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TITLE: Development of Rapid In Vitro and In Vivo Assays to Detect and Quantify MYC Network Protein Associations

PRINCIPAL INVESTIGATOR: Edward V. Prochownik, M.D., Ph.D.

CONTRACTING ORGANIZATION: Children’s Hospital of Pittsburgh
Pittsburgh, Pennsylvania 15213

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We have performed high-throughput screening of low molecular weight chemical compounds to identify those that specifically inhibit the productive interaction between the c-Myc oncoprotein and its obligate heterodimeric partner, Max. The screening was facilitated by the use of a micro version of a yeast two-hybrid assay in which the c-Myc-Max interaction induces the expression of β-galactosidase. A screen of approximately 10,000 compounds identified seven that were able to inhibit β-galactosidase activity. Testing of these in over 30 yeast strains expressing different transcription factors and their dimerization partners indicated that the observed c-Myc-Max inhibition was highly specific. Several of the compounds were also able to inhibit the growth of c-Myc oncoprotein-expressing Rat1 fibroblasts in vitro. Our results indicate that we have identified several low molecular weight compounds that are able to inhibit the activity of c-Myc-Max heterodimers. Plans for the next year include testing of these compounds to demonstrate in vivo activity against c-Myc induced tumors, as well as determining whether the compounds are effective at inhibiting the activities of the N-Myc and L-Myc oncoproteins.
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INTRODUCTION

Members of the Myc oncprotein family (c-Myc, N-Myc, and L-Myc) have been implicated in the pathogenesis of a large number of human cancers (1). For example, the c-Myc gene is translocated in nearly all cases of Burkitt’s lymphoma, is amplified in nearly one-third of cases of breast cancer and about 25% of prostate cancers. The N-myc and L-myc genes are amplified in a substantial fraction of neuroblastomas and small cell lung cancers, respectively (1).

Myc oncproteins are members of the basic-helix-loop-helix-leucine zipper (bHLH-LZ) family of DNA binding transcription factors, which recognize specific sequences termed E-box elements in their target genes (2). For all Myc proteins, DNA binding, subsequent target gene regulation, and the ability to carry out all of known biological functions, require formation of a heterodimer with another bHLH-LZ protein, Max (3,4). In turn, Max can heterodimerize with another group of bHLH-ZIP proteins, dubbed the Mad family (Mad1, Mxi1, Mad3, and Mad4)(ref. 5-7). These counter the effects of Myc-Max heterodimers by competing for DNA binding sites and exerting the opposite transcriptional effect (refs.8-10 and Fig. 1).

A large number of putative target genes for c-Myc have been identified, most recently through the use of cDNA microarrays (8-11). Many of these gene products encode proteins involved in the control of the cell cycle, growth and metabolism, cellular adhesion, and apoptosis.

One of the concepts that drives the field is that a comprehensive cataloging of c-Myc target genes would provide a means by which the former protein’s ability to cause cancer could be understood in molecular terms. Another is that the inhibition of some of these target gene products might provide a relatively specific and non-toxic way of treating tumors with Myc deregulation. Unfortunately, because a number of Myc target gene products have already been shown to be transforming, it seems unlikely that targeting any one of them will be of significant benefit.

With these concepts in mind, we have attempted to identify a pharmacologic means of inhibiting the c-Myc oncoprotein itself. Because the interaction between c-Myc and Max is necessary for all of c-Myc’s biological properties, we devised a screening method that depends upon a putative inhibitor's ability to disrupt the productive interaction between c-Myc and Max.

The basis for this assay rests on the well-known yeast two-hybrid screen (Fig. 2). In this assay, the bHLH-LZ regions of c-Myc was fused to the DNA binding moiety of the Gal4 protein. Similarly, the bHLH-LZ domain of Max was fused with the Gal4 transcriptional activation domain. Both were expressed in a yeast strain harboring a β-galactosidase gene containing a Gal4 binding site. In his strain, the bHLH-LZ-mediated interaction between c-Myc and Max reconstitutes a productive Gal4 transcription factor that is capable both of DNA binding and
transcriptional activation of the β-galactosidase gene (12). Disruption of the c-Myc-Max interaction would be expected to result in the loss of enzyme activity, which can be readily assayed.

Fig. 2. The basis for the yeast two-hybrid screen used to identify low molecular weight compounds that disrupt the c-Myc-Max interaction. The bHLH-LZ regions of c-Myc and Max were amplified by PCR and cloned into the pGBT-9 and pGAD424 plasmid, respectively. In the former case, the cloning resulting in the c-Myc bHLH-LZ region being expressed in-frame with the yeast Gal4 DNA binding domain (red oval). In the latter case, the Max bHLH-LZ domain was expressed in-frame with the Gal4-transactivation domain (blue circle). Co-expression of these in the Y153 yeast strain results in the interaction between the two bHLH-LZ domains and the functional reconstitution of Gal4 DNA binding and transcriptional activation moieties. This results in the activation of β-galactosidase gene under the control of a promoter containing a Gal4 DNA binding site. Activation of enzyme expression can be readily detected by a simple colorimetric assay. Loss of this activity in the presence of a low molecular weight compound indicates that the compound may potentially be capable of inhibiting the c-Myc-Max interaction (Red arrow #1). Trivial reasons for loss of enzyme activity include non-specific toxicity to the yeast, inhibition of DNA binding (arrow #2) or transcriptional activation (arrow #3), or interference with the β-galactosidase enzyme assay (arrow #4). All of these possibilities can be ruled out by showing that the compound neither prevents the growth of the yeast nor inhibits the interaction between another, unrelated pair of dimerization domains.

In addition to the above-described specific pharmacologic inhibition of β-galactosidase activity, there are several “trivial” ways by which loss of enzyme activity could be achieved as well. For example, a compound might simply kill the yeast, resulting in no enzyme activity. Alternatively, the compound might inhibit DNA binding or transcriptional activation, respectively, by the two separate Gal4 moieties, or might directly interfere with the β-galactosidase enzyme itself. In order to control for all of these contingencies, we created a “control” yeast strain that expressed the bHLH proteins Id2 and E47 (13). Compounds that specifically disrupted the c-Myc-Max interaction should permit expression of β-galactosidase in this Id2-E47 yeast strain. We report below our results using this assay.

BODY

The above-described assay was miniaturized to a 96 well plate format. Each of the two described yeast strains was diluted to an OD600 of approximately 0.1 and aliquoted robotically into a series of separate 96 well plates (50 μl/well). Individual chemical compounds (Chembridge, Inc., San Diego, CA) were prepared at an average stock concentration of approximately 1 mM each in DMSO. 0.5 μl of each compound was then added to a corresponding well of both the “Myc-Max” plates and “Id2-E47” plates (final concentration of each compound approximately 10 μM), and the yeast were grown overnight at 30° C. The next day, the density of each culture was determined on a microplate reader. In no case did a compound inhibit yeast growth by >60% and in the vast majority of cases, no inhibition was observed. β-galactosidase assays were then performed essentially as described using the chromogenic substrate chlorophenol red-β-D-galactopyranoside (CPRG, Molecular Probes, Eugene, OR)
The results of β-galactosidase indicated that, of the approximately 10,000 compounds screened in this manner, 7 showed selective inhibition of enzyme activity only in the “c-Myc-Max” yeast, 6 were selective only in the “Id2-E47” yeast, and 32 inhibited enzyme activity in both yeast strains. Dose-response studies performed on this latter group revealed that 4 additional compounds were selective for Id2-E47 at lower concentrations. No additional Myc-Max-specific compounds were identified in this screen. These results show, at least in the case of the Myc-Max and Id2-E47-specific compounds, that the loss of β-galactosidase activity is not the result of a toxic effect on the yeast, an inhibition of DNA binding, an inhibition of transcriptional activation, or a direct inhibition of enzyme activity.

In order to better define the specificities of each of the above compounds as well as to investigate further those with activity in both yeast strains, we established a large series of strains expressing various combinations of bHLH, bLZ or bHLH-LZ transcription factors (12). Most notably, these included Max in combination with each of the four known members of the Mad family, and other members of the Id family in combination with other E proteins such as E12, HEB, and E2-2. These stains was then grown in the presence of each of the above c-Myc-Max and Id2-E47-specific compounds. As seen in Fig. 3, we confirmed the initial specificity of each compound. We also noted that the overall specificity of both the c-Myc-Max and Id2-E47-specific compounds was, in general, quite high. For example, the c-Myc-Max-specific compound 10058-F4 showed essentially no inhibitory against any of the other heterodimers tested except for an intermediate degree of inhibitory activity (25-75%) against MyoD-E2-2, Mad1-Max, Mxi1-Max, and Mad3-Max. Similarly, the Id2-E47-specific compound 10033-B6 was highly specific for the heterodimer, showing an intermediate degree of inhibition only against Id3-HEB. The characteristic spectrum of activity of each compound suggested that each was inhibiting the heterodimeric interactions in different ways.

As expected, the specificity of those compounds initially identified as inhibiting both c-Myc-Max and Id2-E47 was much less than for the other two classes of compounds. For example, the compound 10049-F2 also significantly inhibited (>75%) enzyme activity of Id2-E12, MyoD-E2-2, MyoD-HEB, Max-Max, Mad1-Max, Mad3-Max, and Mad4-Max. One interesting compound, presented here for heuristic purposes only, was 10039-D8, which inhibited β-galactosidase activity in every yeast strain. This compound likely represents one that inhibits some non-specific aspect of the entire system such as DNA binding, transcriptional activation, or the β-galactosidase enzyme itself.

![Fig. 3. The spectrum of heterodimeric transcription factor interactions by anti-c-Myc-Max and E12-E47 compounds. The indicated compounds were added to microwells containing yeast stains expressing the indicated bHLH, bLZ, or bHLH-LZ transcription factors. The average concentration of the compounds was 10 μM. The yeast were incubated overnight until reaching stationary phase and then β-galactosidase assays were performed. Each assay was performed in triplicate. The degree of inhibition was categorized as either minimal (<25%: expressed as green squares), intermediate (25-75%: yellow), or marked (>75%: red).](image-url)
Having concluded the anti-c-Myc-Max compounds were, in general, highly specific for this heterodimeric pair, we tested each one for its effect on the in vitro growth of mammalian cells. For this purpose, we used a Rat1a fibroblast cell line that expressed c-Myc due to transfection with a c-Myc expression vector (Rat1a-c-Myc cells) or a control cell line that was transfected with the empty parental vector and expresses only endogenous levels of c-Myc (Rat1a-neo cells). The former cell line readily demonstrates anchorage-independent growth in soft agar and undergoes accelerated apoptosis in response to serum withdrawal, two hallmark features of c-Myc overexpression. As seen in Fig. 4, five of the seven drugs tested exerted a significant growth inhibitory effect that was more pronounced in the Rat1a-c-Myc cell line. Most notably, compounds 10050-G5 and 10074-G5 showed little inhibitory effect on the growth of Rat1a-neo cells (<50% growth inhibition), whereas they profoundly inhibited the growth of Rat1a-c-Myc cells (>95% inhibition). Two compounds (1009-G9 and 10075-G5) showed significant inhibition of Rat1a-neo cells (approx. 60-70% inhibition) and a much more marked effect on Rat1a-c-Myc cells (>80-95% inhibition). Finally, one drug (10074-A4) showed a marked inhibition of growth of both cell types.

Together with the results presented in Fig. 3, these findings suggest that we have identified compounds that interfere with a functional interaction between c-Myc and Max. In mammalian cells, this results in a loss of growth not unlike that that has been previously described for cells in which c-Myc in inhibited by various methods.

**Fig. 4.** Response of Rat1a cell lines to each of the c-Myc-Max-specific compounds depicted in Fig. 3. Rat1a or Rat1a-c-Myc cells were seeded into 24 well plates at a density of 2,000 cells/well. Each of the drugs was then added to a final average concentration of approximately 20 μM. The medium and drug were changed every other day. At the indicated times, triplicate wells were trypsinized and total cell number/well was determined by Coulter counting. Each point shows the average number of cell/well. Standard errors were generally <10%. Two of the drugs tested (10031-B8 and 10058-F4) showed no significant of cell growth at the concentrations tested.

**KEY RESEARCH ACCOMPLISHMENTS**

1. Use of a yeast-based, high throughput two hybrid screen to identify compounds that prevent the functional interaction between c-Myc and Max.
2. Five of seven compounds tested so far show significant inhibitory effects on the in vitro growth of cells. In at least four cases, selectivity against c-Myc-overexpressing cells has been demonstrated.
3. The same assay has identified other compounds that selectively prevent the functional interaction between Id2 and E47. Because Id proteins are negative regulators of myogenesis, our findings suggest that such compounds might be used to promote muscle growth or regeneration.
REPORTABLE OUTCOMES

We are currently examining the effect of each of the above c-Myc-specific drugs on in vitro colony formation by Rat1a-c-Myc cells. Based on the observations presented in Fig. 4, we anticipate that most of these drugs will significantly impair the colony-forming ability of these cells.

Additional studies planned for the coming year include:

• A determination of whether any of the compounds can inhibit in vivo tumor growth using a Rat1a-c-Myc-nude mouse model system
• Studies aimed at determining whether these drugs actually function by preventing or disrupting the interaction between c-Myc and Max.
• Determining whether any of the above compounds are more effective in combination than individually
• Determining whether, as would be predicted, any of the known downstream targets for c-Myc are inhibited by the addition of these compounds.
• Determining whether the two other major members of the Myc oncprotein family, N-Myc and L-Myc, are also inhibited by any of these compounds.

CONCLUSIONS

Using a version of the traditional yeast two-hybrid assay, modified to a high throughput, 96 well plate screening format, we have identified seven compounds capable of inhibiting functional c-Myc-Max interactions. Similarly, 10 compounds specific for Id2-E47 have been identified. To date, five of the seven c-Myc-Max-specific compounds are able to inhibit the in vitro growth of Rat1a-neo and/or Rat1a-c-Myc cells. Our results suggest that the compounds we have identified may be useful in the treatment of tumors whose proliferative potential is dependent upon the overexpression of c-Myc. It is also possible that these drugs may be useful in the treatment of neuroblastoma in which one-third of cases are associated with N-Myc gene amplification.

REFERENCES


