mycro2.[10] b) Structural diversity elements of the pyrazolopyrimidine library, and classification of substitution patterns present in the library. All compounds carrying furane or thiophene as R^1 contain CF_3 or CF_2Cl as substituent X.

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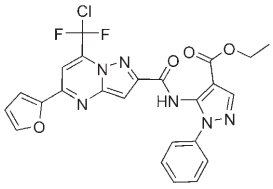
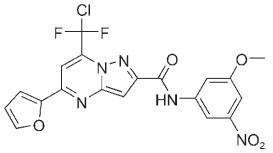
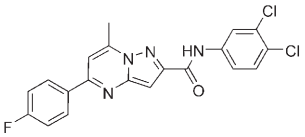
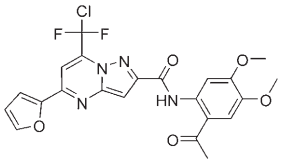
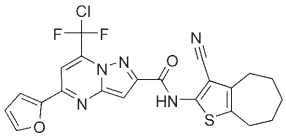
As c-Myc can bind DNA only as dimer with Max, we tested the compounds for their abilities to inhibit DNA binding of c-Myc in a fluorescence polarization assay.[10] Five test compounds (**1**–**5**) inhibited DNA binding of c-Myc/Max with preference over Max/Max DNA binding by more than 50% at a concentration of 100 μM (Table 1). DNA binding of Max/Max dimers is the most stringent specificity control possible, as c-Myc and Max are 59% similar at the protein level in the dimerization domains, and the overall structure of the DNA-bound dimers are very similar to each other.[9,11] As an additional specificity control, we analyzed the effect of compounds **1**–**5** on the function of the Src-homology 2 (SH2) domain of the structurally unrelated transcription factor STAT3. None of the compounds affected the interactions between STAT3 and a phosphotyrosine-containing peptide comprising the STAT3 binding motif to a major extent (Table 1).[12]

Confirmation of compound activities in well-controlled cellular systems would provide a strong argument against the notion that any in vitro data could be influenced by factors which are irrelevant under cellular conditions. In addition, cellular assays allow the analysis of compound specificities against all relevant proteins; therefore, their scope is incomparably larger than any in vitro analysis. We chose a cell proliferation assay to further analyze the effects of inhibitor candidates **1**–**5**. Cell cycle progression and proliferation of almost all cell types, including U-2OS osteosarcoma cells, requires c-Myc function, and hence c-Myc's ability to bind to its activation partner Max.[3,13] However, for reasons not yet fully understood, PC-12 pheochromocytoma cells proliferate independent of c-Myc/Max dimerization, as they express a truncated Max protein which is unable to interact with c-Myc.[14] Therefore, a selective inhibitor of c-Myc/Max dimerization can be expected to inhibit proliferation of the c-Myc/Max-dependent U-2OS cells, without inhibiting the growth of the c-Myc/Max-independent PC-12 cells, provided it is cell permeable and stable in the cellular environment. In contrast, compounds which act unspecifically in the cellular context will either cause a reduction in the proliferative rate of both U-2OS and PC-12 cells, or display toxic effects. Whereas compound **5** appeared to be toxic in both cell lines, compound **4** inhibited proliferation of the cell lines only to a minor extent at 20 μM (Figure S2 in the Supporting Information), possibly for reasons related to cellular uptake or intracellular stability. In contrast, compounds **2** and **3** inhibited proliferation of U-2OS cells with good selectivity over proliferation of PC-12 cells at 10 μM (Figure S2 in the Supporting Information), even though compound **3** was toxic at 20 μM. The best selectivity was observed with compound **1**, which strongly inhibited proliferation of U-2OS cells (70% reduction of cell number after 5 days, Figure 2a), but had no significant effect on the proliferation of PC-12 cells (Figure 2b). These data sug-

Table 1. Structures and activities of compounds **1**–**5**.

Compd	Structure	c-Myc/Max[a] $IC_{50} \pm$ S.D. [μM]	Max/Max[a] $IC_{50} \pm$ S.D. [μM]	STAT3[b] % inhibition at 100 μM	Proliferation U-2OS[c]	Proliferation PC-12[c]
1		40 ± 13	88 ± 8	19 ± 3% inhibition at 100 μM	10 μM: ++ 20 μM: +++	10 μM: - 20 μM: -
2		64 ± 1	94 ± 2	22 ± 1% inhibition at 100 μM	10 μM: ++ 20 μM: +++	10 μM: + 20 μM: +
3		52 ± 8	90 ± 11	0 ± 3% inhibition at 100 μM	10 μM: +++ 20 μM: toxic	10 μM: + 20 μM: toxic
4		29 ± 16	76 ± 14	17 ± 3% inhibition at 100 μM	10 μM: - 20 μM: +	10 μM: - 20 μM: +
5		35 ± 7	24 ± 9% inhibition at 100 μM	8 ± 8% inhibition at 100 μM	10 μM: toxic	10 μM: toxic

[a] Concentrations of compounds at which 50% inhibition of dimerization and DNA binding was observed in fluorescence polarization assays (IC_{50}). [b] % inhibition of binding of a phosphopeptide to STAT3 at 100 μM compound in a fluorescence polarization assay.[12] [c] (+++) strong inhibition; (++) medium inhibition; (+) weak inhibition; (−) no inhibition. Standard deviations (S.D.) were obtained from 3 independent experiments. n.a.: not analyzed.

gest that pyrazolo[1,5-*a*]pyrimidines can have cellular targets other than c-Myc/Max[15,16], and stress the importance of analyzing activities of bioactive compounds identified in vitro also under cellular conditions.

Based on the results of the proliferation assay, we analyzed the activity of compound **1** in more detail. Firstly, we verified the inhibitory effect on the formation of the c-Myc/Max/DNA complex as observed in the fluorescence polarization assay (Table 1) in an electrophoretic mobility shift assay (EMSA). To this end, various concentrations of compound **1** were incubated with c-Myc and Max. After addition of ^{32}P-labeled, double-stranded DNA comprising the c-Myc/Max binding site, the mixture was analyzed by gel electrophoresis using a nondenaturing polyacrylamide gel. Compound **1** inhibited the formation of the c-Myc/Max/DNA complex in a dose-dependent manner (Figure 2c). In contrast, formation of the complex between the related bZip proteins v-Jun and v-Fos and their DNA-binding motif was not significantly inhibited (Figure 2d). To analyze whether compound **1** inhibited the formation of the c-Myc/Max/DNA complex by inhibiting the protein–protein interactions between c-Myc and Max, or by blocking the interaction between c-Myc/Max dimers and DNA, we tested it in a dimerization assay which does not involve DNA. Max was expressed as a fusion protein with GST, and the GST-Max protein was immobilized on glutathione-agarose beads. Subsequently, a fusion protein of c-Myc and cyan fluorescent protein (CFP) was added in the presence of increasing concentrations of compound **1**, and the amount of MycCFP bound to the beads by interacting with Max was determined by Western blot analysis against CFP. Compound **1** reduced the interaction between c-Myc and Max at concentrations similar to the effective concentrations in the c-Myc/Max/DNA binding assay (compare Figure 2c and 2e), suggesting that the compound interferes with the formation of the c-Myc/Max/DNA complex by inhibiting protein–protein interactions between c-Myc and Max.

c-Myc exerts its effects by regulating the transcription of up to 15% of the genes of an organism,[17] and a c-Myc/Max dimerization inhibitor can be expected to specifically interfere with c-Myc-dependent transcription. Indeed, compound **1** inhibited c-Myc dependent transcription in a dose-dependent manner at 10 μM and 20 μM (Figure 2f). In contrast, it did not inhibit transcription dependent on the leucine zipper-mediated dimerization and DNA binding of the structurally related AP-1 family transcription factors (Figure 2g), a significant proportion of which are composed of Jun/Fos dimers in mammalian cells.[18] Thus, **1** displayed a superior cellular specificity profile as compared to the c-Myc inhibitors Mycro1 and Mycro2, both of which also had an effect on AP-1-dependent transcription.[10] A further indication for the specific cellular activity of compound **1** is provided by the results of a soft agar colony assay. Rat fibroblast cells (Rat1a) transformed by overexpression of an oncoprotein such as c-Myc (Rat1a/c-Myc cells) gain the ability to grow as colonies in soft agar. In the presence of compound **1**, a dose-dependent reduction of the number of large agar colonies was observed (Figure 2h). In contrast, anchorage-independent growth of v-

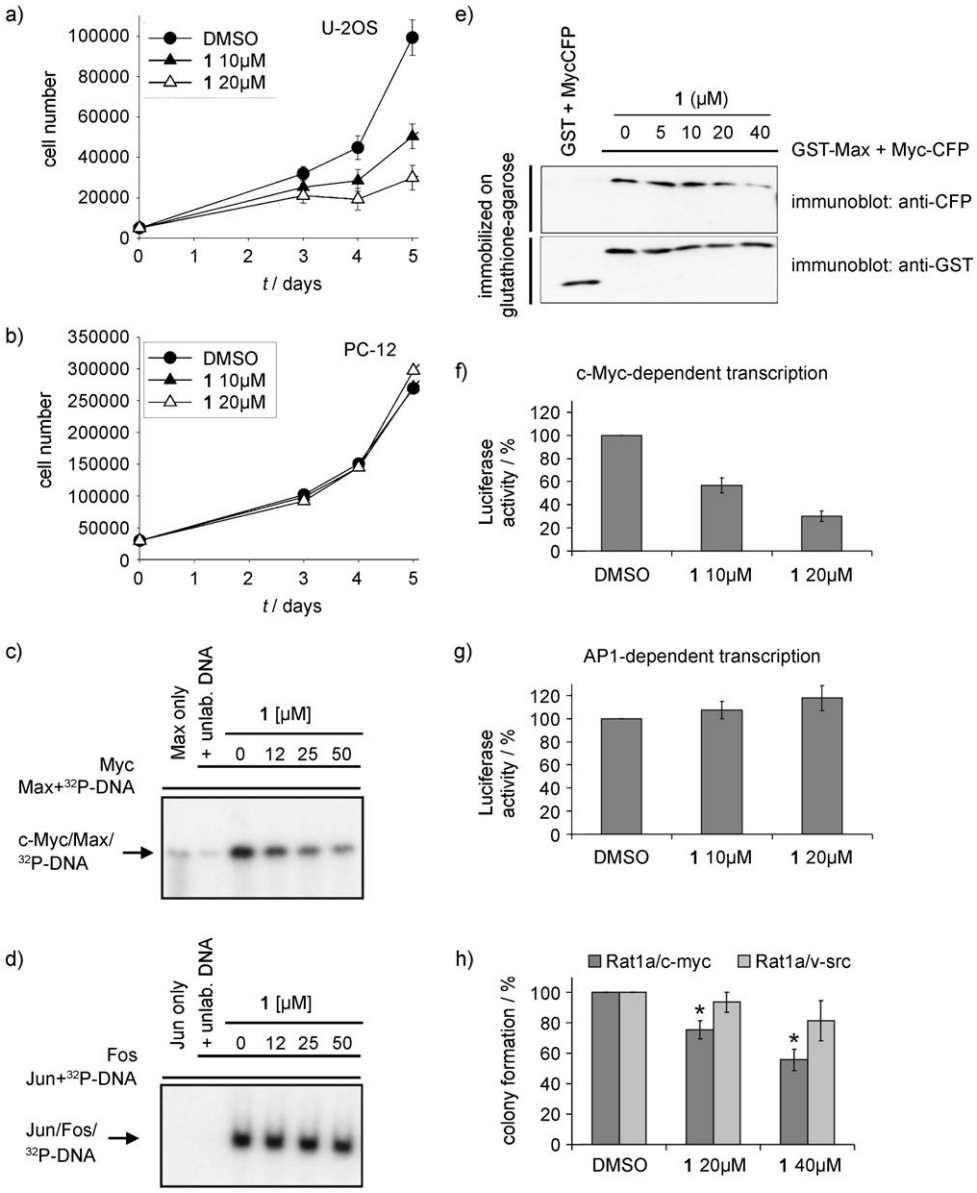


Figure 2. Biological characterization of compound **1**. a–b) **1** inhibits c-Myc-dependent proliferation of U-2OS cells, but not of the c-Myc-independent cell line PC-12. c) DNA binding of c-Myc/Max is inhibited by **1** as analyzed by electrophoretic mobility shift assay (EMSA). All lanes contain ^{32}P-labeled DNA comprising the c-Myc/Max binding site, c-Myc, and Max, except for the first lane, which lacks c-Myc and thereby confirms that it is mostly c-Myc/Max heterodimers which are bound to DNA. c-Myc cannot bind DNA on its own. The second lane contains additional, unlabeled competitor DNA in 50-fold excess to verify the identity of the band. d) DNA binding of Jun/Fos is not significantly inhibited by **1** in EMSA. All lanes contain ^{32}P-labeled DNA comprising the Jun/Fos binding site, Jun, and Fos, except for the first lane, which lacks Fos and thereby excludes the possibility that Jun/Jun homodimers are bound to DNA. Fos cannot bind DNA on its own. The second lane contains additional unlabeled competitor DNA in 50-fold excess to verify the identity of the band. e) **1** inhibits binding between immobilized GST-Max fusion protein and a CFP-tagged Myc-protein in a GST-pulldown assay. All lanes contain GST-Max and Myc-CFP, except for the first lane, in which GST (without Max) and MycCFP were incubated. The absence of a signal for this lane in the CFP blot indicates specific binding between Myc and Max in the other lanes. f–g) **1** selectively inhibits c-Myc-dependent, but not AP-1-dependent, luciferase gene transcription in HEK 293T cells. h) Compound **1** reduces the number of large colonies ($>$ 0.13 mm) of Rat1a/c-myc cells, but only slightly interferes with colony formation of Rat1a/v-src cells. * $p < 0.01$ as compared to DMSO.

Src transformed rat fibroblasts (Rat1a/v-src), which are less c-Myc-dependent,[5,7,10] was reduced only to a lesser extent. Thus, the cellular activity profile of **1** is consistent with the idea that it selectively blocks the interaction between c-Myc and Max. Future efforts will focus on the elucidation of its binding mode.

Structural comparison of Mycro1, Mycro2, and the newly identified compound **1** suggest that the following substitution pattern in pyrazolo[1,5-*a*]pyrimidines appears to be associated with increased likelihood for this class of compounds to be selective, cell-permeable inhibitors of c-Myc: 1) a thiophene or furane in position R^1, 2) a hydrogen as both R^2 and R^3, and 3) an aromatic residue as R^4. This combination of structural features was found in only 11 % of the library members (158/1438) (Figure 1 b, and see Figure S1 in the Supporting Information for an overview on substituents R^1-R^3 and X contained in the screening library). As all of our test compounds which contained a thiophene or furane residue in position R^1 also carried a CF_3 or CF_2Cl group as substituent X, we cannot make a statement on the contribution of CF_3 or CF_2Cl as substituent X towards biological activity. Targeted libraries comprised of properly substituted pyrazolopyrimidines might yield an increased hit rate over that observed in the screen described in this manuscript, and could lead to the identification of more potent inhibitors (see Scheme S1 in the Supporting Information for a synthetic route to pyrazolo[1,5-*a*]pyrimidines). We suggest the newly identified compound **1** as potential tool for the analysis of c-Myc functions in cellular systems and in vivo, as it displayed the best cellular specificity profile amongst the pyrazolopyrimidine-based c-Myc/Max inhibitors identified to date. Our data provide an indication for chemical moieties linked to selective activity of pyrazolo[1,5-*a*]pyrimidines against c-Myc/Max dimerization, and support the idea that transcription factors can be selectively targeted with cell-permeable molecules[19] even in the absence of obvious cavities for small-molecule inhibitors.[20,21]

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