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Cell-Based Methods for the Identification of MYC-Inhibitory Small Molecules

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Abstract

Oncoproteins encoded by dominant oncogenes have long been considered as targets for chemotherapeutic intervention. However, oncogenic transcription factors have often been dismissed as “undruggable.” Members of Myc family of transcription factors have been identified as promising targets for cancer chemotherapy in multiple publications reporting the requirement of Myc proteins for maintenance of almost every type of tumor. Here, we describe cell-based approaches to identify c-Myc small molecule inhibitors by screening complex libraries of diverse small molecules based on Myc functionality and specificity.

Keywords

c-Myc; MYCN; Small molecules; Cell-based assays; Functional screening

1 Introduction

c-Myc is a member of the Myc family of transcription factors that regulate expression of multiple genes involved in many cellular processes, including promotion of proliferation, enhancement of cellular metabolism, and induction of apoptosis [1]. Among Myc proteins, c-Myc and MYCN are most frequently implicated in tumorigenesis [2]. Moreover, these proteins are structurally and functionally very similar such that replacement of the *c-myc* gene with the *MYCN* gene creates a viable mouse with no developmental abnormalities, suggesting that the c-Myc and MYCN proteins share all critical functions [3]. Because a large number of human tumors exhibit deregulated expression of Myc-family members (more than 50 % of all human malignancies [4]) and because of the high dependency of tumor growth on elevated Myc levels in several experimental systems [5, 6], Myc proteins are attractive targets for cancer chemotherapy. Accordingly, it has been demonstrated recently that whole-mouse genetic inhibition of transactivating properties of c-Myc resulted in rapid regression of incipient and established tumors, whereas the side effects to normal tissues were well tolerated and completely reversible even over extended time periods of c-Myc inhibition [7]. Thus, the inhibition of Myc appears to be a safe and efficient method to eliminate cancer. Several approaches have been pursued to develop anti-MYC therapeutics

²The readout cell line should be selected as a single cell clone rather than a population of cells such that there is a significant differential in luciferase activity between cells prior to transduction with *c-myc* or *MYCN* and after (ideally five- to tenfold). In our experience, the Myc reporter in the SHR6-17 cells was increasingly silenced the longer it was maintained in culture (i.e., drop in fold activation of reporter over time) so it is important to maintain a substantial collection of early passages in liquid nitrogen.

[8]; however, no drugs targeting c-MYC or MYCN have reached clinical trials. Therefore, identification of anti-Myc pharmaceutical agents capable of either direct tumor elimination or sensitization of a tumor to conventional chemotherapy is an important goal for anticancer drug development.

2 Materials

2.1 Cell Lines

Grow all cell lines in DMEM medium supplemented with 10 % fetal bovine serum:

1. SHR6-17 (SH-EP human neuroblastoma cell line that expresses low level *C-MYC* and no *MYCN* stably transfected with a MYC-responsive luciferase reporter to follow the effects of library compounds on MYC transactivation).
2. SH-CMV-luc (SH-EP cells with a constitutive luciferase reporter for identification of luciferase inhibitors or general transcription inhibitors).
3. HO15.19 (Rat-1 fibroblasts with both alleles of *c-myc* gene deleted via somatic recombination) [9].

2.2 Lentiviral and Retroviral Vectors

The plasmids used for the cell-based readout system and described in this chapter are as follows:

1. pTZV3 vector (kindly provided by Tranzyme, Inc).
2. pTZV3-eGFP-N3i (*N3i*; shRNA against human *MYCN* sequence GCAGCAGTTGCTAAAGAAA in replication-incompetent lentiviral vector TZV3-*eGFP*).
3. pTZV3-*eGFP-GFPi* (control shRNA).
4. pTZV3-CMV-*hMYCN* (generated by replacement of eGFP in vector pTZV3-eGFP with human *MYCN* cDNA (kindly provided by Dr. William Weiss, University of California at San Francisco, USA)).
5. Lentiviral packaging plasmids (pTRE-gag-pro-RRE-poly A, pCMV-vpr-RT-IN-poly A, pCMV-VSV-G-poly A, pCMV-tetoff-poly A, and pCMV-tat/rev) (kindly provided by Tranzyme, Inc).
6. The Myc-responsive reporter plasmid, pR6mHSP-luc: consisting of a minimal heat shock protein promoter containing six E-box sequences that were cloned from a modified ornithine decarboxylase (*ODC*) promoter construct (kindly provided by Mary Danks of St. Jude Children's Hospital, USA).
7. pLXSH-FLAG-c-MYCN and pLXSH-FLAG-N-myc (kindly provided by Dr. Michael D. Cole, Dartmouth College, USA).

2.3 Other Reagents

1. Bright-Glo™ Luciferase Assay System (Promega Corporation).

2. Polybrene (Sigma-Aldrich).
3. Propidium iodide (Sigma-Aldrich).
4. Methylene blue (US Biological).
5. Methanol.
6. Sodium dodecyl sulfate (SDS).
7. Phosphate buffer saline (PBS).
8. DMSO.

3 Methods

3.1 Overview

The readout system described here (SHR6-17, *see* **Notes 1 – 3**) is based on measuring anti-Myc activity by following the effect of small molecules on MYCN-mediated transactivation of an MYC-responsive luciferase reporter in neuroblastoma cells that are transduced with *MYCN* lentivirus prior to the addition of the small molecule library (Fig. 1). In this system, SHR6-17 cells, which express low levels of c-Myc and no MYCN, are transduced with lentivirus for human MYCN. After 24 h, when MYCN levels begin to increase, library compounds are added to the cells. At 48 h, luciferase activity is measured. If a library compound is inactive, the luciferase activity will continue to increase; however, if a compound is active, the luciferase activity will remain low. Strong hits are classified as those that reduce Myc-responsive luciferase activity to levels comparable to wells transduced with *MYCN* shRNA (*N3i*). However, because positive compounds may be quenchers or direct inhibitors of luciferase, “weak” hits are also validated (40–60 % reduction in luciferase activity). The readout cell line is validated by the demonstration of dose-dependent response of the Myc-responsive reporter to *MYCN* lentivirus (Fig. 2a) and a dose-dependent inhibition of MYCN-induced luciferase reporter by *N3i MYCN* shRNA (Fig. 2b), but not by nonspecific shRNA (Fig. 2c). To verify activity, “hit” compounds are passed through a series of filters to eliminate false positives, including dose-dependent effects on *MYC*-mediated transcription, luciferase inhibition/quencher, and general transcription inhibition assays followed by a cell-based assay for specific inhibition of proliferation of HO15.19 *myc*-null cells ectopically expressing mouse *c-myc* or *MYCN* cDNAs. HO15.19 is the only cell line that is capable of continuous proliferation, albeit slowly, in the absence of any Myc protein expression, a feature that makes this line a standard for studying Myc-dependent phenotypes [9]. Reconstitution of these cells with ectopically expressed c-Myc or MYCN completely reverses the slow-growth phenotype [9].

¹This type of system can be set up in any cancer cell line of interest. As a general rule, however, the cell line used for the readout should be adherent, transduced easily (i.e., ~100 % transduction efficiency), and should form a single cell suspension following trypsinization (i.e., not a cell line prone to clumping) so that it can be uniformly delivered across 96-well plates. These characteristics will enable a more reproducible readout across days of screening.

³For some cell lines, prolonged incubation with viruses can be toxic. If this is the case, virus containing medium can be removed and replaced with virus-free medium prior to addition of compounds.

3.2 Procedure for Screening Small Molecules for Myc Inhibition

1. Prepare a bulk batch of *MYCN*, *N3i*, and *GFPi* lentiviruses in sufficient quantities to cover the entire library screening. Titrate the amount of *MYCN* virus needed for optimal induction of the MYC-responsive reporter and the amount of *N3i* needed to block MYC-mediated transcription. The volumes of each lentivirus applied to the readout cells will depend on this optimization procedure.
2. Divide the viruses into aliquots, with the size of each aliquot being sufficient for 1 day of screening based on the titrations performed above (*see Note 4*).
3. For day 1 of screening, seed 7,500 SHR6-17 cells in each well of a 96-well plate in a volume of 100 μ l per well.
4. Remove cell medium and add *MYCN* and/or *N3i* or *GFPi* viruses plus 2 μ g/ml Polybrene following the template in Fig. 3. The amount of virus required will be determined from **item 1** above, and the volume adjusted to 100 μ l with DMEM + 10 % FBS. Each readout plate should include nontransduced cells and cells transduced with only *MYCN* lentivirus as well as positive control, *N3i* and nonspecific shRNA.
5. 24 h after infection, treat cells with library compounds at a final concentration of $\sim$ 10 μ M and incubate for additional 24 h.
6. Prior to determining luciferase activity, view each well under the microscope to identify compounds that are generally cytotoxic, based on the complete rounding up of cells and detachment from the well surface. These molecules are eliminated from the “hit” list as potential false positives.
7. Add 10 μ l Bright-Glo Luciferase Assay System reagent to each well and gently tap side of plates to mix the Bright-Glo with cell medium (*see Note 5*).
8. Read plates on a luminometer.
9. To identify hits, calculate the inhibition ratio (ratio of luciferase measurement values of test compound divided by that of the average *MYCN*-only controls on the same plate). Compounds with a ratio of $\leq$ 0.6 are classified as hits with compound evaluation prioritized based on their ratio (i.e., strong hits—inhibition ratios $<$ 0.4, similar to that of *MYCN* shRNA; weak hits—inhibition ratio 0.4–0.6). An example plate of screening results is presented in Fig. 3.
10. To validate the hits, the above procedure should be performed for selected compounds at three different doses to establish the dose-dependence of each hit compound. This is typically done by taking an aliquot of each putative hit directly from the library and testing it at 0.1, 1 and 10 μ M.

⁴Do not freeze-thaw the virus.

⁵We titrated the amount of Bright-Glo required to give optimal signal in our system (10 μ l). This may vary with different cell lines and from batch to batch so it is important to purchase sufficient Bright-Glo to cover the screen and to optimize for particular batches. Aliquot the Bright-Glo as repeated freeze-thaw cycles can affect activity.

11. Hits that demonstrate dose-dependence can be ordered from the library source for further characterization, pending filtering for false positives (*see* Subheading 3.3).

3.3 Filtering for False Positives

3.3.1 Luciferase Inhibition/Quenchers

1. Plate SHR6-17 cells in 96-wells and transduce with *MYCN* lentivirus as described in Subheading 3.2.
2. 48 h after transduction (i.e. when maximum luciferase expression is reached), add “hit” compounds to the cells at a final concentration of 10 μ M in duplicate. Incubate at 37 °C for 30 min. This time period is sufficient to inhibit the enzyme but too short to affect expression of luciferase.
3. After the incubation, add 10 μ l Bright-Glo Luciferase Assay System reagent to each well, mix, read plate, and analyze as in Subheading 3.2 . If the results still indicate inhibition of luciferase activity for a compound, then that compound is considered a false positive due to quenching of the signal or direct inhibition of luciferase. It is not necessary to distinguish between those two conditions.

3.3.2 General Transcription Inhibition—This filter can be performed with any cell line containing a luciferase reporter under the control of a constitutive promoter. As an example, we use SH-CMV-luc cells, which are SH-EP human neuroblastoma cells that contain a luciferase reporter driven by the CMV promoter.

1. Seed 10,000 SH-CMV-luc cells in each well of a 96-well plate.
2. The next day, add “hit” compounds to the cells at a final concentration of 10 μ M in duplicate. Incubate at 37 °C for 24 h.
3. After the incubation, add 10 μ l Bright-Glo Luciferase Assay System reagent to each well, mix, read plate, and analyze as in Subheading 3.2 . If the results still indicate inhibition of luciferase activity for a compound, then that compound is considered a false positive due to general transcription inhibition. Those compounds that are not toxic during screening and pass previous filtering are deemed validated hits and proceed to specificity testing as described below to evaluate their anti-Myc properties.

3.4 Cell-Based Assays for Inhibition of Endogenous Myc-Dependent Phenotypes

Analyze structures of confirmed hits and divide compounds into classes when two or more compounds share significant structural similarity. Based on the dose-response data from MYCN transactivation assay described in Subheading 3.2 , derive IC₅₀ values (the inhibitory dose resulting in 50 % decrease in Myc-specific luciferase activity) for each compound. Rank compounds within each chemical class by their IC₅₀ value for MYCN transactivation and identify, where possible, several best compounds within each class. The best of each of the structural classes and compounds with unique structures are subjected to the Myc specificity filter that uses HO15.19 *myc* -null cells transduced with pLXSH–vector, pLXSH–c-Myc, or pLXSH–N-Myc.

3.4.1 Proliferation Assay

1. Seed 4,000 HO15.19–vector cells and 2,000 pLXSH–c-Myc or pLXSH–N-Myc cells in each well of a 96-well plate.
2. The next day, prepare two fold serial dilutions of the compounds selected in Subheading 3.3 to achieve a range of concentrations (e.g. 0.08–20 μ M). Add the compounds to the cells and incubate at 37 °C for 72 h.
3. Remove medium from plates and add 100 μ l of 0.5 % methylene blue/50 % methanol in water, incubate for 30 min, wash three times with water, and air dry. Add 100 μ l of 1 % SDS in PBS per well for 10 min, measure optical density of each well at 650 and 540 nm, and then subtract the background at 540 nm from the absorbance at 650 nm (*see Note 6*).
4. Calculate an IC₅₀ value based on the above measurements (a dose of the compound that inhibits proliferation by 50 % under the above experimental conditions). These calculations can be done using GraphPad Prism software (Fit Spline–Lowess) or equivalent program.

3.4.2 The Cell Cycle Distribution Assay—From the above data, a MYC Index can be calculated for each compound by dividing the IC₅₀ value of a compound in HO15.19–vector cells by the average of the IC₅₀ of that compound in HO15.19–N-myc cells and HO15.19–c-Myc cells (active compounds possess a MYC Index >1). Rank compounds within each chemical class by their MYC Index (the higher the MYC Index, the better the compound). The best of each of the structural classes and compounds with unique structures are subjected to filtering based on the cell cycle distribution of cells treated with the compound.

1. Seed 7,000 HO15.19–vector cells or 3,000 pLXSH–c-Myc or pLXSH–N-Myc cells in each well of a 96-well plate.
2. The next day, add vehicle (DMSO) or the compounds to the cells at concentrations equal to the IC₅₀, 0.5 $\times$ IC₅₀ and 0.25 $\times$ IC₅₀. Incubate at 37 °C for 48 h.
3. Remove medium, trypsinize the cells, and subject them to standard propidium iodide FACS analysis.
4. Calculate the percentage of cells in G₀/G₁, S, and G₂/M phases of the cell cycle in treated and untreated populations. Based on the above measurements, calculate a Cell Cycle Index: divide an average ratio between the proportion of G₀/G₁ cells in treated versus untreated HO15.19–c-myc and HO15.19–N- myc cells by a similar ratio in HO15.19–vector cells (*see Note 7*).

⁶In the event that a plate reader does not have a 650 nm filter, a 595 nm filter can be used. The background corrected values will be lower but should not affect the overall results as long as the controls (i.e., vehicle control treated wells) are approaching confluence at the time the assay is harvested.

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⁷The procedures described in Subheading 3.4 utilize HO15.19 cells and their derivatives, since these have for a long time been considered as the standard in the field. With the obvious goal to develop c-Myc-targeting therapeutic agents, the next step should include testing of the compounds in the transformed cells. However, due to the extreme variability in the phenotypes caused by genetic inhibition of Myc in different tumor cell lines [10], it is becoming increasingly difficult to provide a detailed protocol for testing the compounds beyond the described systems. We therefore suggest further characterization of specific phenotypes caused by the compounds and vis-à-vis genetic inhibition of c-Myc using siRNA technology in the cell line of choice. This may include comparison of changes in global cellular transcription or the ability to induce a specialized form of proliferation arrest such as differentiation or senescence or cell death such as apoptosis or mitotic catastrophe.

Fig. 1.

Schematic representation of MYCN primary screen. SHR6-17 cells with low basal levels of luciferase reporter activity are transduced with *MYCN* lentivirus. 24 h post-transduction, library compounds are added to cells while *MYCN* levels are still low (~twofold induction), and then luciferase activity of cells is measured ~24 h after incubation with compounds. N3i, an *MYCN* shRNA lentivirus, serves as a positive control for inhibition of *MYCN*-driven reporter activity. Two categories of hits are obtained: strong hits return luciferase activity back to baseline or to N3i shRNA levels, and weak hits reduce luciferase activity to ~40–60 %

Fig. 2.

Induction of luciferase activity by transduction of *MYCN* lentivirus. **(a)** *MYCN* lentivirus induces luciferase activity in a dose-dependent manner. SHR6-17 cells containing the MYC-responsive luciferase reporter were transduced with increasing concentrations of *MYCN* lentivirus for 24, 48, and 72 h in 96-well plates. At each time point, cells were assayed for luciferase activity. *Numbers* next to each curve correspond to the amount of lentivirus (μ l) added per well in a total volume of 200 μ l. **(b, c)** *MYCN* shRNA lentivirus (N3i, **b**) but not GFP shRNA (GFPI, **c**) block *MYCN* induction of luciferase activity in SHR6-17 cells. Reporter cells were transduced with *MYCN* lentivirus (10 μ l) alone or in combination with increasing amounts of N3i or GFPI lentivirus for 24, 48, and 72 h as described above. *Numbers* next to curves correspond to the amount of shRNA lentivirus used in the experiment

		Small Molecule Library Compounds											
		1	2	3	4	5	6	7	8	9	10	11	12
MYCN only	A	0.86	1.02	1.21	1.29	1.23	2.18	1.28	1.73	1.38	1.46	1.86	1.11
	B	1.09	1.11	1.34	1.42	0.99	1.62	1.78	1.47	1.66	1.08	1.20	1.20
	C	0.87	0.86	1.66	0.73	0.79	1.28	0.88	1.36	2.13	1.03	Strong Hit	0.94
	D	0.83	2.85	1.06	0.83	0.83	1.48	1.50	1.31	1.30	1.39	Strong Hit	1.10
untransduced	E	0.19	0.97	0.89	1.03	0.33	1.65	1.59	1.15	1.16	1.1	1.14	0.31
	F	0.18	1.08	0.42	0.88	0.88	1.17	1.15	1.21	2.02	0.28	1.92	0.64
	G	0.19	0.82	0.71	1.02	0.99	Strong Hit	1.1	1.00	1.18	0.94	1.01	0.82
	H	0.17	0.89	0.94	1.14	0.92	1.33	1.44	1.08	1.07	0.98	1.00	0.84

5X N3i
1X N3i
5X Gi
1X Gi

Weak Hit

Fig. 3.

Representative plate from a small molecule library screening for MYC inhibitors. 80 library compounds were tested per 96-well plate, and the level of luciferase activity in the presence of compound was compared to that of the average of the luciferase activity in cells transduced with *MYCN* lentivirus (MYCN only) to produce an inhibition ratio as presented in the table. Strong hits represent those compounds that reduce the luciferase activity back to basal levels or to a level equivalent to that obtained with the highest *MYCN* shRNA (*N3i*) dose. Weak hits reduce the luciferase levels to 40–60 % of the control. Gi represents wells transduced with an shRNA control virus. Two *strong hits* and one *weak hit* are shown on this plate