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Cell-based high-throughput screening using a target–NanoLuc fusion construct to identify molecular glue degraders of c-Myc oncoprotein

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Oncoprotein c-Myc (Myc) plays a critical role in regulating cellular gene expression. Although Myc dysregulation is found in more than 70% of cancers and can facilitate tumor initiation and progression, it is still considered to be an ''undruggable'' oncotarget years after its first discovery. Recent advances in the field of targeted protein degradation provide alternative Myc-targeting strategies. Here, we develop the first Myc-NanoLuc fusion plasmid transfected cell-based high-throughput screening assay to identify Myc-downregulating small molecules. We verified the effectiveness of our assay by demonstrating that previously known Myc-downregulating compounds (**G9** and SY-1365) were successfully identified from a library of bioactive compounds with established biological function. Next, we screened another 108 800 compounds from the diverse ChemDiv library collection, and 14 novel Myc-downregulating compounds were identified after cherry-pick triplicate confirmation, counter-screening, dose–response and western blotting experiments. A cellular thermal shift assay further demonstrated that five out of the 14 Myc-downregulating compounds bound to endogenous Myc protein in crude 293T whole-cell lysate. Subsequently, compound **C1** was shown to selectively degrade Myc protein at a DC_{50} value of around 5 μM. Further characterization showed that **C1** killed cancer cells with high Myc expression at a lower dose than it killed cancer cells with low Myc expression. Moreover, **C1** selectively reduced the expression of various Myc-target genes. Intriguingly, co-immunoprecipitation showed that **C1** functionally acted like a molecular glue to aggregate Myc proteins and block Myc/Max interaction. The self-aggregation of Myc and the dissociation of the Myc/Max dimer by **C1** promoted Myc degradation. Using a target–NanoLuc fusion strategy in our novel cell-based high-throughput screening system, we identified a molecular glue-like small molecule degrader of Myc.

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Introduction

The c-Myc oncoprotein (Myc) is a DNA-binding transcription factor/activator with a basic helix-loop-helix leucine zipper domain that dimerizes with its binding partner Max. The Myc/Max dimer regulates around 15–20% of cellular gene expression (direct regulation plus indirect regulation of secondary responsive genes).[1,2] Myc plays critical roles in regulating various cellular functions, including but not limited to amplification, cell cycle, growth, differentiation, development, and stemness.[3] The *MYC* oncogene family includes c-Myc, N-Myc and L-Myc, all of which have different expression timings and tissue specificities[2]. Due to its pivotal role in gene regulation, Myc activity is usually tightly controlled. However, Myc was found to be dysregulated in more than 70% of cancers with

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a mainly over-expressed signature, including in lymphomas, melanomas, breast, ovarian, prostate, lung and liver cancers. Strong evidence indicates that aberrantly expressed Myc is involved in both tumor initiation and progression.[2] Thus, therapeutics targeting Myc have great market and healthcare potential.

Although dysregulated Myc enables cancer cells to evade growth suppression, avoid immune destruction, promote tumor-associated inflammation, and cause genome instability/mutation, no direct Myc-targeting drug has been approved in the 40 years since its discovery.[2,3] Thus, Myc is considered an "undruggable" target in drug discovery. The failure to develop effective Myc-targeted drugs can be attributed to several factors including: (i) Myc is structurally flexible and does not have a traditional ligand binding pocket; (ii) Myc functions inside the nucleus and is surrounded by chromatin DNA and other protein–protein interactions (PPIs); and (iii) Myc is also considered to be essential for cellular activity, and thus, it should not be completely knocked out.[2,4] Nevertheless, scientists from both the industrial and academic communities have successfully developed numerous pre-clinical and clinical compounds that directly or indirectly target Myc through diverse approaches.[2] Back in 2003, 10058-F4/10074-G5, two small molecules inhibiting Myc/Max dimerization, were identified through yeast two-hybrid screening.[5] Other compounds that can similarly disrupt Myc/Max interactions, such as Mycro3,[6] KJ-Pyr-9,[7] sAJM589,[8] MYCMI-6[9] and MYC975i,[10] were subsequently discovered. However, their development stopped in the pre-clinical stage due to either high toxicity or low efficacy *in vivo*. Recently, UNC10112785,[11] a small molecule promoting Myc protein degradation by inhibiting CDK9 kinase activity on Ser62 of Myc, was identified by fluorescence-based screening. WBC100[12] and PLX6117,[13] a triptolide analog and a BRD4 BET domain inhibitor, are currently under clinical investigation. OmoMyc,[14–16] a low-toxicity polypeptide blocker of Myc/Max dimerization and Myc/Max-DNA binding, has recently completed its phase I clinical trial with a promising outcome. However, more efforts are still needed to ensure the approval of a Myc-targeting drug.

With the development of small-molecule degraders such as protein-targeting chimeras (PROTACs) and molecular glue degraders (MGDs) for targeted protein degradation, numerous "undruggable" targets have now become "druggable".[17] Recently, two Myc-targeting PROTACs based on either TNA-DNA-pomalidomide or MYC361i-VHL-ligand bivalent binders were successfully invented to target "undruggable" Myc. However, such PROTACs have high molecular weights with complex chemical structures, which have hindered their development as orally deliverable drugs.[10,18,19] Compared to PROTACs, MGDs do not require a pre-existing ligand binding pocket on the surface of the target protein and can enable the degradation of previously inaccessible/undruggable targets by inducing/enhancing novel PPIs on their surface.[17] MGDs generally have low MWs (usually <500 Dalton), can easily pass membranes, and are orally deliverable.[20] Thus, MGDs have intrinsic advantages as oral drugs for the targeting of flexible transcription factors. As flexible Myc does not have a ligand binding surface and is frequently over-expressed in different cancer cells, it is an ideal target for an MGDs. Additionally, Myc is also thought to have an essential function in regulating normal cellular transcriptional activity and should not be completely knocked out in normal cells.[2,3] It is essential to determine the optimal concentration of an MGD that reduces Myc protein levels incompatible with cancer cell survival, while maintaining viability in normal cells. However, most MGDs were discovered serendipitously and are hard to design rationally.[17,20] An automatic high-throughput screening (HTS) robot system can screen tens of thousands of compounds from compound libraries in a short time and is a vital tool in drug discovery to quickly identify early hits for medicinal chemistry optimization.[21,22] Advancements in HTS technologies can facilitate the discovery of Myc MGDs by targeted library screening.

Here, we developed the first Myc-NanoLuc fusion plasmid transfected cell-based HTS assay to identify Myc-downregulating small molecules. We verified the effectiveness of our assay by proving that previously known Myc-downregulating compounds (**G9** and SY-1365) could be successfully screened out from a Bioactive library with known functional compounds. Next, we screened another 108 800 compounds from the diverse ChemDiv library collection and 14 novel Myc-downregulating compounds were confirmed after screening. A cellular thermal shift assay (CETSA) further demonstrated that five out of the 14 Myc-downregulating compounds bound endogenous Myc protein in whole cell extract. Subsequently, compound **C1** was shown to selectively degrade Myc protein at a DC_{50} of around 5 μM. Further characterization showed that **C1** killed high-Myc–expressing cancer cells at a lower dose than low-Myc–expressing cancer cells, with a >5.2 fold increase in selectivity. Moreover, **C1** selectively reduced the expression of various Myc-target genes. Intriguingly, Co-immunoprecipitation (Co-IP) and fluorescence microscopy imaging showed that **C1** functionally acted like a molecular glue to aggregate the Myc protein and block Myc/Max interaction. The self-aggregation of Myc and the dissociation of the Myc/Max dimer occurred *via* **C1**-promoted Myc degradation. Together, we identified a molecular glue-like small molecule degrader of Myc through our novel cell-based HTS system.

Results

Identification of Myc-downregulating small molecules using the f-Myc-Nluc-fusion plasmid transfected cell-based HTS assay

MGDs bind and degrade target proteins.[17,20] To identify potential MGDs of the Myc oncoprotein, the first step is to identify small molecules that can reduce Myc protein levels. A fluorescence-based dual color HTS assay has been previously utilized to screen Myc-downregulating small molecules.[11] A NanoLuc (Nluc) luciferase-based assay was found to be much more robust and stable (t-half $>$ 4 h) than assays based on other luciferases (*e.g.* firefly luciferase).[23,24] Both an Nluc-fusion and 11-amino acid HiBiT-tagged protein degradation assays have been developed to monitor protein degradation activities.[25] The smaller HiBiT-tag fusion was more physiologically

relevant than the whole Nluc-fusion, but the Nluc-fusion assay was much more cost-effective and operationally friendly because the HiBiT-tagged Myc required CRISPR editing and stable cell line generation. To establish a cell-based luminescence HTS degradation assay (Fig. 1A), we constructed two plasmids: one encoding an f-Myc-Nluc-fusion protein and the other encoding f-Nluc alone. These plasmids were transiently transfected separately into 293T cells.[26] Any compound that reduced Myc protein stability within 4–5 h should also downregulate the luminescence signal from f-Myc-Nluc. Meanwhile, the luciferase inhibitors and toxic compounds could be excluded by reverse screening with the f-Nluc only construct without Myc-fusion. UNC10112785, a small molecule Myc-downregulating compound previously identified by fluorescence-based degradation screening,[11] was utilized as the positive control for assay optimization (Fig. 1A and B). A library of 17 452 bioactive compounds with well-known biological functions was utilized as the ideal small molecule library to verify the efficiency of our newly constructed Myc-degradation assay (Fig. 1A). Two cancer-related pre-clinical compounds were identified from the pilot screening: including a deubiquitinase (DUB) inhibitor that promotes Myc protein degradation (EOAI3402143/**G9**) and a CDK7 inhibitor that also inhibits CDK9 kinase activity (SY-1365).[27–29] Notably, UNC10112785 promoted Myc degradation through the inhibition of CDK9 kinase activity on Myc Ser62, as reduction of Ser62 phosphorylation made the Myc protein unstable.[11] Thus, our pilot screening of the library of bioactive compounds identified two known indirect Myc-downregulating compounds **G9** and SY-1365, a Myc-DUB inhibitor and a CDK7/9 kinase inhibitor that similarly promotes Myc degradation as UNC10112785 (Fig. 1A and B and Fig. S1A–C). Together, these results demonstrated that our HTS assay successfully identified Myc-downregulating small molecules that directly or indirectly reduced Myc protein levels.

As Myc-DUB inhibitor **G9** showed a stronger degradation effect on Myc and a better *Z*-factor index[30] than UNC10112785 and SY-1365 (Fig. S1A–C, *Z*-factors 0.913 for **G9**, 0.638 for UNC10112785 and 0.893 for SY-1365), **G9** was employed as the new positive control and another 108 800 compounds were screened from the ChemDiv library. A total of 89 primary hits were identified with $>30\%$ reduction of the luminescence signal from 293T cells transfected with pCDNA3-f-Myc-Nluc (primary HTS hit rate: 0.08% (89/108 800), Fig. S1D and E). Subsequently, cherry-pick triplicate confirmation and counter-screening by f-Nluc (without Myc-fusion) were performed to exclude the hits with high cellular toxicity or hits that were merely luciferase inhibitors (hit confirmation threshold: f-Myc-Nluc reduction $>$ 40% and f-Nluc reduction $<$ 20%; toxicity or luciferase inhibitors: f-Nluc reduction $\geq$ 20%, 64 hits excluded, Fig. S1D). A total of 25 hits were confirmed and subjected to a nine-point dose–response assay (maximal dose at 10 μM, 2-fold dose) to determine the DC_{50} of each compound (Fig. S1E and F). The results showed that the DC_{50} for f-Myc-Nluc of these 25 compounds ranged from 0.9 μM to 14.8 μM. There was little reduction in the signals of the f-Nluc vector-transfected cells (Fig. S2). Thus, all 25 compounds were subjected to a Myc-degradation test by western blotting in the next step. Similar to **G9**, 14 out of the 25 compounds showed a significant degradation effect on ectopically expressed f-Myc-Nluc at a 10 μM concentration after treatment, while no reduction of the GAPDH control was observed. We thus re-named these 14 compounds as **C1** to **C14** for simplicity (Fig. 1C). Markedly, 11 out of the 25 luciferase-confirmed hits were unable to degrade ectopic f-Myc-Nluc, as assessed by western blotting (Fig. 1C, top), which suggested that false-positive primary hits might be identified by this NLuc-fusion assay (11 hits out of 25 in this study). However, all the remaining 14 compounds (chemical structures in Fig. 1D) showed strong degradation of endogenous Myc protein in Ramos cells (Fig. 1C, bottom), which demonstrated that the true novel Myc-downregulating hit rate from screening was still relatively high (the confirmation rate of our HTS campaign was 15.7% (14/89) and the final hit rate was 0.01% (14/108 800) in this study). Notably, rt-PCR data showed that only **C2** and **C3** reduced *MYC* mRNA levels while the other compounds (**G9**, **C1**, **C4–C14**) either increased *MYC* mRNA or did not significantly affect *MYC* mRNA expression, suggesting that most of the identified compounds regulated Myc at the protein level (Fig. 1C and Fig. S3A), otherwise Myc protein levels would increase due to the upregulation of *MYC* mRNA. Induction of *MYC* mRNA by Myc-DUB inhibitor **G9** and most of the other Myc protein-downregulating compounds could be a compensation mechanism of cancer cells whereby they produce more Myc proteins for survival due to a sharp reduction of Myc protein inside cells in a short time (within 5 h). This is particularly relevant in Ramos cancer cells, which expressed the highest Myc protein levels among all of the cells tested (Fig. S3B).

CETSA analysis shows that the C1, C3, C7, C8 and C11 Myc degraders could bind and stabilize endogenous Myc in 293T cell lysate

The identification of the false-positive pan-assay interference compounds (PAINS) as primary hits is a common issue in HTS, particularly in campaigns using protein-based biochemical assays.[31] Because our primary hits were identified using a cell-based NanoGlo assay and their Myc-specific degradation was confirmed by western blotting, the likelihood of false-positive hits was low (Fig. 1C). Nonetheless, the 14 hits were tested using the online PAINS testing tool SwissADME (**https://www.swissadme.ch/index.php**, based on PAINS ref. 31). The results showed that out of the 14 hits only **C3**, **C13** and **C14** contained PAINS alerts, and these three hits are marked in red in Fig. 1D.

MGDs bind and degrade target proteins.[17,20] To identify potential MGDs of the Myc oncoprotein, the second step was to screen out from these 14 primary hits the Myc-downregulating compounds that also bind the Myc protein. Although **C3**, **C13** and **C14** were shown to contain PAINS alters (Fig. 1D), we included all 14 primary Myc-downregulating compounds in subsequent Myc-binding tests. Because Myc is structurally flexible[2] and recombinant purified Myc was not available at the early stage of this study, the CETSA was first adapted to detect the interactions between Myc and the 14 Myc-downregulating hits. The CETSA can detect interactions between small molecules and structurally flexible proteins

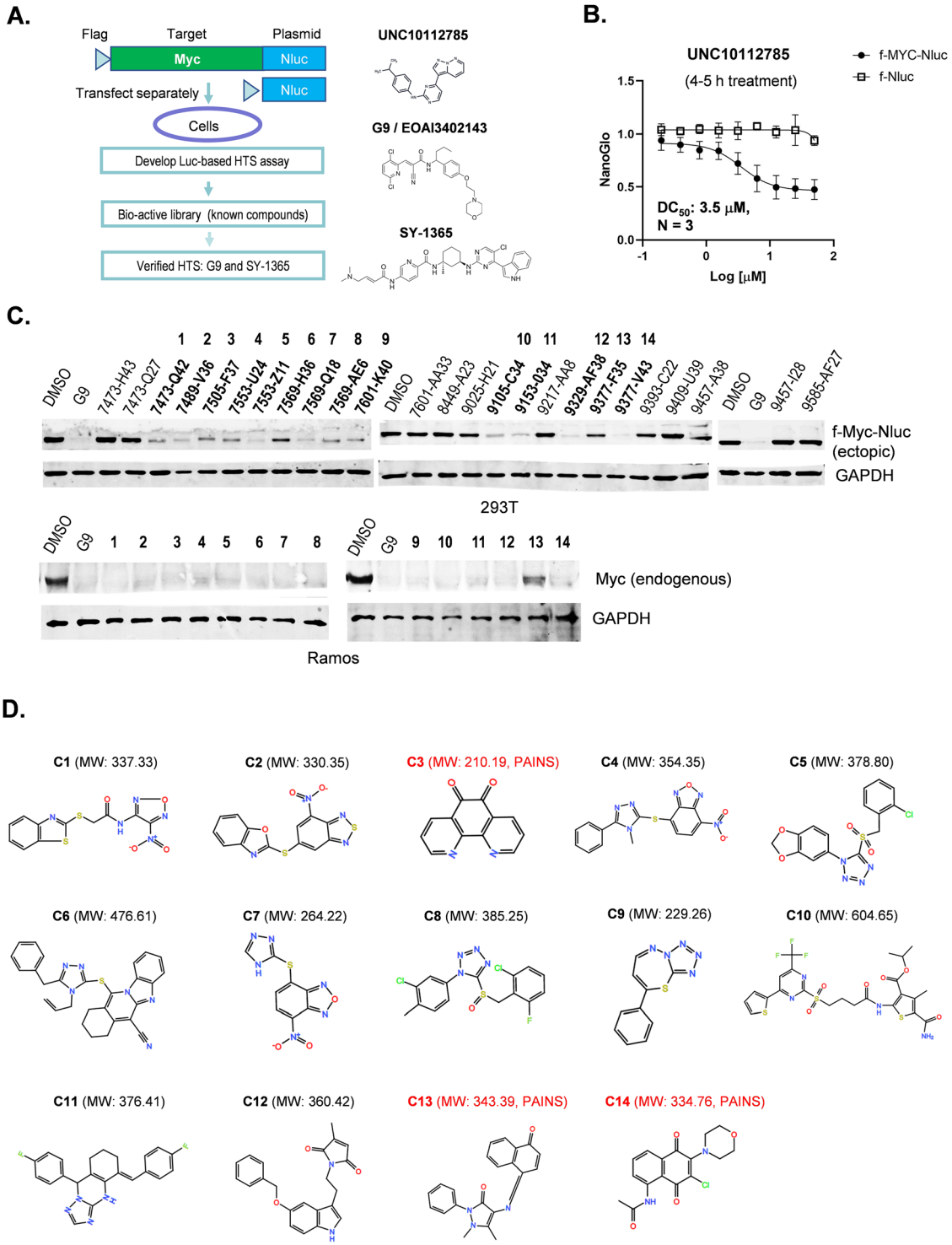


Fig. 1 Identification of 14 novel Myc-downregulating small molecules using a cell-based high-throughput screening assay. (A). Graphical description of the NanoLuc (Nluc) fusion plasmid constructions (f-Myc-Nluc and f-Nluc) and the HTS verification assay after screening of a library of 17 452 well-known bioactive annotated compounds. UNC10112785 served as the positive control. The NanoGlo assay was performed 4–5 h after compound treatments. **G9** and SY-1365 were obtained after screening. (B). NanoGlo titration curves of UNC10112785 (4–5 h treatment) in f-Myc-Nluc or f-Nluc transfected 293T cells, respectively. Curves were averaged from three independent experiments. (C). Top, western blotting test of the 25 hits selected after dose–response. The degradation effect on f-Myc-Nluc was shown by the anti-flag antibody. The names of the compounds represent their locations in our compound library. Compounds with good degradation effect are shown in bold and numbered as **1–14**. Bottom, western blotting test of the 14 degraders screened from the top panel. Their degradation effect on endogenous Myc was confirmed using E1A lysate from Ramos cells. The compound treatment time was 4–5 h. Notes: UNC10112785: CDK9 inhibitor, positive control for HTS assay verification; **G9**/EOAI3402143: Myc-DUB inhibitor; SY-1365: CDK7/9 inhibitor. (D). Chemical structures of all 14 novel Myc-downregulating small molecules obtained from screening assay. The molecular weights (MW) of the 14 novel hits are shown next to their name on the right and their structures are shown in color. The names of **C3**, **C13** and **C14** are colored red because these compounds contained PAINS alerts from the PAINS testing online tool: **https://www.swissadme.ch/index.php**.[31]

(such as Myc) in a crude extract.[32] MYC361i, a small molecule inhibitor that was previously shown to disrupt Myc/Max dimerization and directly bind Myc,[10,19] was utilized as our positive control for the CETSA. The optimal heating temperature for the CETSA on Myc stability was pre-determined by adding DSMO or 100 μM of MYC361i to 293T whole cell lysate, followed by heating at different temperature gradients for 3 min. The results showed that the optimal temperature to achieve soluble Myc proteins (not over-precipitated to the level of being non-detectable) preserved by MYC361i from heating (relative to the DMSO control) was 56 °C (Fig. S4A). Next, MYC361i and **C1–C14** were titrated from 0.01 μM up to 100 μM over 10-fold escalation gradients, heated at 56 °C for 3 min, and the preservation effects of the compounds were analyzed using the CETSA (Fig. 2 and Fig. S4B). The results showed that additions of the control compound MYC361i, **C1**, **C3**, **C7**, **C8** or **C11** had the tendency to gradually increase the soluble Myc protein levels in the 293T cell lysate after heating at 56 °C for 3 min (*i.e.* increased Myc thermal stability) while most of the other Myc degraders tested did not have any effect on Myc thermal stability (Fig. 2: **C2**, **C4–C6**, **C9–C10** and **C12–C14**; Fig. S4B), suggesting that **C1**, **C3**, **C7**, **C8** and **C11** might bind and stabilize the Myc protein in the extract. We noticed that a strong stabilization effect only occurred at high doses for some compounds (Fig. 2 and Fig. S4B), which was consistent with the dose–response results found using cell lysates when CETSA first invented.[32] To sum up, only five out of the 14 Myc-downregulating compounds might bind Myc and qualify as candidate MGDs of Myc that warrant further investigation. The other nine Myc-downregulating compounds might regulate the Myc protein level through other indirect molecular mechanisms, similar to UNC10112785, SY-1365 and **G9**.[11,27–29]

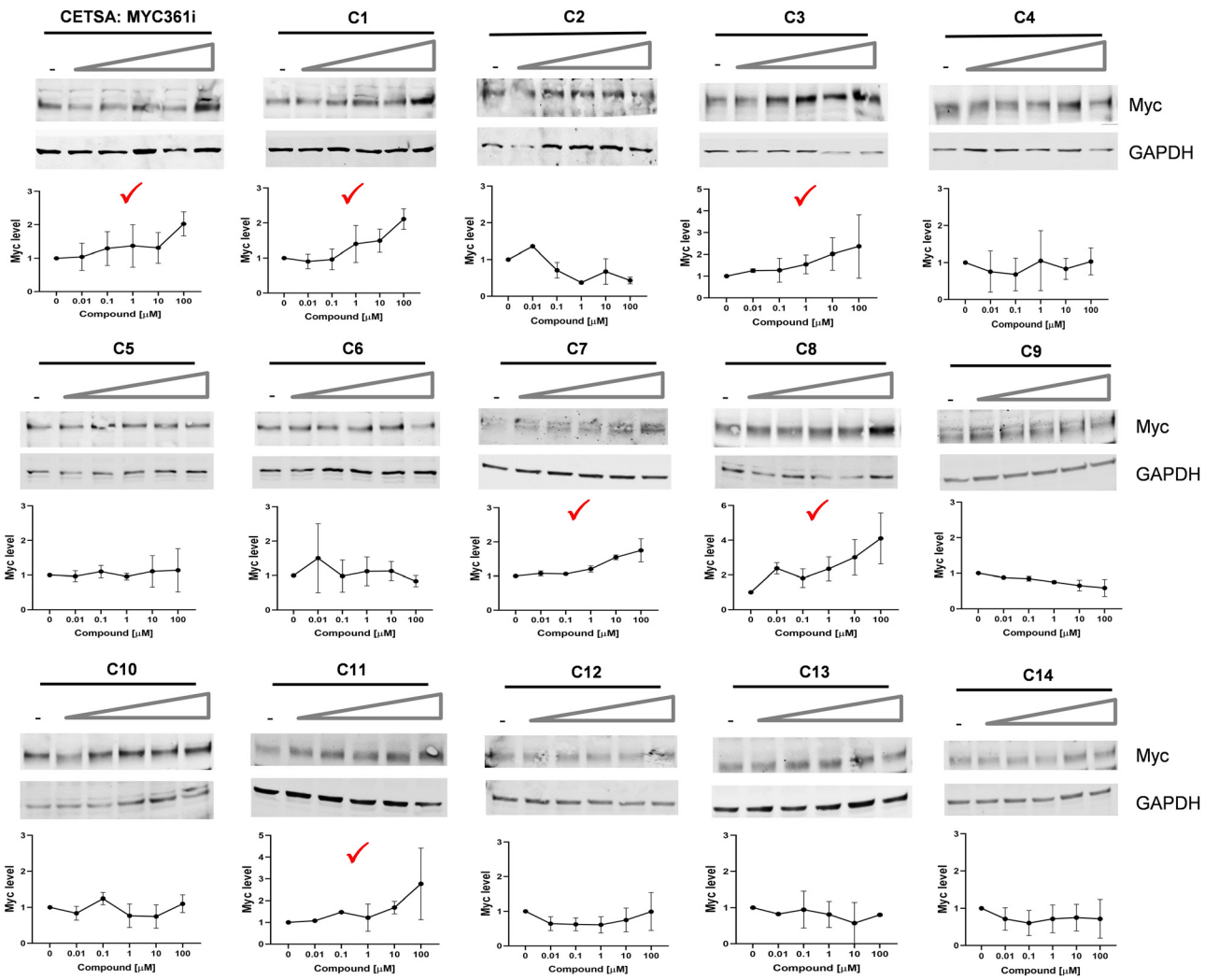


Fig. 2 CETSA analysis shows that the **C1**, **C3**, **C7**, **C8** and **C11** Myc-downregulating compounds bind and stabilize Myc protein in crude cell lysate. Representative CETSA results using 293T E1A lysate for MYC361i and the 14 Myc-downregulating small molecules. The heating temperature for CETSA was pre-determined as shown in Fig. S4A and was set to 56 °C for 3 min. MYC361i and **C1** to **C14** were titrated from 0.01 μM to 100 μM with 10-fold increases in dose. The symbol "-" represents DMSO-treated samples. Compounds MYC361i, **C1**, **C3**, **C7**, **C8** and **C11** increased the amount of soluble Myc protein and are marked with a red checkmark. The intensity curves under the blots are averaged from two or more independent CETSA experiments. The intensity of the Myc blots was calculated by dividing by the intensity of the GAPDH blots. Protein intensity was calculated using ImageJ.

Small molecule degrader C1 selectively degrades endogenous Myc, preferentially kills cancer cells with high Myc expression and selectively reduces Myc-target gene expression

The five Myc-binding degraders (**C1**, **C3**, **C7**, **C8** and **C11**) screened out by CETSA were further narrowed down according to their PAINS-alert testing results and pharmacological potential (Fig. 2). The chemical structure of **C3** contained PAINS alerts and the rigid ring structures and chemical structure of **C11** is complicated with more than four rings inside the molecule (Fig. 1D). Thus, **C3** and **C11** were excluded from immediate follow-up investigation. The remaining three compounds **C1**, **C7** and **C8** were either purchased or synthesized and subjected to label-free quantitative (LFQ) mass spectrometry analysis to test their general degradation of cellular proteins. LFQ data from Ramos following 8 h of compound treatment demonstrated that 269 cellular proteins (including Myc) were significantly downregulated by **C1**, 533 proteins by **C7**, 1709 proteins by **C8**, and 1783 proteins by **G9** at 10 μM (Fig. S5A; SI Excel files S1–S4). To further assess the specificity of these compounds, we analyzed the overlap between compound-downregulated proteins and 3458 direct Myc-binding genes previously identified by ChIP-PET from P493 B cells.[33] (Note: Of the total 4296 Myc-binding loci reported in Table S7 of Zeller *et al.*, 2006,[33] 3458 unique Myc-binding genes were obtained after removing the redundant binding loci). A Venn diagram tool (**https://bioinformatics.psb.ugent.be/webtools/Venn**) was utilized for the overlap analysis and the results showed that the overlap percentages of the compound-downregulated proteins with the 3458 Myc-binding genes were 11.7% for **G9**, 15.2% for **C1**, 15.3% for **C7** and 13.3% for **C8** (Fig. S5B). Considering that the LFQ analysis was conducted at an early stage of 8 h and at the protein level but not at the mRNA level (which responded faster), the 15% overlap with the direct Myc-binding genes by **C1** and **C7** was relatively high. Higher overlap percentages indicate a higher possibility that the compounds function through Myc-dependent pathways and have better Myc-targeting specificity. However, the fact that the majority of downregulated proteins did not overlap with the direct Myc-binding genes indicated that Myc-dependent secondary transcriptional changes had likely occurred by 8 h and that off-target effects occur for all compounds tested. Notably, several protein degradation-related biological processes were identified from a gene ontology analysis using the **C1** significantly-altered protein list from our proteomic data (Fig. S5C, red arrows), which enhanced the possibility that **C1** functions *via* protein degradation. Furthermore, previous data showed that **C1** affected Myc at the protein level rather than by reducing mRNA transcription (Fig. 1C and Fig. S3A, Myc mRNA level was increased but not decreased by **C1**). Therefore, we first focused our interest on compound **C1** due to the limitation of our research resources (Fig. 1D). NanoGlo assay re-testing from multiple titration experiments showed that **C1** had a DC_{50} for f-Myc-Nluc of around 9.1 μM and did not inhibit vector f-Nluc (Fig. 3A for purchased **C1**; Fig. S5D for synthesized **C1**). Subsequent western blotting showed that **C1** selectively degraded Myc protein at a DC_{50} of around 5 μM, while it had no significant reduction effect on the other cellular proteins tested, such as GAPDH, BRD4, c-FOS and TBP (Fig. 3B). Furthermore, the Myc-interacting partner protein Max could also be degraded by **C1** and the degradation started around 2 h after the addition of **C1** in Ramos cells (Fig. 3C). Western blotting analysis of **G9**, **C7** and **C8** showed that they had similar Myc DC_{50} values (5 μM) to **C1** but much stronger non-specific degradation of other control cellular proteins tested, such as BRD4, TBP and c-FOS (Fig. 3D and Fig. S1B). By this point, we had identified that novel compound **C1** could selectively degrade Myc/Max with a DC_{50} of around 5 μM in cells.

Next, we wanted to check whether **C1** could selectively kill Myc-dependent cancer cell lines. Myc mRNA expression levels of different cell lines from The Human Protein Atlas are listed in Fig. 4A. Six cell lines were selected: including Ramos lymphoma cancer cells (nTPM: 461.8), colorectal cancer cells HCT116 (nTPM: 361.8), leukemia cells K562 (nTPM: 260.5), pancreatic cancer cells Mia-PACA2 (nTPM: 204.6), 293T cells (nTPM for HEK293: 154.0) and lung cancer cells A549 (nTPM: 109.6). CTG cell viability assay results showed that the GI_{50} values of **C1** were inversely correlated with the Myc mRNA expression levels of these cells (Fig. 4A and B and Fig. S6). Ramos lymphoma cancer cells were killed with the lowest GI_{50} value of around 7.6 μM, followed by HCT116 and K562 at 14.7 μM and 27.6 μM, respectively. The GI_{50} values for MIA-PACA2, 293T and A549 were all greater than 40 μM, with **C1** showing more than 5.2-fold selectivity for Ramos cells relative to these lines (Fig. 4C). Intriguingly, Myc-DUB inhibitor **G9** also showed selectivity (maximum 5.2-fold selectivity) in killing cancer cells with high Myc expression, while the other positive control Myc degrader MYC975i[10] did not show any selectivity (maximum 1.3-fold selectivity) for killing high Myc expression cancer cells (Fig. S6A–C).

As **C1** exhibited decent selectivity for killing Myc-dependent cancer cells, we rationalized that it would also preferentially reduce Myc-target gene expression. Indeed, the expression of Myc-target genes, such as CAD, CCNB1, CDK4, TERT, UBE2C and GAPDH (note: The *t*-half of the GAPDH protein is greater than 20 h, thus it can still serve as the loading control in our western blotting experiments where we generally treat compounds for less than 8 h, even though its mRNA expression was decreased by **C1**), were significantly reduced by **C1** while the non-Myc-target genes ACTB and HPRT1 were not affected by **C1** treatment (Fig. 4D). Together, our data suggested that **C1** was an excellent Myc degrader that not only preferentially killed Myc-dependent cancer cells but also selectively reduced the expression of Myc-target genes.

C1 functionally acts like a molecular glue to aggregate Myc protein, block Myc/Max interaction, and cause Myc/Max degradation

CETSA analysis of crude extracts was used previously to show that **C1** binds Myc (Fig. 2 and Fig. S4). To test whether **C1** directly bound purified Myc, recombinant Myc and Max were separately purified from *E. coli*, and the Myc/Max dimer was reconstituted for additional analysis using a regular thermal shift assay (also called differential scanning fluorimetry: DSF) to

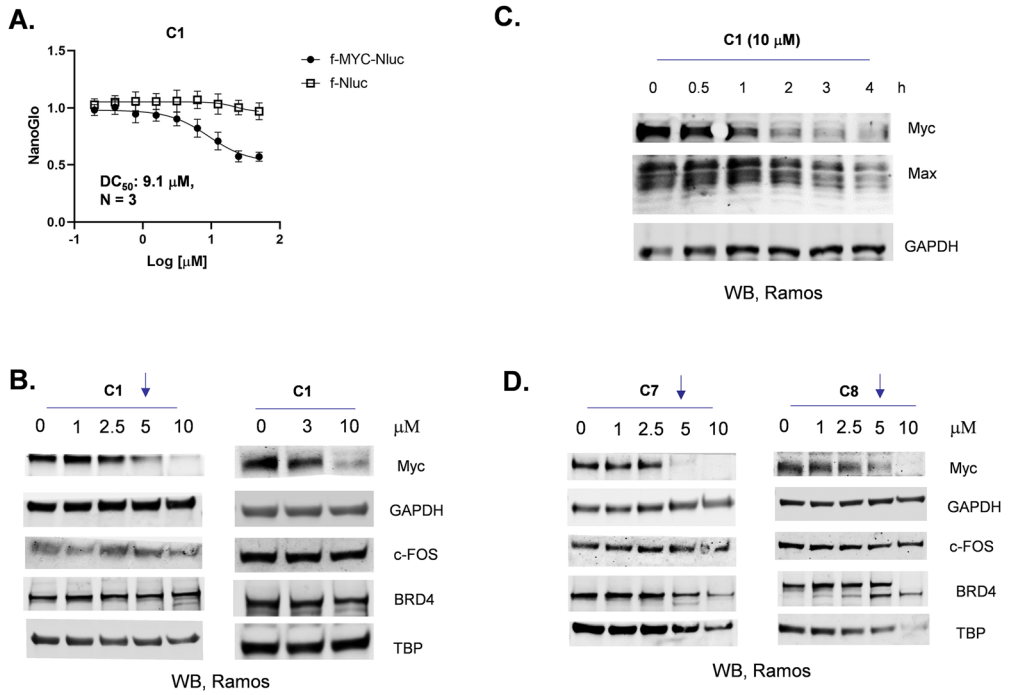
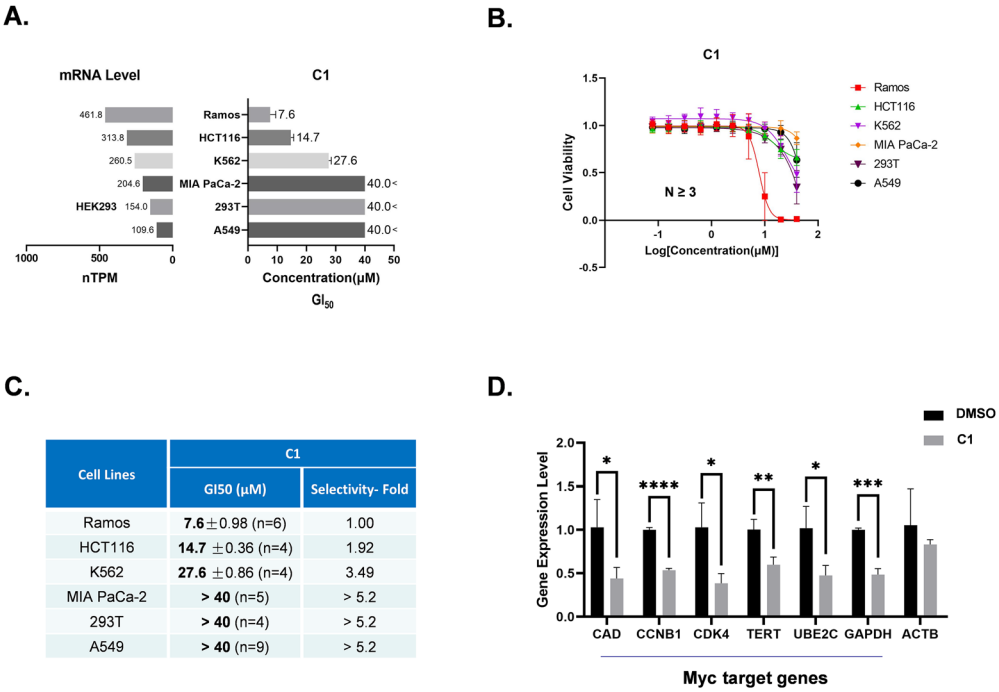


Fig. 3 Characterization of small molecule Myc degrader **C1** by NanoGlo assay and western blotting. (A). NanoGlo titration curves of **C1** in f-Myc-Nluc and f-Nluc transfected 293T cells. Curves were averaged from three independent experiments. **C1** was purchased from screeningcompound.com. **C1** had a f-Myc-Nluc DC_{50} value of 9.1 μM similar to the value obtained for **C1** from the Chemdiv. library (DC_{50} of 12.9 μM by NanoGlo). (B). Dose–response western blotting test of **C1** using lysate from Ramos cells after 8 h of compound treatment. The proteins were detected using the indicated antibodies. The arrow indicates that western blotting analysis shows that **C1** has a DC_{50} of around 5 μM. (C). Western blotting test as a function of time for **C1** Myc and Max protein degradation using lysate from Ramos cells. GAPDH was the loading control. (D). Dose–response western blotting test of Myc degraders **C7** and **C8** using Ramos lysate after 8 h of compound treatment. The proteins were detected by the indicated antibodies. The arrows indicate that western blotting analysis shows that **C7** and **C8** have similar DC_{50} values to **C1** of around 5 μM.

show direct small molecule protein binding (Fig. S7A). Regular thermal shift analysis showed that the addition of compounds MYC361i, **C1**, **C8** but not **C7** to the system could significantly shift the curves to the right, suggesting that MYC361i, **C1** and **C8** but not **C7** increased Myc thermal stability and interacted with the purified Myc protein (Fig. S7B). To sum up, CETSA and DSF both indicated that Myc degrader **C1** directly binds the Myc protein.

Conventional MGDs not only bind and degrade target proteins but also interact with E3 ubiquitin ligases.[17,20,34] Thus, we generated a Flag-Myc (f-Myc) construct to perform an f-Myc co-immunoprecipitation (Co-IP) assay in the presence of increasing doses of **C1**, testing whether conventional Myc E3 ligases, such as FBXW7[35] and CHIP/STUB1,[36] could be pulled down by f-Myc in the presence of **C1** to form a ternary complex. An unrelated E3 ligase TOPORS[37] served as the interacting control for the Co-IP. The Co-IP results showed that FBXW7 interacted with f-Myc with medium affinity, CHIP weakly interacted with Myc, and as expected, TOPOR3 did not interact with Myc (Fig. 5A). Unexpectedly, Myc itself was shown to be pulled down by f-Myc in a **C1** dose-dependent manner (Fig. 5A and B), *i.e.*, more and more Myc protein could be pulled when the dose of **C1** to input cell lysate aliquots of the same batch was increased from 5 μM up to 125 μM (Fig. 5A and B and Fig. S8A and B). These data indicated that **C1** not only bound Myc but also glued two or more Myc proteins together; otherwise, the same amount of Myc protein would be pulled down by the same amount of Co-IP resins from equal amounts of lysate aliquot, even in the presence of increasing amounts of **C1**. Intriguingly, the bound Max (relative to Myc) pulled down by the Co-IP resins gradually decreased in the presence of increasing doses of **C1**, displaying an inverse relationship with Myc levels (Fig. 5A and B and Fig. S8B), suggesting that Myc/Myc self-aggregation caused by **C1** could block Myc/Max interactions. Notably, the interactions of Myc with the other Myc-interacting proteins FBXW7 and CHIP slightly increased and remained unchanged, respectively, (Fig. 5A). In addition, we performed the same f-Myc Co-IP experiments on another Myc-downregulating compound **C7** (Fig. 1D and 2), and we did not observe the aggregation of the Myc protein by **C7**, suggesting that the Myc gluing molecular degradation mechanism was unique to **C1** (Fig. S8C). Furthermore, to observe Myc aggregation/multimerization using fluorescence microscopy, 293T cells were transfected with pCDH-MycGFP-IRES-mCherry so that they could simultaneously express the Myc-eGFP fusion protein and mCherry alone without Myc-fusion in the same cells at the IRES site. Confocal fluorescence scanning imaging showed that there were many more cells showing bright green dots and that the green light in the 10 μM **C1**-treated cells after 4 h was relatively darker than in the DMSO-treated cells (green lights were evenly distributed inside the DMSO-treated cells and were relatively brighter after 4 h of treatment). Meanwhile, the mCherry without Myc-fusion was not aggregated into bright red dots and was not brighter in the



Cell Lines	C1	
	GI50 (μM)	Selectivity- Fold
Ramos	**7.6** ±0.98 (n=6)	1.00
HCT116	**14.7** ±0.36 (n=4)	1.92
K562	**27.6** ±0.86 (n=4)	3.49
MIA PaCa-2	**> 40** (n=5)	> 5.2
293T	**> 40** (n=4)	> 5.2
A549	**> 40** (n=9)	> 5.2

Fig. 4 **C1** preferentially kills high-Myc expression cancer cells at lower doses and selectively reduces Myc-target gene expression. (A). GI_{50} obtained values from CTG cell viability assays in the presence of **C1**. The mRNA expression levels (nTPM) of different cell lines (Ramos, HCT116, K562, MIA-PaCa-2, HEK293, A549) are obtained from The Human Protein Atlas (**https://www.proteinatlas.org**). The GI_{50} values on the right side were averaged from three or more independent CTG experiments. (B). CTG titration curves of **C1** topped at 40 μM with double-dilution on the six indicated cell lines. The curves were averaged from three or more independent experiments. Cells with 100% viability after DMSO treatment were set to 1. (C). Table listing the GI_{50} values and the decrease in selectivity of **C1** toward different cancer cells relative to the Ramos cells. The change in selectivity of **C1** toward the Ramos cells was set to 1.0. (D). Q-rt-PCR results for the indicated genes. Total RNA for reverse transcription was extracted from the Ramos cells 8 h after treatment with 15 μM of **C1**. Myc-target genes: CAD, CCNB1, CDK4, TERT, UBE2C, GAPDH; Non-Myc target: ACTB; PCR internal control: HPRT1 (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). Representative data from at least two independent experiments is shown.

DMSO-treated cells (Fig. S8D). These observations indicated that **C1** caused MycGFP to aggregate/polymerize but not the mCherry alone, which suggested that **C1** could cause Myc protein aggregation/multimerization.

It had been shown that the disassociation of Myc/Max dimer caused by compounds makes Myc and Max unstable and prone to degradation through the proteasome pathways.[8,10] Thus, we inhibited the proteasome pathways using MG132 to test how this treatment would affect Myc degradation by **C1**. Interestingly, inhibition of the proteasome pathway by MG132 not only prevented Myc degradation by the **G9** DUB inhibitor but also restored **C1**-mediated Myc reduction (Fig. 5C), suggesting that proteasomes might be involved in the degradation of Myc by **C1**. Although Myc destruction by **C1** could be blocked by MG132, aggregated proteins might also be degraded and cleared up through the autophagy pathways.[38,39] Together, the data indicated that our newly identified Myc degrader **C1** could bind and aggregate Myc protein, block Myc/Max interaction and promote Myc and Max protein degradation. Thus, **C1** might be a novel atypical MGD of Myc (Fig. 5D).

Discussion

Dysregulated in more than 70% of cancer cells, Myc oncoprotein is considered a critical regulator in cancer initiation and progression.[2] Thus, tremendous efforts have been invested with the hope of developing an effective drug targeting this traditionally "undruggable" regulator. Diverse targeting strategies have been developed, including blocking Myc transcription by inhibition of upstream BRD4,[13,40,41] targeting Myc mRNA with siRNA,[42] disrupting Myc/Max dimerization,[5–10] blocking Myc/Max-DNA binding and Myc translation,[14,43] destroying the Myc protein[12] *etc.* Of the known direct Myc inhibitors, WBC100 and OmoMyc have entered clinical trials.[12,16] Direct inhibition and destruction of the Myc protein seem to be effective approaches. After the development of a cell-based HTS assay by constructing a target-NanoLuc-fusion plasmid, more than 100 K compounds were screened with the hope of identifying Myc MGDs (Fig. 1 and Fig. S1). Through our screening activity, 14 small Myc-downregulating molecules that directly or indirectly targeted Myc were identified (Fig. 1 and 2), which suggested that our target-Nluc-fusion HTS technology worked well in identifying target-downregulating small molecules of proteins of interest (POIs). The reason for this was likely that the innovative NanoLuc was small, very stable with a *t*-half of > 2 h[23] while the Myc has a much shorter *t*-half of around 0.5 h.[8] Any compound that affected the stability of the Myc protein could be selectively picked up early within the treatment process and toxic primary hits could be excluded through reverse-screening using an f-Nluc only construct without Myc-fusion (Fig. 1, within 5 h; Fig. S1D–F). Although there was a possibility that

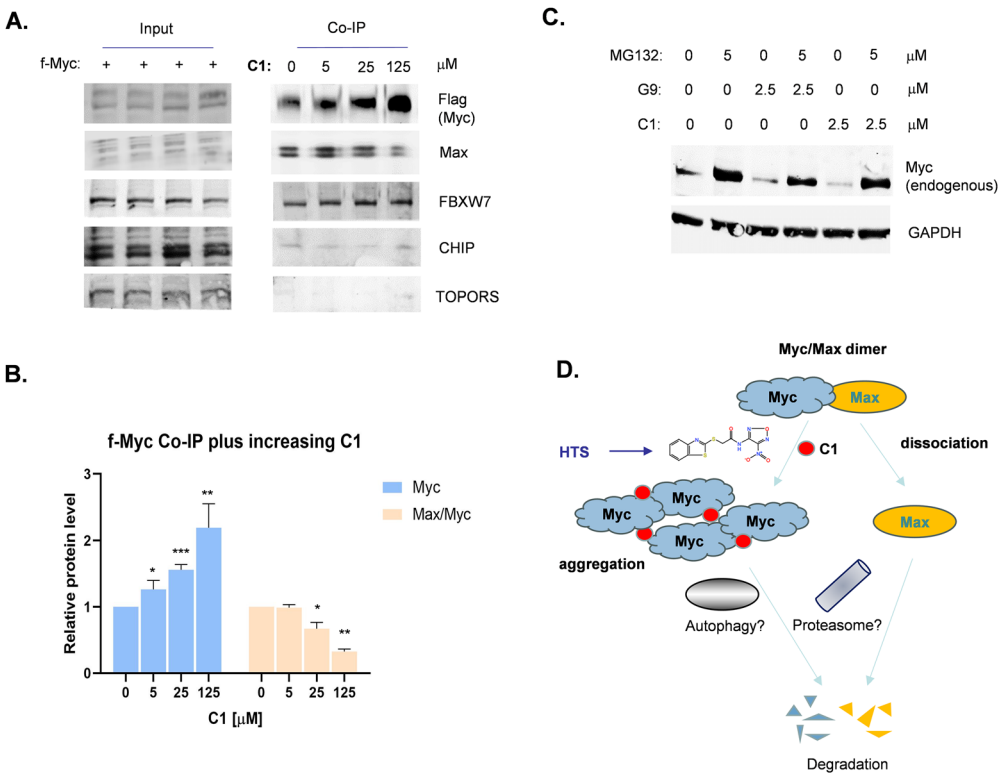


Fig. 5 **C1** functions as a molecular glue to aggregate Myc proteins, block Myc/Max interaction, and cause Myc/Max protein degradation. (A). Results from an F-Myc Co-IP in the presence of increasing concentrations of **C1** using input lysate aliquots from the same batch with the abundance of the indicated protein bands shown by western blotting. (B). The averaged intensity bar graph of Myc (normalized to 1 for **C1** at 0 μM: DMSO-only) and bound Max relative to Myc (Max/Myc: Max intensity was divided by Myc intensity at the corresponding doses of **C1**). The intensity was calculated using Image J (the one-sided *p* values of Myc at 5 μM of **C1**: $*p = 0.015 < 0.05$, at 25 μM: $***p = 0.0001 < 0.001$, at 125 μM: $**p = 0.0023 < 0.01$; the one-sided *p* values of Max/Myc: at 5 μM of **C1**: $p = 0.35 > 0.05$, at 25 μM: $*p = 0.019 < 0.05$, at 125 μM: $**p = 0.0001 < 0.001$). The values were averaged from three independent f-Myc Co-IP experiments (the original data blots of the other two independent Co-IP experiments are shown in Fig. SB: Co-IP Exp. 1 and Exp. 3). The *p* values were calculated from the website: **https://www.graphpad.com/quickcalcs/ttest1.cfm**. (C). Western blot testing of Myc protein level after 8 h of the indicated compound treatments. The levels of the proteins were detected using the indicated antibodies. (D). A graphical summary of the molecular mechanisms of how **C1** functions as a molecular glue to promote Myc/Max complex degradation.

compounds that selectively inhibited the f-Myc-Nluc construct but did not downregulate endogenous Myc might be falsely picked out from our assay (Fig. 1), more than 50% of the final confirmed hits demonstrated true Myc-downregulating abilities as tested by western blotting and also had a strong down-regulating effect on endogenous Myc (Fig. 1C, bottom). Thus, this target–Nluc-fusion HTS strategy was very useful and also transferable to the identification of small molecules that can downregulate target protein levels of other POIs.

After screening more than 100 K compounds, we identified that **C1** could not only bind and degrade the Myc protein but also disrupt Myc/Max interactions (Fig. 2, 3 and 5). Although the DC_{50} of **C1** was around 5 μM (Fig. 3B), it showed high selectivity in killing Myc-dependent cancer cells (Fig. 4 and Fig. S6). Western blotting results (Fig. 3B) showed that the levels of two other short-life proteins c-FOS (*t*-half: 1 h) and BRD4 (*t*-half: 4 h)[44,45] were not reduced by **C1** after 8 h of treatment, suggesting that the degradation of Myc by **C1** was likely not caused by artifacts arising from the toxicity of the compound to short-life proteins.[46] Compounds such as 10058-F4/10074-G5, Mycro3, KJ-Pyr-9, sAJM589 and MYC361i, have previously been shown to disrupt Myc/Max interactions.[5–8,10] Our molecular mechanism studies showed that **C1** could similarly disrupt Myc/Max interaction. Moreover, **C1** could also aggregate the Myc protein (Fig. 5A and B and Fig. S8B and D). The ability of **C1** to promote the self-aggregation of the Myc protein had not been previously observed for the other pre-clinical Myc/Max inhibitors. Furthermore, another novel Myc-downregulating compound **C7** identified from our screening could not aggregate Myc like **C1** (Fig. 2 and Fig. S8C), suggesting that the molecular mechanism by which **C1** functions was different from other Myc inhibitors/degraders. On the one hand, **C1** disrupted Myc/Max to make the dimer unstable; on the other hand, **C1** aggregated Myc to accelerate its clearance. These two effects together made **C1** a specific Myc degrader.

Typically, MGDs can enhance/induce interactions between target proteins and E3 ligases.[17,20] Unexpectedly, we found Myc can be pulled down by itself in the presence of increasing concentrations of **C1** during our search for potential E3 ligases (Fig. 5A and B). Given the standard way that MGDs function, *via* the induction of novel PPIs and by causing target protein degradation,[17,20] **C1** could still qualify as an atypical MGD for

Myc. Indeed, a previous discovery has shown that simply gluing target proteins to form aggregates could also promote target protein degradation. For example, BI-3802, an MGD that induces the polymerization and degradation of transcription factor BCL6,[20,47] functions in a similar manner to **C1**. Our findings suggested that **C1** might also be an atypical MGD for Myc (Fig. 5 and Fig. S8). Further investigation is needed to understand whether aggregation of Myc by **C1** enhances its interactions with conventional E3 ligases of Myc (*e.g.* FBXW7 interactions with Myc were slightly increased by **C1**, as shown in Fig. 5A) or whether it induces novel interactions with other unrelated E3 ligases. Proteomics technologies using an f-Myc Co-IP in conjugation with mass spectrometry or CRISPR screenings can facilitate the discovery of potential E3 ligases that bind the aggregated Myc. Notably, aggregation of proteins can promote their degradation through autophagy pathways.[38,39,48,49] Thus, without further investigation, we cannot rule out the possibility that aggregation of Myc by **C1** might also promote Myc degradation through autophagy pathways.

It is worth noting that around 5% of FDA-approved drugs contained PAINS alerts.[50] The three Myc-binding degraders **C1**, **C7** and **C8** do not have traditional PAINS alerts (Fig. 1D and Fig. 2). However, both **C1** and **C7** contain a reactive nitro group and might be considered as ''bad actors'' in potential drug development.[31,50] We first focused on **C1** because the **C1**-downregulated proteins showed greater overlap with direct Myc-binding genes[33] and are involved in protein stability-regulation pathways (Fig. S5B and C). Moreover, the limitations of our research resources also hindered us from simultaneously investigating all three hit compounds. Therefore, it would also be very interesting to study the potential molecular mechanisms of how Myc was degraded by **C7** and **C8**. LFQ mass spectrometry showed that **C1**, **C7** and **C8** also affected other cellular proteins besides direct or indirect Myc-regulated proteins (Fig. S5A and B), indicating the existence of off-target effects and thus, extensive chemical modifications in the future might also be needed to improve the potency and specificity of **C1**, **C7** and **C8** because they have similar DC_{50} values in the single digit μM ranges, as shown by western blotting experiments (Fig. 3). Although **C1** contains a reactive nitro group, it has a relatively low molecular weight of 337.3 Da. Thus, there is still huge potential for medicinal chemistry optimization to replace the nitro group to further improve the pharmacological potential of **C1** in the future. Notably, it might also be very interesting to further investigate the other nine Myc-downregulating compounds that did not bind the Myc protein, as we only focused on direct Myc-binding compounds in this study.

In summary, a Myc-NanoLuc fusion plasmid transfected cell-based HTS assay was developed to identify small molecules that could downregulate Myc oncoprotein. This goal was achieved once the 14 primary hits had been identified. Therefore, an alternative approach to identify novel Myc-targeting small molecules through HTS was demonstrated in this study. Co-IP and other small molecule-protein binding assays indicated that **C1** might function as a novel atypical MGD for Myc. More efforts are needed to better understand the detailed molecular mechanisms by which **C1** functions and to further test the efficacy/stability of **C1** *in vivo* with the hope of making it a clinical drug.

Methods

Commercial antibodies used in this study

Myc (Ab32072, Abcam), Max (10426-1-AP, ProteinTech), GAPDH (5174S, CST), c-Fos (66590-1-1g, ProteinTech), BRD4 (28486-1-AP, ProteinTech), TBP (22246-1-AP, ProteinTech), Flag (14793S, CST), STUB1/CHIP (55430-1-AP, ProteinTech), FBXW7 (55290-1-AP, ProteinTech), TOPORS (ab86383, Abcam), DZIP3 (Abs148295, Absin).

Compounds and libraries used in this study

UNC10112785 (AOBIOUS, AOB13455), G9/EOAI3402143 (TargetMol, 1699750-95-2), SY-1365 (MedChem, HY-128587), MYC361i and MYC975i (Selleckchem, S8905 and S8906), and MG132 (Selleckchem, S2619). Compounds **C1**, **C7** and **C8** (**https://screeningcompound.com**, 8015-8353, 8018-6021 and 8018-5652, respectively) were purchased from the indicated vendors. All compounds were diluted with DMSO according to the manufacturer's instructions. Bioactive and ChemDiv compound libraries were constructed and prepared by the HTS and CMG team of Shenzhen Bay Laboratory.

HTS of *MYC* small molecule degraders using a NanoGlo Assay

A large amount of 293T cells was transiently transfected with pCDNA3-f-Myc-Nluc or pCDNA3-f-Nluc for screening and prepared in advance. For preparation, around 20 μg of plasmids (either pCDNA3-f-Myc-Nluc or pCDNA3-f-Nluc) were mixed with 50 μL of Lipofectamine^{2000} and transfected into one 15-cm plate of 293T cells. Ten 15-cm plates were transfected in one preparation. Twenty-four hours after transfection, cells were detached by trypsin, and an aliquot of 1 mL of each was placed into a storage tube (around 20 million cells/tube), and cryopreserved inside a −80 °C freezer for future usage. The HTS assay was optimized to 1536-well plates (Corning 3727) from 384-well plates (Corning 3570). The HTS assay indexes in the 1536-plates for the **G9** positive control (luciferase readings of lines 3–4 from **G9**-treated cells *vs.* readings of lines 45–46 from DMSO-treated cells on 1536 screening plate) were *Z*-factor = 0.913 (> 0.5 for all screening plates), assay windows = 3.5 and CV% < 10%. The *Z*-factor was calculated through the website: **https://www.screeningunit-fmp.net/tools/z-prime.php**. When performing the HTS assay, the cell density was first adjusted to 500 cells per μL, 5 nL of library compounds (final at 12.5 μM) were added by Echo to columns 5–44 of the Corning 3727 plate. Additionally, 5 nL of the positive control compounds (final concentration of 12.5 μM) were also added to columns 1–4 and the negative control DMSO was added to columns 45–48, respectively. A total of 4 μL of medium-diluted cells (250–500 cells per μL, 1000–2000 cells per well) was dispensed to each well of the 1536-plate from column 1 to 48 by the EL406cell dispenser (note: one tube of frozen 293T can be diluted with about 40–80 mL

of medium and is sufficient for the screening of 5–10 Corning 3727 plates). Cells should be filtered with a 40–70 μm cell filter-top before seeding to reduce cell dispenser clogging. The plates were incubated inside a 37 °C cell incubator for 4–5 h. After incubation was completed, 4 μL of the NanoGlo substrate (1 : 2 water further diluted plus regular dilution, Promega N1120) was added to each well of the 1536-plate by the liquid dispenser. The luminescence was measured instantly by a Neo2 or Envision plate reading system (shaken at 200 rpm for 30 s then the whole plate was measured). Data was recorded in Excel files for further analysis. Please see the SI for other methods used in this study.

Author contributions

Conceptualization: Weijun Shen, Seung H. Choi, Muyu Xu; data curation: Muyu Xu, Lin Tan, Hongda Liao; formal analysis: Muyu Xu, Jeffrey Hill, Weijun Shen; funding acquisition: Weijun Shen; investigation: Muyu Xu, Jinying Qiu, Lin Tan, Jiayu Xu, Yi Wang, Wenyue Kong, Hongda Liao, Anran Chen, Xiaolan Chen, Jiying Zhang, Cookson K. C. Chiu, Meiying Zhang, Yingying Tian, Caohui Li, Biao Ma, Leiming Wang, Jingpeng Fu, Seung H. Choi, Jeffrey Hill, Weijun Shen; methodology: Muyu Xu, Lin Tan, Cookson K. C. Chiu, Yingying Tian, Biao Ma, Leiming Wang, Jingpeng Fu; project administration: Muyu Xu, Jeffrey Hill, Weijun Shen; supervision: Jeffrey Hill, Weijun Shen; writing – original draft: Muyu Xu, Jiying Zhang; writing – review & editing: Muyu Xu, Jeffrey Hill, Weijun Shen.

Conflicts of interest

There are no conflicts of interest to declare. The newly identified Myc-downregulating compounds (**C1–C14**) were not under patent application while publishing this manuscript.

Data availability

All relevant data are contained within the paper and its SI. Supplementary information: Fig. S1–S8 and Supplemental methods are included in the SI. LFQ data of **C1**, **C7**, **C8** and **G9** are shown in SI Excel files S1–S4. See DOI: **https://doi.org/10.1039/d5cb00093a**.

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