A selective high affinity MYC-binding compound inhibits MYC:MAX interaction and MYC-dependent tumor cell proliferation

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MYC is a key player in tumor development, but unfortunately no specific MYC-targeting drugs are clinically available. MYC is strictly dependent on heterodimerization with MAX for transcription activation. Aiming at targeting this interaction, we identified MYCMI-6 in a cell-based protein interaction screen for small inhibitory molecules. MYCMI-6 exhibits strong selective inhibition of MYC:MAX interaction in cells and in vitro at single-digit micromolar concentrations, as validated by split Gaussia luciferase, in situ proximity ligation, microscale thermophoresis and surface plasmon resonance (SPR) assays. Further, MYCMI-6 blocks MYC-driven transcription and binds selectively to the MYC bHLHZip domain with a $K_D$ of 1.6 ± 0.5 μM as demonstrated by SPR. MYCMI-6 inhibits tumor cell growth in a MYC-dependent manner with IC50 concentrations as low as 0.5 μM, while sparing normal cells. The response to MYCMI-6 correlates with MYC expression based on data from 60 human tumor cell lines and is abrogated by MYC depletion. Further, it inhibits MYC:MAX interaction, reduces proliferation and induces massive apoptosis in tumor tissue from a MYC-driven xenograft tumor model without severe side effects. Since MYCMI-6 does not affect MYC expression, it is a unique molecular tool to specifically target MYC:MAX pharmacologically and it has good potential for drug development.

The MYC family of oncogenes (MYC, MYCN and MYCL, here collectively referred to as “MYC”), encodes basic helix-loop-helix leucine zipper (bHLHZip) transcription factors. Through the HLHZip domain, MYC heterodimerizes with the bHLHZip protein MAX, which enables the MYC:MAX complex to bind E-box regulatory DNA elements throughout the genome, thereby controlling transcription of a large group of specific genes1–6. The direct target gene products in turn influence global RNA and protein synthesis, thereby coordinating multiple fundamental cellular processes, including cell cycle progression, cell growth, apoptosis, senescence, metabolism and stem cell functions2,7–11. Deregulation of expression of MYC family genes/proteins occurs in over half of all human tumors, and is often correlated with aggressive disease, resistance to therapy and poor prognosis3,4,12,13. MYC is therefore considered as one of the most important drivers of tumor development and has been highlighted as a key therapeutic target for cancer therapy for a number of tumor types. However, so far there are no specific anti-MYC drugs available in the clinic. A number of attempts have been made to target MYC indirectly by interfering with transcription of the MYC gene, translation or turnover of the MYC protein or by inhibiting downstream effectors of MYC14–16. Due to the diversity of signals regulating the MYC genes/proteins and the pleiotropic functions of MYC, tumor cells have multiple ways of escaping these pathways to maintain MYC-family expression and activity. The most reliable strategy is therefore probably to target the MYC proteins directly. Since MYC is strictly dependent on MAX for binding E-boxes, targeting MYC:MAX interaction is a conceivable approach to target MYC. Several examples of successful targeting of protein–protein interactions (PPIs) with small molecules, including Nutlin-3a (targeting p53:MDM2)17, BET inhibitors such as JQ118,

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Figure 1. Identification and validation of small molecules targeting the MYC:MAX protein interaction using a cell-based Bimolecular Fluorescence Complementation assay (BiFC). (A) Schematic representation of the principle of the BiFC assay. MYC and MAX were fused to two inactive fragments of YFP, generating MYC-YFP-C and MAX-YFP-N, respectively. Upon MYC:MAX heterodimerization, the two YFP fragments refold into a functional YFP protein. (B) Nuclear expression of MYC-eGFP (left panel) and MYC-YFP-C and MAX-YFP-N BiFC (right panel). (C) HEK293 cells cotransfected with the wt MYC-YFP-C or the MYCΔbHLHZip-YFP-C mutant (lacking the MAX-interacting bHLHZip region of MYC) and MAX-YFP-N BiFC constructs together with CMV-CFP used as internal reference. (D) Images of cells treated with compound MYCMI-6 (lower panel) or vehicle (upper panel). CFP channel (left panel) and YFP (BiFC) channel (right panel). (E) HEK293 cells cotransfected with MYC-YFP-C and MAX-YFP-N together with CMV-CFP and treated with NCI/DTP Diversity set library (25 μM of each compound) or vehicle (DMSO) for 24 hours in 96-well plates. The ratio of YFP/CFP was calculated relative to DMSO-treated cells. With a cut off of 40% inhibition, six hit compounds (MYCMIs) were identified (F) Effect of MYCMIs on MYC:MAX, MYCN:MAX and GCN4:GCN4 interactions using the Gaussia luciferase fragment complementation (GLuc) assay. The GLuc fusion protein constructs...
were transfected into the cells together with the CMV-Luc plasmid and treated with the indicated compounds for 17 hours and analyzed in a dual luciferase assay. The ratio of Gaussia/Firefly luciferase luminescence were calculated and normalized to DMSO-treated cells. Data are shown as mean ± standard deviation of 3–7 biological experiments with each with 3–6 technical repeats. Significant p-values are indicated. (G) Western blot analysis of endogenous MYC expression in HeLa cells after 24 hours of treatment with the indicated MYCMIs (10 μM), the experimental MYC:MAX inhibitor 10058-F4 (64 μM) and the BET-inhibitor JQ1 (5 μM). Actin was used as loading control. Full length blots are presented in Suppl. Fig. S9A.

(bromodomains:histones) and the BH3 mimetic compound Navitoclax/ABT-263 (BCL-2 family interactions) have been reported recently. These compounds, or improved versions thereof, are now in clinical trials, which have encouraged further research on PPIs as drug targets. Several groups have attempted to find compounds targeting the MYC:MAX interaction by screening small-molecule libraries using FRET, fluorescence polarization, or yeast-two-hybrid (Y2H). As a result, a number of small molecules have been reported to target the MYC:MAX or MYC:MAX:DNA interaction. However, none of these compounds have made their way for clinical studies due to a number of limitations including low potency in vitro or in cells, poor specificity or inadequate bioavailability in vivo. Development of more potent and selective MYC:MAX inhibitors is therefore much warranted. The aim of this work was to identify bioactive molecules that (1) efficiently and selectively inhibit the MYC:MAX interaction in vitro and in cells, that (2) bind MYC with high affinity, that (3) inhibit MYC-dependent tumor cell growth with high efficacy, that (4) do not affect MYC expression, and that (5) are active in vivo. In a cellular MYC:MAX PPI inhibitor screen, we identified the compound MYCMI-6 that fulfills all these criteria.

Results
Establishment of a cell-based Bimolecular Fluorescence Complementation (BiFC) assay for screening for small molecules interfering with MYC:MAX protein interactions. To screen for inhibitors of the MYC:MAX protein interaction, we utilized BiFC to monitor the interaction in living cells. Two constructs encoding complementary BiFC fragments of yellow fluorescent protein (YFP) fused to full length MYC (MYC-YFP-C) and MAX (MAX-YFP-N), respectively, were cotransfected into cells, resulting in a nuclear BiFC signal as a result of MYC:MAX interaction bringing the YFP fragments in close proximity. (Fig. 1B, left panel). To optimize the system for screening purposes, MYC-YFP-C and MAX-YFP-N were transiently coexpressed in HEK293 cells together with a full-length cyan fluorescent protein (CFP) vector as internal control (Fig. 1C, upper panels), after which the ratio of BiFC/CFP fluorescence intensities was calculated. Replacing wt MYC-YFP with a MYC mutant lacking the bHLHZip domain (∆bHLHZip) required for interaction with MAX, did not produce BiFC signals, thus demonstrating that the MYC:MAX BiFC signal is highly specific (Fig. 1C, lower panels). A strictly standardized mean difference (SSMD) of −5 was calculated from cells expressing CFP together with MYC:MAX BiFC and MYCΔbHLHZip:MAX BiFC, respectively, suggesting a strong threshold for hit selection. We concluded that this system was suitable for screening small molecule libraries to identify inhibitors of MYC MAX interaction in cells.

Identification and validation of small molecules selectively targeting the MYC:MAX interaction in cells. We next utilized a diversity set library from the NCI/DTP Open Chemical Repository (http://dtp.cancer.gov) consisting of 1990 compounds to screen for small molecules reducing the MYC:MAX interaction 15,16,22,24–33. However, none of these compounds have made their way for clinical studies due to a number of limitations including low potency in vitro or in cells, poor specificity or inadequate bioavailability in vivo. As a result, a number of small molecules have been reported to target the MYC:MAX or MYC:MAX:DNA interaction. However, none of these compounds have made their way for clinical studies due to a number of limitations including low potency in vitro or in cells, poor specificity or inadequate bioavailability in vivo. Development of more potent and selective MYC:MAX inhibitors is therefore much warranted. The aim of this work was to identify bioactive molecules that (1) efficiently and selectively inhibit the MYC:MAX interaction in vitro and in cells, that (2) bind MYC with high affinity, that (3) inhibit MYC-dependent tumor cell growth with high efficacy, that (4) do not affect MYC expression, and that (5) are active in vivo. In a cellular MYC:MAX PPI inhibitor screen, we identified the compound MYCMI-6 that fulfills all these criteria.

MYCMI-6 selectively inhibits endogenous MYC:MAX interaction and MYC-induced gene expression. To validate the inhibitory effects of the selected MYCMIs on endogenous MYC:MAX interaction...
in cells, \textit{in situ} proximity ligation assay (isPLA) \cite{10} was performed using MYC and MAX antibodies. The interactions were visualized as fluorescent dots mainly localized in the cell nucleus by fluorescence microscopy (Fig. 2B) as previously reported \cite{10}. Treatment of breast cancer cells with the MYCMI-6, MYCMI-11 and MYCMI-14 for 24 hours significantly decreased MYC:MAX isPLA signals to 7%, 23% and 23% of DMSO-treated controls, respectively (Fig. 2B and C). Titration showed an IC$_{50}$ for inhibition of MYC:MAX of less than 1.5 $\mu$M for MYCMI-6 and of approximately 6 $\mu$M for MYCMI-11 and MYCMI-14 by isPLA (Fig. 2D). Further, coimmunoprecipitation

Figure 2. MYCMIs inhibit endogenous MYC:MAX interaction in breast cancer cells and repress MYC-induced target gene expression. (A) Chemical structures of MYCMI-6, MYCMI-11 and MYCMI-14. (B–D) \textit{in situ} Proximity Ligation Assay (isPLA). (B) Endogenous MYC:MAX (upper panel) and FRA1:JUN (lower panel) interactions visualized by isPLA as fluorescent red dots in cell nuclei (blue) after treatment with indicated compounds (10 $\mu$M) or DMSO for 16 hours. isPLA was performed using pairs of MYC and MAX and of FRA1 and JUN antibodies, respectively. As negative control, one primary antibody was used together with the pair of secondary antibodies. The isPLA results are based on three biological experiments for MYC:MAX and two for FRA1:JUN. One representative experiment for each is shown. (C) Quantification of MYC:MAX (left panel) and FRA1:JUN (right panel) isPLA, representing an average number of nuclear dots per cell from three microscopic fields normalized to corresponding values for DMSO-treated cells. $p$-values are indicated. (D) Titration of indicated compounds in MCF7 cells for 24 hours prior to MYC:MAX isPLA assay. Quantification was performed as in (C). (E) Coimmunoprecipitation of endogenous MAX with MYC from MDA-MB231 cells treated with 5 $\mu$M MYCMI-6 or DMSO for 3.5 hours. 1$^{st}$–4$^{th}$ lanes from top; coimmunoprecipitated MAX, immunoprecipitated MYC, total levels of MAX and ACTIN, respectively, as determined by western blot analysis. Note that the gels have been cropped. The uncropped, full length versions are presented in Suppl. Fig. S9B. (F) Inhibition of MYC transactivation of target genes ODC1, RSG16, and CR2 as determined by RT-qPCR analysis, based on three biological experiments with three technical repeats each. U2OS-MYC-ER cells were treated with or without 100 nM 4-hydroxy-tamoxifen (HOT) for 4 hours, after which DMSO or indicated compounds (10 $\mu$M) were added for 24 hours before total RNA was extraction. Fold changes in mRNA expression are presented relative to DMSO in non-HOT-treated cells after normalization to GAPDH, used as reference gene. Significant $p$-values are indicated.
of endogenous MYC:MAX proteins showed that MYCMI-6 reduced the MYC:MAX protein interaction already at 3 hours post treatment (Fig. 2E). In contrast, the three compounds did not significantly affect the interaction between the bZip transcription factors FRA1 and JUN (Fig. 2B and C) or the interaction between MAX and the bHLHZip protein MXD1 (MAD1) – an intracellular competitor of MYC for MAX41,42 (Suppl. Fig. S2B), thus further supporting MYC:MAX selectivity of the compounds.

To investigate the effect of the compounds on MYC-driven transcription, we utilized U2OS cells expressing a MYC-estrogen receptor (MYC-ER) fusion protein, the activity of which is regulated by the ligand 4-hydroxytamoxifen (HOT)43. Treatment with MYCMI-6 significantly reduced HOT-induced expression of three previously described direct MYC target genes, ODC1, RSG16 and CR210,11, while MYCMI-11 and -14 in general reduced expression of the genes to a lesser extent, being significant only for RSG16 (Fig. 2F). Taken together, these results highlight MYCMI-6 as the most potent and selective inhibitor of endogenous MYC:MAX protein interactions and of MYC-driven transcription among the three MYCMIs.

**MYCMI-6 is a potent and selective inhibitor of the MYC:MAX bHLHZip interaction in vitro.** We next investigated the capacity of the small molecule inhibitors to directly target the MYC:MAX interaction in vitro. Two biophysical interaction assays, microscale thermophoresis (MST)44 and surface plasmon resonance (SPR)45, were developed using recombinant MYC and MAX proteins (Suppl. Fig. S1C) (manuscript in prep.). MST is based on the shift of fluorescent molecules moving in a temperature gradient (thermophoresis), which...
can be perturbed by interactions. In the MST MYC:MAX interaction assay, fluorescently labeled MAXbHLHZip was combined with MYCbHLHZip pre-mixed with compound. All three MYCMIs, and in particular MYCMI-6, shifted MAX thermophoresis relative to DMSO, indicating that the compounds affected the MYC:MAX conformation (Fig. 3A). Titration of MYCMI-6 in mixture with MYC and labeled MAX resulted in a thermophoresis shift with a $K_d$ of $4.3 \pm 2.9 \mu M$, while having minor effects on labeled MAX when pre-mixed with unlabeled MAX (Fig. 3B), suggesting that MYCMI-6 discriminates well between MYC:MAX and MAX:MAX interactions.

The SPR method is a highly sensitive assay to determine the affinity between protein and ligand and to measure the kinetics of the interaction. In the SPR MYC:MAX interaction assay, MAXbHLHZip was immobilized by amino coupling on a CM5 sensor chip. MYCMI-6 was injected at various concentrations in a kinetic experiment. The reference surface was subtracted from the analyte surface to generate a sensorgram. Association and dissociation rates ($k_a = 9294 M^{-1} s^{-1}, k_d = 0.02293 s^{-1}$) were determined using the Langmuir 1:1 model in the Biacore Evaluation program fitting curves with a constant $R_{max}$ of 43 RU (theoretical $R_{max}$), thereby suggesting a $K_d$ of 2.5 $\mu M$ with a $\chi^2$ value of 0.073. The sensorgrams display one representative experiment. Four kinetic experiments were carried out on two different sensor chips and an average $K_d$ of $1.6 \pm 0.5 \mu M$ was calculated. Four MYC equilibrium binding experiments with MYCMI-6 summarized in an equilibrium binding plot, carried out on two different sensor chips. Binding affinities were estimated from the plot as 50% of $R_{max}$ suggesting a $K_d$ of approximately 1.5–2 $\mu M$ with an experimental $R_{max}$ of 25–30 RU (theoretical $R_{max}$ = 23 RU). SPR experiments of MYCMI-6 binding to immobilized MXD1 (MAD1) and MAX protein, respectively were included in the graph. Sensorgrams are shown in Suppl. Fig. S5A,C and D. (D) Reference subtracted sensorgram from the p53 protein SPR assay. p53 core protein was immobilized to 3000 RU and MYCMI-6 was injected at various concentrations (theoretical $R_{max}$ of 56 RU).

Figure 4. MYCMI-6 binds selectively and with high affinity to the MYC bHLHZip domain. (A) MST assay measuring the effect of MYCMI-6 on MYC and MAX, respectively. Recombinant MYC bHLHZip and MAX bHLHZip proteins were titrated, respectively, in a fixed concentration (3 M) of MYCMI-6. Changes in fluorescence were measured and normalized to control (buffer). Data are shown as mean ± standard deviation of 6–8 biological repeats. (B) SPR assay to determine the affinity of MYCMI-6 to MYC. MYC bHLHZip protein was immobilized by amino coupling on a CM5 sensor chip. MYCMI-6 was injected at various concentrations in a kinetic experiment. The reference surface was subtracted from the analyte surface to generate a sensorgram. Association and dissociation rates ($k_a = 9294 M^{-1} s^{-1}, k_d = 0.02293 s^{-1}$) were determined using the Langmuir 1:1 model in the Biacore Evaluation program fitting curves with a constant $R_{max}$ of 43 RU (theoretical $R_{max}$), thereby suggesting a $K_d$ of 2.5 $\mu M$ with a $\chi^2$ value of 0.073. The sensorgrams display one representative experiment. Four kinetic experiments were carried out on two different sensor chips and an average $K_d$ of $1.6 \pm 0.5 \mu M$ was calculated. (C) Four MYC equilibrium binding experiments with MYCMI-6 summarized in an equilibrium binding plot, carried out on two different sensor chips. Binding affinities were estimated from the plot as 50% of $R_{max}$ suggesting a $K_d$ of approximately 1.5–2 $\mu M$ with an experimental $R_{max}$ of 25–30 RU (theoretical $R_{max}$ = 23 RU). SPR experiments of MYCMI-6 binding to immobilized MXD1 (MAD1) and MAX protein, respectively were included in the graph. Sensorgrams are shown in Suppl. Fig. S5A,C and D. (D) Reference subtracted sensorgram from the p53 protein SPR assay. p53 core protein was immobilized to 3000 RU and MYCMI-6 was injected at various concentrations (theoretical $R_{max}$ of 56 RU).
MYCMI-6 binds directly and selectively to the MYC bHLHZip domain with high affinity. To determine whether MYCMI-6 binds directly to MYC (or MAX), MST and SPR were applied. The bHLHZip domains of MYC or MAX, respectively (Suppl. Fig. S1C), were titrated and mixed with 3 μM of MYCMI-6. There were clear changes in thermophoresis at 1–15 μM of MYC while no obvious changes were observed for MAX, indicating binding of MYCMI-6 to MYC but not to MAX at these concentrations (Fig. 4A). Further, MYCMI-6 did not bind the control proteins BCL-XL (Suppl. Fig. S4A) and bovine serum albumin (BSA) (Suppl. Fig. S4B). Similar results were obtained in a reverse MST experiment, keeping labeled MYC at a fixed concentration while titrating MYCMI-6, showing relative changes in thermophoresis at concentrations above 100 nM, supporting that MYCMI-6 binds MYC with affinity in the low micromolar range (Suppl. Fig. S4C).

Direct binding of MYCMI-6 to MYC was also analyzed by SPR, where MYC bHLHZip was immobilized on the chip and different concentrations of MYCMI-6 were injected in kinetic experiments. Association and dissociation rates of the compound were obtained from a 1:1 Langmuir model after subtraction of reference cell values, suggesting a Kd value of 1.6 ± 0.5 μM (Fig. 4B). From equilibrium binding experiments (Suppl. Fig. S5A), a Kd value of approximately 1.5–2 μM was determined by an equilibrium binding plot (Fig. 4C). The previously identified MYC:MAX interaction inhibitors 10074-G5 and #474 (an analogue of 10058-F4)38 were used as reference compounds were found to bind to MYC with Kd's of 28 and 15 μM, respectively (data not shown). We could, however, not detect any binding of KJ-Pyr-9 up to 8 μM in this assay (Suppl. Fig. S5B). Further, MYCMI-6 binding to MAX, MXD1 (MAD1) (Figs 4C and 5C and D), p53 (Fig. 4D), BSA (Suppl. Fig. S5E) and YFP (data not shown) were negligible. Binding to BCL-XL was only 10% of the theoretical maximum at 2 μM MYCMI-6 (Suppl. Fig. S5F). In summary, the MST and SPR experiments suggest that MYCMI-6 binds the bHLHZip domain of MYC directly, selectively and with affinity in the low micromolar range.

MYCMI-6 selectively suppresses MYC-driven tumor cell growth with high efficacy. We next addressed whether treatment with MYCMI-6, -11 and -14 inhibits tumor cell growth and if this correlates with the MYC status of the cells. For this purpose, we first utilized a panel of neuroblastoma cell lines with or without MYCN-amplification. Since a subset of MYCN-non-amplified neuroblastomas have been shown to exhibit deregulated expression of (c)-MYC46, we investigated the expression of MYCN and MYC as well as total level of MYC-family proteins in the six cell lines using antibodies specific for MYCN, MYC or pan-MYC. While the three cell lines with MYCN-amplification all expressed high levels of MYCN protein, two out of three of the MYCN-non-amplified cell lines expressed MYC, although the total level of MYC-family proteins were much lower in these cells than in the MYCN-amplified cell lines (Fig. 5A). The cell lines were exposed to MYCMI-6 (6.25 μM), MYCMI-11 or MYCMI-14 (25 μM) or the reference 10058-F4 (64 μM) for 48 hours. All four compounds reduced growth of the MYCMI-6-amplified cell lines significantly stronger than the MYCN-non-amplified cell lines (Fig. 5A). Titration experiments confirmed that MYCMI-6 discriminates between MYCN-amplified and MYCN-non-amplified neuroblastoma cell lines cells, with average growth inhibition of 50% (GI50) values of 2.5–6 μM for MYCN-amplified cell lines and around 20 μM or higher for MYCN-non-amplified cell lines (Fig. 5B). Notably, in the SK-N-F1 cell line, which essentially did not respond at all to MYCMI-6, MYCN or MYC were hardly detectable. These results demonstrate that the response to MYCMI-6 and the other MYCMIs in general correlated well with the total level of MYC-family proteins. Further, the MYCMI-6, MYCMI-11 and MYCMI-14 efficiently inhibited anchorage-independent growth of MYCN-amplified neuroblastoma cells with GI50 values of <0.4, 5 and 0.75 μM, respectively (Fig. 5C and D). These results demonstrate that MYCMI-6 is clearly the more potent and selective of the MYCMIs, and we therefore focused the remainder of this work on MYCMI-6.

To verify that MYCMI-6 inhibited MYCN:MAX interaction and the transcriptional output of MYCN in MYCN-amplified neuroblastoma cells, we performed MYCN:MAX isPLA and measured the expression of a panel of verified MYC family target genes in neuroblastoma47 in response to MYCMI-6. Figure 5E–G shows that MYCMI-6 significantly inhibited MYCN:MAX interaction and the expression of MYCN target genes already at a concentration of 2.5 μM, while maintaining the expression of MYCN and MAX (Suppl. Fig. S6). This is consistent with the results from MYCN:MAX Gluc interaction assays after MYCMI-6 treatment (Fig. 1F).

Moreover, MYCMI-6 significantly inhibited growth of Burkitt's lymphoma (BL) cells - another classical example of a MYC-driven tumor, having translocations of the MYC gene in 80% of cases. As expected, MYCMI-6 significantly inhibited growth of Burkitt's lymphoma (BL) cells - another classical example of a MYC-driven tumor, having translocations of the MYC gene in 80% of cases. As expected, MYCMI-6 significantly inhibited growth of Burkitt's lymphoma (BL) cells - another classical example of a MYC-driven tumor, having translocations of the MYC gene in 80% of cases. As expected, MYCMI-6 significantly inhibited growth of Burkitt's lymphoma (BL) cells - another classical example of a MYC-driven tumor, having translocations of the MYC gene in 80% of cases.
responsive, while the probability of a “lower MYC” cell line to be less responsive was significantly higher than being responsive (Fig. 6B), thus demonstrating a strong correlation between MYC expression and growth inhibitory response to MYCMI-6 in human cancer cell lines.
MYCMI-6 treatment reduces cell growth and survival in a MYC-dependent manner but is not cytotoxic to normal human cells. To further explore the MYC-dependence of MYCMI-6 cell growth inhibitory responses, we utilized immortal H015.19 MYC null rat fibroblasts derived from Tgr1 (parental cells).
and H0Myc3 cells, which has been reconstituted with MYC. MYCMI-6 strongly inhibited growth of wt and reconstituted cells, but did not significantly affect growth of the MYC null cells, thus showing a clear difference in response between MYC expressing and MYC-deficient cells (Fig. 6C), thus further supporting the conclusion that the cellular response after MYCMI-6 treatment is MYC-dependent.

We next addressed the question whether cellular responses to MYCMI-6 treatment differentiate between MYC-dependent tumor cells and normal primary human cells. As shown in Fig. 6D, MYCMI-6 (12.5 μM) dramatically increased cell death and reduced cell number in MYCN-amplified SK-N-DZ neuroblastoma cells after 24 hours treatment but had only marginal effects on the number of viable cells and the percentage of dead cells in normal lung (IMR-90) and foreskin (BJ) human fibroblasts. We concluded that MYCMI-6 was well tolerated by the normal human cells at a concentration that was highly toxic to MYC-dependent tumors cells, showing good discrimination between cancer cells and normal cells in response to MYCMI-6 resulting in a good therapeutic window for this compound.

**MYCMI-6 induces massive apoptosis and reduces tumor cell proliferation, tumor microvascular density and MYC:MAX interaction in a MYC-dependent xenograft tumor model in vivo.** To analyze the effects of MYCMI-6 on tumor physiopathology in vivo, we utilized a mouse xenograft tumor model based on human MYCN-amplified SK-N-DZ neuroblastoma cells. Tumor cells were injected into the flank of athymic nude mice and allowed to form tumors after which MYCMI-6 or vehicle were administered by daily intraperitoneal injection at a dose of 20 mg/kg body weight for 1–2 weeks. TUNEL-staining of tumor sections revealed a dramatic increase in the extension of apoptotic areas in the tumors (Fig. 7A and B) and a significant increase in non-proliferative areas as determined by Ki67 staining (Fig. 7C and D) in tumors from MYCMI-6–treated mice compared with vehicle-treated mice. CD31 staining of endothelial cells revealed a significantly reduced microvascular density (MVD) in MYCMI-6–treated mice compared with vehicle-treated mice (Fig. 7E and F). Further, tumors from MYCMI-6–treated mice displayed signs of necrosis and hemorrhage and exhibited scar tissue to a larger extent than vehicle-treated mice (Suppl. Fig. S8A). Importantly, isPLA analysis showed a significant reduction in MYCN:MAX interaction in tumors from MYCMI-6–compared to vehicle-treated mice (Fig. 7G and H), indicating that MYCMI-6 reaches, and is active against, its target in vivo. MYCMI-6 administration was well tolerated in mice with only slight and temporal effects on body weight (Suppl. Fig. S8B).

In summary, administration of MYCMI-6 to MYC-dependent tumor-bearing mice resulted in the reduction of MYCN:MAX interaction, tumor cell proliferation and MVD and in induction of apoptosis in the tumor tissue without causing severe side effects.

**Discussion**

Targeting the interaction between MYC and its obligatory heterodimerization partner MAX, which is required for specific DNA-binding of the MYC:MAX complex, has been a desired goal ever since the discovery of MAX. A number of efforts from several laboratories has been made to identify small molecule inhibitors targeting this interaction, but many of these suffer from either poor efficacy, weak affinity for MYC, poor selectivity (or the lack of information about selectivity) and/or discrepancy between activity in vitro and in vivo. While these compounds were all identified through biochemical in vitro protein interaction assays or on the yeast2hybrid system, we utilized a cell-based BiFC assay to screen for MYC:MAX interaction inhibitors. BiFC has previously been used successfully to identify inhibitors of p65:p50 NFkB subunit interactions in cells. Cell-based screens have the advantage that hit compounds exhibit cell compatible features and interact with targets in their physiological state, thereby increasing the probability of finding hit molecules with higher bioactivity already at the screening level.

Here we describe three “hits” from our BiFC screening approach using a diverse library of small molecules from DTP/NIH (http://dtp.cancer.gov), MYCMI-6, MYCMI-11 and MYCMI-14. We could not find any obvious structural resemblance between the identified compounds, nor between these and previously described MYC:MAX inhibitors. The three MYCMIs were validated using a number of cell based and biophysical in vitro validation assays with the aim to identify molecules fulfilling the following criteria: (1) inhibition of MYC:MAX interaction in cells and in vitro with high efficacy and selectivity; (2) direct binding to MYC with high affinity and selectivity; (3) inhibition of tumor cell growth with high efficacy in a MYC-dependent manner; (4) MYC:MAX inhibition occurring under maintained MYC expression, thereby enabling specific pharmacological studies of the role of MYC:MAX in MYC biology; and (5) MYC:MAX inhibitory activity in tumor tissue in vivo. To our knowledge, no previously reported MYC:MAX inhibitor has fulfilled all of these five criteria.

Regarding the first criterion, MYCMI-6, MYCMI-11 and MYCMI-14 significantly inhibited MYC:MAX and MYCN:MAX interactions in human cells as verified by GLuc and isPLA assays (Figs 2 and 3), showing IC_{50} for inhibition of endogenous interactions of 1.5, 6 and 6 μM, respectively. Importantly, at active concentrations, the three compounds did not significantly inhibit cellular interactions between MAX and the intracellular bHLHZip domains of MYC and MAX competitor MXD1 (MAD1), or hetero- or homodimer interactions between the bZip transcription factors FOXJ1, GNCN4, GNCN4 and FRA1-JUN, which form similar dimeric α-helical structures as interacting bHLHZip proteins (Figs 2 and 3 and S2). As comparison, the IC_{50} of MYC:MAX inhibition in cells for previously reported compounds range from 10–100 μM in different cellular MYC:MAX interaction assays. Apart from the MYCMIs, only 10058-F4 has been shown to be selective for MYC:MAX vs. MXD:MAX (in Y2H assays in yeast cells), and for most inhibitors data on selectivity in general in cells have been poor or are lacking.

In the biophysical PPI validation assays based on MST and SPR, MYCMI-6 inhibited the interaction between recombinant bHLHZip domains of MYC and MAX with a K_{D} of 4.3 and 3.8 μM, respectively (Fig. 3), which is well in line with the IC_{50} of 1.5 μM in the cellular isPLA assay. In contrast, MYCMI-11 and MYCMI-14 were less effective at inhibiting the MYC:MAX interaction in the MST assay as well as in the isPLA. Further, MYCMI-6
Figure 7. MYCMI-6 inhibits MYC:MAX interaction, induces apoptosis and reduces tumor cell proliferation and microvascularity in a MYCN-amplified neuroblastoma mouse tumor model in vivo. SK-N-DZ MYCN-amplified neuroblastoma xenograft tumors reaching a volume of 100–200 mm³ were treated with MYCMI-6 (20 mg/kg body weight) or vehicle injected i.p. daily for 1–2 weeks. (A) Apoptosis was determined by TUNEL staining (green) of tumor tissues from mice treated with MYCMI-6 (upper two panels) or vehicle (lower two panels), counterstained with DAPI (blue). Representative images are shown at 1.25X (panel 1 and 3 from top, bar = 800 μM) or 40X (panel 2 and 4, bar = 50 μM) magnification. (B) Quantification of TUNEL staining normalized to whole tumor areas as determined by DAPI from three MYCMI-6- and three vehicle-treated mice. (C) Cell proliferation and microvascular density determined by Ki67 (green) and CD31 (red) staining, respectively, of tumor tissues from mice treated with MYCMI-6 (upper two panels) or vehicle (lower two panels), respectively, and counterstained with DAPI (blue). Representative images taken at 1.25X (panel 1 and 3 from top, bar = 800 μM) or 20X (panel 2 and 4) magnification. (D) Quantification of Ki67 negative areas normalized to whole tumor areas by DAPI from three MYCMI-6- and three vehicle-treated mice. (E) Microvascular density visualized by CD31 staining in the red channel at 1.25X magnification as in (C). (F) Quantification of CD31 staining normalized to whole tumor areas from three MYCMI-6- and three vehicle-treated mice. (G) Detection of MYCN:MAX protein interaction by isPLA performed on tumor tissue from mice treated with MYCMI-6 (upper panel) or vehicle (middle panel) using antibodies against MYCN and MAX. Representative images were...
not inhibit MAX:MAX homodimerization at active concentrations, thus showing selectivity for MYC:MAX. As comparison, the reference 10058-F4 inhibited the MYC:MAX interaction less well, with an IC50 of >25 μM in the SPR assay. KJ-Pyr-9 has been shown to inhibit recombinant MYC:MAX:DNA and MAX:MAX:DNA interaction with an IC50 of approximately 10 and 30 μM, respectively, in fluorescence polarization assays25,27. In our hands, KJ-Pyr-9 did, however, not affect the MYC:MAX interaction in the SPR assay at concentrations up to 10 μM. The reason for this discrepancy between the results obtained by these two methods is unclear. Using a GLuc screening assay in vitro, Choi et al.26 identified the molecule sAJM589, which inhibited MYC:MAX interaction with an IC50 of 1.8 μM while not affecting MAX:MAX and other interactions at 20 μM, which is comparable with our data for MYCMI-6. However, inhibition of MYC:MAX in cells by sAJM589 seemed to require higher concentrations (10–30 μM), which coincided with downregulation of MYC expression25.

Regarding the second criterion, direct binding of MYCMIs to the recombinant bHLHZip domain of MYC in vitro, MST and SPR analyses showed that MYCMI-6 binds to MYC with a Kd of 1.6 ± 0.5 μM (Figs 4 and S4 and S5), again in good agreement with both MYC:MAX in vitro MST, SPR and cellular isPLA data. MYCMI-6 did not bind to MAX in vitro in the MST or SPR assays. Further, binding of MYCMI-6 to the bHLHZip domain of MAD1, the p53 core domain, BSA and YFP were negligible by SPR (Figs 4 and S5), thus demonstrating selectivity for MYC bHLHZip domain. By comparison, 10074-G5, 10058-F4 and the 10058-F4 analogue #474 were reported to have affinities for MYC with a Kd around 32, 40 and 17 μM as monitored by SPR, respectively54. The selectivity of these compounds with respect to direct binding to reference proteins by SPR has, however, not been described. KJ-Pyr-9, was reported to bind MYC, but not MAX, with a Kd of 6.5 nM in a Backscattering Interferometry (BSI) assay27. We could not detect any binding of KJ-Pyr-9 to MYC up to 8 μM in our SPR assay (Suppl. Fig. S5B). The reason for this discrepancy is unclear at present.

Park and coworkers previously published work describing MYCMI-6 (BXI-61) as an inhibitor of BCL-XL:BAK interactions by binding BCL-XL at a concentration of 14 nM based on an in vitro fluorescence polarization assay26. We did not observe any binding of MYCMI-6 to BCL-XL in the MST assay up to 10 μM, but detected some binding in the SPR assay (Suppl. Figs S4A and S5F), indicating that MYCMI-6 has at least a 5-fold lower affinity for BCL-XL than for MYC. In the light of our findings, MYC:MAX inhibition is likely to contribute to the reported inhibition of lung tumor cell growth by MYCMI-6 in a mouse xenograft model56. MYCMI-6 (NSC354961) was also reported as a hit in vitro screens for inhibitors of hTERT, S-adenosylmethionine decarboxylase (AdoMetDC) and of HDMD2, in all these cases with approximately 10-fold higher IC50 than what we found for inhibition of MYC:MAX in vitro57–59. Notably, the hTERT inhibitory activity was found to be artificial due to unspecific binding to DNA in vitro at these concentrations58. While the activity towards AdoMetDC was not validated in cells26, MYCMI-6/NSC354961 did cause increased expression of p53 in cells at concentrations from 5 μM. However, in the light of our findings, inhibition of MYC:MAX by MYCMI-6 is likely to contribute to p53 activation since inhibition of MYC by the dominant negative OmoMYC protein, which interferes with the MYC:MAX complex, is known to activate the p53 pathway60. One should also point out that in none of these studies direct binding of MYCMI-6 to the proposed target was demonstrated.

Regarding the third criterion, MYC-dependent tumor cell inhibition, MYCMI-6, MYCMI-11 and MYCMI-14 inhibited growth of neuroblastoma cells with MYCN-amplification to a significantly larger extent than MYCN-non-amplified cell lines, which correlated with the much higher expression of MYCN in the MYCN-amplified cell lines than of MYC in the non-amplified cell lines as determined by western blot analysis. Regarding the fourth criterion, effects on MYC expression, it is important to keep in mind that many MYC:MAX inhibitors, including 10058-F4, 10074-G5 and sAJM589 down-regulate MYC-family protein levels, while MYCMI-6, MYCMI-11, MYCM-14 (Figs 2 and S6) and #474 do not25,54,61. For the former category, it is therefore not clear if these effects are primarily linked to MYC:MAX inhibition or reduced MYC expression27,28,61,62. In these studies it was not demonstrated...
whether the compounds inhibited MYC:MAX interaction in vivo, but Mycro3 was shown to reduce expression of MYC. Utilizing a MYCN-amplified neuroblastoma xenograft tumor model, we could show that MYCMI-6 treatment significantly reduced MYCN:MAX interaction in tumor tissue, suggesting that MYCMI-6 reached and was active against its target in vivo (Fig. 7). This correlated significantly with massive induction of apoptosis and reduction of tumor cell proliferation in large areas of the tumors, which is typically observed after blocking MYC function in MYC ON/OFF and in the dominant negative Omomyc mouse tumor models63–66, as well as by 10008-F4 and by Mycro3 in neuroblastoma61 and pancreatic62 tumor models, respectively. Other features of MYCMI-6 treatment were reduction in microvasculature and signs of increased hemorrhage and necrosis, which might reflect collapse of tumor vasculature previously observed in the Omomyc tumor model65. The effects of MYCMI-6 treatment therefore resemble the effects of MYC depletion by genetic means in mouse tumor models. Importantly, MYCMI-6 treatment was well tolerated by the mice and did not result in severe side effects. As mentioned above, MYCMI-6 has previously been shown to inhibit lung tumor growth in a mouse xenograft model56. Further validation of the efficacy of MYCMI-6 in MYC-dependent mouse tumor models is needed in the future.

In conclusion, we have identified three new MYC:MAX inhibitors in a cell-based protein interaction screen. Among these, MYCMI-6 fulfills all the criteria we set up for a useful MYC:MAX inhibitor: (1) Potent and selective inhibition of MYC:MAX interaction in cells and in vitro; (2) selective direct binding to MYC; (3) inhibition of tumor cell growth in a MYC-dependent manner, all at similar single-digit micromolar concentrations, while sparing normal cells; (4) In contrast to many other MYC:MAX inhibitors, MYCMI-6 did not affect MYC or MYCN expression, and thus could be used as a specific molecular tool to explore role of the MYC:MAX and MYCN:MAX interactions in normal and tumor cells; and (5) Exhibiting MYC:MAX inhibitory activity in tumor tissue in vivo. For future research, it will be important to determine the precise mechanism of action of MYCMI-6 to enable the development of more potent and selective molecules towards clinically relevant direct MYC inhibitors.

**Methods**

All experiments were performed in accordance with the guidelines and regulations of the Karolinska Institutet.

**Cell lines.** HEK293A, MCF7, MDA-MB231, SK-N-F1, SK-N-RA, SK-N-AS, COS-7, HeLa, BJ cells, and Rat1 fibroblasts TGR-1 (wt), HO15.19 (MYC knockout) and HOmyc3 (MYC reconstituted HO15.19) were maintained in DMEM. The neuroblastoma cell lines Kelly, IMR-32 and SK-N-DZ, and the Burkitt’s lymphoma cell lines Mutu, Daudi and ST486 were kept in RPMI. SK-N-BE(2) cells were maintained in a 1:1 mixture of EMEM and F12 media, supplemented with 1% non-essential amino acid solution (NEAA) and 0.5% Glutamine. U2OS-MYC-ER cell lines were cultured in phenol-red free DMEM and treated with 100 nM 4-hydroxytamoxifen (HOT) (Sigma-Aldrich) to activate MYC-ER. IMR-90 was maintained in MEM. All media were supplemented with 10% FBS and 1% penicillin and streptomycin and kept at 37 °C and 5% CO₂. All cell lines were mycoplasma negative.

**Compounds.** A screening library of small molecules, 10 mM of each compound in DMSO delivered in a 96 well plate format, from the NCI/DTP Open Chemical Repository Diversity set library (http://dtp.nci.nih.gov) was used. After hit selection, three independent compound batches were obtained from NCI/DTP to confirm activity. In addition, compound MYCMI-6 was synthesized in larger quantities by Latvian Institute of Organic Synthesis, Riga, Latvia. All compounds were dissolved in DMSO (Sigma-Aldrich) to a final concentration of 10 mM, verified by mass spectrometry (LC-MS) and stored in −80 oC for further use. DMSO, IQ1, 10058-F4 and 10074-G5 were purchased from Sigma-Aldrich. KJ-Pyr-9 was purchased from Cayman Chemical and was verified by LC-MS.

**Bimolecular fluorescence complementation assay (BiFC).** The BiFC assay using MYC fused to the C-terminal fragment of YFP and MAX fused to the N-terminal fragment of YFP has been described previously48. Fluorescence intensity was analyzed using an automated Axiovert 200M inverted microscope (Zeiss). Images were captured and processed using a Hamamatsu ORCA-ER camera together with software from Improvision (OpenLab 4.0.1). CMV-CFP was cotransfected as an internal standard and the ratio between YFP and CFP fluorescence intensity was calculated and normalized to the value of the DMSO treated cells.

**Gaussia luciferase protein fragment complementation assay (GLuc).** The protein fragment complementation assay using the Gaussia luciferase has been described13. 0.2–0.4 μg of each GLuc-construct (see Supplementary Information) together with 0.05–0.2 μg pCMV-Luc (Firefly luciferase) were transiently transfected into HEK293 or COS-7 cells in 6-well plates. 24 hours later cells were treated with compound (10–25 μM) or DMSO. After another 17 hours, the cells were harvested and lysed in passive lysis buffer (Promega) supplemented with complete protease inhibitor (Roche). After 60 min incubation at room temperature 20 μM D-luciferin was added (substrate of Firefly luciferase) and luminescence was measured in a Lumat LB9501 (Berthold) or OmegaFluostar (BMG Labtech) luminometer. Directly after, Gaussia luciferase substrate Coelenterazine (Promega) was added to a final concentration of 20 μM in a mixture with “Stop n’ Glow” (Promega) and the luciferase activity was measured. The ratio between Gaussia and Firefly luciferase values were calculated and normalized to DMSO-treated control cells.

**isPLA.** The isPLA assay has been described49. Antibodies used are listed in Supplementary Information. Briefly, cells were grown on collagen-coated chamber slides (Falcon), and treated with 1–10 μM of compounds, respectively, for 16 hours, then washed twice with PBS and fixed in ice cold methanol for 5–15 min at room temperature. Slides were washed in PBS with 0.05% Tween 20 and incubated in blocking buffer after which isPLA was performed using the Duolink® in situ PLA kit (Sigma-Aldrich) according to the manufacturer’s protocol. DNA was stained with DAPI. Incubation with primary antibodies were performed at +4 °C overnight. Images
were taken using an Axiovert 200M inverted microscope (Zeiss) and fluorescent dots were quantified using semi-automated analysis in ImageJ (http://imagej.net) and averaged to number of dots per cell.

isPLA for tumor tissue was performed using 4% formaldehyde fixed paraffin embedded material. The tissue slides were deparaffinized at 65 °C for an hour, rehydrated in 1X Aqua De Par (Biocare Medical) and incubated at 80 °C for 10 min in a pressure chamber (Decloaking Chamber – Biocare Medical). Slides were soaked in antigen retrieval buffer (10 mM sodium citrate, pH 6.0) and heated in the pressure chamber to 100 °C for 20 minutes, after which the temperature was lowered to 65 °C.Slides were removed from the chamber and cooled down to room temperature. The antigen retrieval buffer was replaced gradually by deionized H2O for 15 min. Slides were placed in blocking buffer (Duolink® isPLA kit) for 1.5 hours at 37 °C followed by an extra blocking step using the Mouse on Mouse (M.O.M.™) basic kit (Vector Laboratories) according to manufacturers’ protocol. isPLA was performed as above.

**Coimmunoprecipitation, western blot, immunohistochemistry and RT-qPCR analyses.** These assays were performed essentially as described in[67,68]. For further information on immunohistochemistry, and on primers and antibodies, see Supplementary Information.

**Plasmids and recombinant proteins.** For information about plasmids and production and purification of recombinant proteins see Supplementary Information.

**Microscale thermophoresis (MST).** MST was carried out on a Monolith NT.115 with blue/green filters, kindly provided by Luca Jovine, Karolinska Institutet Huddinge, according to manufacturer’s protocol (NanoTemper). MYCbHLHZip, MaxbHLHZip, BSA and BCL-X, recombinant proteins and compounds were diluted and titrated in PBS supplemented with 0.05% Tween-20. Titration of molecules was carried out in 16 PCR tubes into which a fixed concentration of fluorescent molecule was added. The mixture was applied to capillaries (standard treated, NanoTemper), and scanned in the Monolith NT.115 to measure initial fluorescence of molecule. MST was induced and fluorescence was measured during 40 seconds. Double measurements were carried out (MST power of 20% and 40%, or 40% and 60%) for each sample. Delta fluorescence or relative fluorescence of fluorescent molecule normalized to control vehicle was plotted against titrated molecule concentration. The protein labeling kit GREEN-NHS (NanoTemper) was used to label primary amines of proteins according to manufacturer’s protocol.

**Surface plasmon resonance (SPR).** The SPR experiments were performed at 25 °C using a Biacore T200 (GE Healthcare) instrument kindly provided by SciLifeLab Solna. An amino coupling procedure was used to immobilize protein on a CM5 sensor chip (GE Healthcare). Sensorgrams were generated by subtraction of the reference (blank immobilized) surface. All details will be described in Castell et al. (manuscript in preparation).

In general, for the MYC:MAX interaction assay, 200–500 RU of MAXbHLHZip was immobilized and 100 nM MYCbHLHZip pre-incubated with compound in PBS supplemented with 0.05% Tween-20 and 1% DMSO was injected with a flow rate of 30 µl/min and allowed to dissociate. MAX was regenerated by injection of PBS buffer supplemented with Urea.

The MYC SPR assay was carried out as described above with MYCbHLHZip immobilized to a level of 800–1000 RU. For kinetic binding experiments, a Langmuir 1:1 binding event was applied using the Biacore T200 Evaluation Software 2.0 (GE Healthcare) to determine association (kₐ) and dissociation (k₈) constants of the compound and to calculate affinity (K_D) by the formula; K_D = (MW analyte/MW ligand) × Rmax × (RU)/(immobilized ligand level on the chip (RU)).

**Statistical analyses.** Proportional data corresponding to Gluc, isPLA in cells for MYC:MAX and qPCR experiments in U2OS cell line were analyzed with a non-parametric Kruskal-Wallis test, while comparisons across treatments were performed with Bonferroni post hoc or Wilcoxon tests, to account for heteroscedasticity. Proportional data corresponding to MYCN:MAX isPLA and RT-qPCR experiments in neuroblastoma cell lines were initially transformed (square root and arcsin) and analyzed with generalized linear models (GLMs) with a normal distribution. Pairwise comparisons were performed with Bonferroni post hoc tests. Proportional data corresponding to isPLA in vivo experiments were log-transformed and analyzed with a GLM. The effect of MYC mRNA and protein levels on the response of the NCI-60 cancer cell lines to MYCMI-6 was analyzed with GLM assuming a normal distribution, while the hypothesis on the probability of a cell line with “high MYC” or “low MYC” protein levels to respond to MYCMI-6 was tested with the binomial exact test. The rest of the data were analyzed with the Student’s t-test. All analyses were carried out in R (v. 3.3.3; R Foundation for Statistical Computing, Vienna, AT), at a level of significance α = 0.05, with packages car, agricolae, multcomp and MASS.
References

1. Blackwood, E. M. & Eisenman, R. N. Max: a helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with Myc. Science 251, 1211–1217 (1991).

2. Dang, C. V. MYC on the path to cancer. Cell 149, 22–35, https://doi.org/10.1016/j.cell.2012.03.002 (2012).

3. Eilers, M. & Eisenman, R. N. Myc's broad reach. Genes Dev 22, 2755–2766, https://doi.org/10.1101/gad.1712408 (2008).

4. Larsson, L. G. & Henriksson, M. A. The Yin and Yang functions of the Myc oncoprotein in cancer development and as targets for therapy. Exp Cell Res 316, 1429–1437, https://doi.org/10.1016/j.yexcr.2010.06.011 (2010).

5. Meyer, N. & Penn, L. Z. Reflecting on 25 years with MYC. Nat Rev Cancer 8, 976–990, https://doi.org/10.1038/nrc2331 (2008).

6. Nair, S. K. & Burley, S. K. X-ray structures of Myc-Max and Mad-Max recognizing DNA. Molecular bases of regulation by proto-oncogenic transcription factors. Cell 112, 193–205, doi:10.1016/j.cell.2005.02.003 (2003).

7. Kress, T. R., Sabo, A. & Amati, B. MYC: connecting selective transcriptional control to global RNA production. Nat Rev Cancer 15, 593–607, https://doi.org/10.1038/nrc3984 (2015).

8. Lin, C. Y..

9. Nie, Z.

10. Sabo, A.

11. Walz, S.

12. Babcock, F. A.

13. Li, M.

14. Castell, A. & Larsson, L. G. Targeting MYC Translation in Colorectal Cancer. Cell 112, 22–35, https://doi.org/10.1016/j.cell.2012.03.003 (2012).

15.  McKeown, M. R. & Bradner, J. E. Therapeutic strategies to inhibit MYC.

16. Whitfield, J. R., Beaulieu, M. E. & Soucek, L. Strategies to Inhibit Myc and Their Clinical Applicability. Oncogene 33, 844–848, https://doi.org/10.1038/s41598-018-28107-4 (2014).

17. Vassilev, L. T.

18. Filippakopoulos, P.

19. Arkin, M. R., Tang, Y.

20. Arkin, M. R., Tang, Y.

21. Tang, Y.

22. Berg, T.

23. Kiessling, A., Wiesinger, R., Sperl, B. & Berg, T. Selective inhibition of c-Myc/Max dimerization by a pyrazolo[1,5-a]pyrimidine.

24. Yin, X., Giap, C., Lazo, J. S. & Prochownik, E. V.

25. Kiessling, A., Sperl, B., Hollis, A., Eick, D. & Berg, T. Selective inhibition of c-Myc/Max dimerization and DNA binding by small molecules. Chem Biol 13, 745–751, https://doi.org/10.1016/j.chembiol.2006.05.011 (2006).

26. Yin, X., Gnap, C., Lazlo, J. S. & Prochownik, E. V. Low molecular weight inhibitors of Myc-Max interaction and function. Oncogene 22, 6515–6519, https://doi.org/10.1038/sj.onc.1206641 (2003).

27. Choi, S. H. et al. Targeted Disruption of Myc-Max Oncoprotein Complex by a Small Molecule. ACS Chem Biol 12, 2715–2719, https://doi.org/10.1021/acschembio.7b00799 (2017).

28. Whitfield, J. R., Beaulieu, M. E. & Soucek, L. Strategies to Inhibit Myc and Their Clinical Applicability. Oncogene 33, 844–848, https://doi.org/10.1038/s41598-018-28107-4 (2014).

29. Arkin, M. R., Tang, Y.

30. Kiessling, A., Wiesinger, R., Sperl, B. & Berg, T. Selective inhibition of c-Myc/Max dimerization by a pyrazolo[1,5-a]pyrimidine.

31. Mo, H. & Henriksson, M. Identification of small molecules that induce apoptosis in a Myc-dependent manner and inhibit Myc-driven transformation. Proc Natl Acad Sci USA 103, 6354–6349, https://doi.org/10.1073/pnas.0601418103 (2006).

32. Wang, H.

33. Wang, H.

34. Wang, H.

35. Wang, H.

36. Wang, H.

37. von der Lehr, N.

38. Remy, I. & Michnick, S. W.

39. Remy, I. & Michnick, S. W.

40. Remy, I. & Michnick, S. W.

41. Soderberg, O. et al. Direct observation of individual endogenous protein complexes in situ by proximity ligation. Nat Methods 3, 995–1000, https://doi.org/10.1038/nmeth947 (2006).

42. Ayer, D. E., Keetelm, L. & Eisenman, R. N. Mad: a heterodimeric partner for Max that antagonizes Myc transcriptional activity. Cell 72, 211–222 (1993).

43. Larsson, L. G., Pettersson, M., Oberg, F., Nilsson, K. & Luscher, B. Expression of mad, mxi1, max and c-myc during induced differentiation of hematopoietic cells: opposite regulation of mad and c-myc. Oncogene 9, 1247–1252 (1994).
43. Adhikary, S. & Eilers, M. Transcriptional regulation and transformation by Myc proteins. *Nat Rev Mol Cell Biol* 6, 635–645 (2005).
44. Seidel, S. A. *et al.* Microscale thermophoresis quantifies biomolecular interactions under previously challenging conditions. *Methods* 59, 301–315, https://doi.org/10.1016/j.ymbeth.2012.12.005 (2013).
45. Schasfoort, R. B. M. & Tudos, A. J. *Handbook of surface plasmon resonance* (RSC Pub., 2008).
46. Zimmerman, M. W. *et al.* MYC Drives a Subset of High-Risk Pediatric Neuroblastomas and Is Activated through Mechanisms Including Enhancer Hijacking and Focal Enhancer Amplification. *Cancer Discov* 8, 320–335, https://doi.org/10.1158/2159-8290.CD-17-0993 (2018).
47. Jung, M. *et al.* A Myc Activity Signature Predicts Poor Clinical Outcomes in Myc-Associated Cancers. *Cancer Res* 77, 971–981, https://doi.org/10.1158/0008-5472.CAN-15-2906 (2017).
48. Baxevanis, A. D. & Vinson, C. R. Interactions of coiled coils in transcription factors: where is the specificity? *Curr Opin Genet Dev*, 811–822, https://doi.org/10.1016/j.gde.2012.12.005 (2013).
49. Mateyak, M. K., Obaya, A. J., Adachi, S. & Sedivy, J. M. Phenotypes of c-Myc-deficient rat fibroblasts isolated by targeted homologous recombination. *Cell Growth Differ* 8, 1039–1048 (1997).
50. Yu, H. *et al.* Measuring drug action in the cellular context using protein-fragment complementation assays. *Assay Drug Dev Technol* 1, 811–822, https://doi.org/10.1089/154287103772613444 (2003).
51. Park, D. *et al.* Novel small-molecule inhibitor of c-Myc/Max dimerization. *J Pharmacol Exp Ther* 335, 715–727, https://doi.org/10.1124/jpet.117.150553 (2010).
52. Muller, I. *et al.* Targeting of the MYCN protein with small molecule e-MYC inhibitors. *PloS One* 9, e97285, https://doi.org/10.1371/journal.pone.0097285 (2014).
53. Clausen, D. M. *et al.* In vitro cytotoxicity and in vivo efficacy: pharmacokinetics, and metabolism of 10074-G5, a novel small-molecule inhibitor of c-Myc/Max dimerization. *J Pharmacol Exp Ther* 370, 278–285 (1999).
54. Sassano, M. F., Schlesinger, A. P. & Jarstfer, M. B. Identification of G-Quadruplex Inducers Using Simple, Inexpensive and Rapid High Throughput Assay, and Their Inhibition of Human Telomerase. *Open Med Chem J* 6, 20–28, https://doi.org/10.2174/1874104512061001020 (2012).
55. Weissman, A. M. *et al.* Inhibiting Hdm2 and ubiquitin-activating enzyme: targeting the ubiquitin conjugating system in cancer. *Ernst Schering Found Symp Proc* 171–190 (2008).
56. Soucek, L. *et al.* Inhibition of Myc family proteins eradicates KRas-driven lung cancer in mice. *Genes Dev* 27, 504–513, https://doi.org/10.1101/gad.205542.112 (2013).
57. Zirath, H. *et al.* MYC inhibition induces metabolic changes leading to accumulation of lipid droplets in tumor cells. *Proc Natl Acad Sci USA* 110, 10258–10263, https://doi.org/10.1073/pnas.1222404110 (2013).
58. Brooks, W. H. *et al.* In silico chemical library screening and experimental validation of a novel 9-aminoacridine based lead-inhibitor of human S-adenosylmethionine decarboxylase. *J Chem Inf Model* 47, 1897–1905, https://doi.org/10.1021/ci7000051 (2007).
59. Curtis, E. *et al.* Novel small molecule inhibitor of Bcl-XL to treat lung cancer. *Cold Spring Harb Perspect Med* 5, 110, https://doi.org/10.1101/cshperspect.a014365 (2014).
60. Soucek, L. *et al.* Endogenous Myc maintains the tumor microenvironment. *Cancer Discov* 25, 907–916, https://doi.org/10.1101/gad.2038411 (2011).
61. Zirath, H. *et al.* Modelling Myc inhibition as an cancer therapy. *Nature* 455, 679–683, https://doi.org/10.1038/nature07264 (2008).
62. Bahram, F. *et al.* Interferon-gamma-induced p27kip1 binds to and targets MYC for proteasome-mediated degradation. *Oncotarget* 7, 2837–2854, https://doi.org/10.18632/oncotarget.6693 (2016).
63. Tabor, V., Bocci, M., Alikhani, N., Kuiper, R. & Larsson, L. G. MYC synergizes with activated BRAFV600E in mouse lung tumor development by suppressing senescence. *Cancer Res* 74, 4222–4229, https://doi.org/10.1158/0008-5472.CAN-13-3234 (2014).

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Additional Information
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