Chemical Genetic and Genomic Approaches Reveal a Role for Copper in Specific Gene Activation

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Received June 22, 1999

Unbiased approaches toward exploring biological systems can yield unexpected, but useful results. Genetic screening of random mutations is a powerful tool for uncovering the molecular basis of biological processes.[1] Chemical genetic screening of small molecules may prove to be similarly useful.[2] Genetic and chemical genetic screens identify mutations and small molecules, respectively, that affect a biological process of interest.[3,4] However, a potential barrier to the widespread use of the chemical genetic approach is the need to identify the molecular mechanisms by which small molecules affect biological systems.

Genomics-based techniques provide a possible solution to this problem. Transcriptional profiling with cDNA microarrays reveals the expression levels of a large number of genes simultaneously.[5] For example, this technique can measure the change in expression of nearly every gene in the model organism *Saccharomyces cerevisiae* under various conditions,[6] such as in response to treatment with a small molecule.[7] The pattern of such transcriptional changes may reveal the mechanistic basis of a compound's activity, illuminating the small molecule-sensitive pathway.

We developed a primary screen for small molecules that activate the reporter gene p3TPLux[8] in a stably transfected mink lung epithelial cell line.[9] This reporter is composed of a regulatory sequence of DNA optimized for its sensitivity to TGF-β,[8] and the coding sequence for luciferase, which has a readily detectable enzymatic activity. We determined the extent of reporter gene activation by measuring the amount of luciferase that had been synthesized in these cells. We also used a secondary cytoblot screen[10] to characterize the effects of the active compounds on DNA synthesis, expecting that TGF-β-like compounds would block this process. Our goal was to find compounds that activate the reporter gene, determine how these compounds act mechanistically, and then formulate and test hypotheses regarding the relevance of these mechanisms to TGF-β signaling.

More than 16000 structurally diverse small molecules[11] were assayed in the primary screen, and four active compounds were found. 2,2′-(Methylimino)bis(8-quinolinol) (**1a**) and the related dimeric 8-quinolinols **1b**−**c** strongly activated the reporter (Figures 1 and 2, and data not shown). In addition, 4-hydroxybenzoic acid [(2-hydroxyphenyl)methylene]hydrazide (**2**) modestly activated the reporter (Figures 1 and 2). **1a** and **2** were used in subsequent studies. Both **1a** and **2** induced a dose-dependent increase in reporter activity and acted synergistically with TGF-β (Figure 2). At higher concentrations **1a** caused cell detachment, which resulted in a loss of apparent activity. Neither **1a** nor **2** activated a transiently transfected NFκB-responsive luciferase reporter gene (data not shown), indicating a degree of promoter specificity. In an S-phase progression cytoblot assay[10] used for a secondary screen, **1a** and **2** inhibited 5-bromodeoxyuridine incorporation in Mv1Lu mink lung epithelial cells with EC_{50} values of 5 and 15 μM, respectively (data not shown). Thus, these compounds mimic two aspects of TGF-β signaling, namely reporter gene activation and inhibition of DNA synthesis.

A large number of proteins and pathways are conserved between mammals and *S. cerevisiae.* Since the entire genome of *S. cerevisiae* is available in microarray format, one can perform a comprehensive analysis of gene expression in this organism. We wondered if transcriptional profiling in *S. cerevisiae* would reveal anything about the mechanisms by which **1a** and **2** act.

We purified messenger RNA (mRNA) from cultures of *S. cerevisiae* treated with either nothing or a small molecule, then converted this mRNA to complementary DNA (cDNA) labeled with one of two fluorescent dyes. We simultaneously hybridized these two cDNA populations to a 2 cm × 2 cm microarray containing over 5800 distinct *S. cerevisiae* genes (>90% of the total number of genes in *S. cerevisiae*). By comparing the amount of the two fluorescent dyes at each spot in the array, we determined the effect of a particular compound on the expression of each gene in *S. cerevisiae*.

Compound **2** had no significant effect on the expression of any gene in *S. cerevisiae* (data not shown). On the other hand, **1a** reproducibly increased the expression of five genes (*HSP26*, *ZRT1*, *FET3*, *YDR534C*, and *YOL155C*), out of 5800, more than 2-fold. Of these five genes, only three have been previously characterized (*HSP26*, *ZRT1*, and *FET3*). *HSP26* is a heat shock protein induced by osmotic stress,[12] *ZRT1* is a high-affinity zinc transporter,[13] and *FET3* is a multicopper iron oxidase required for iron transport.[14] Thus, these genes are all involved in metal ion homeostasis. Given our results (see below), it is likely that the two uncharacterized genes (*YDR534C* and *YOL155C*) are somehow regulated by the level of intracellular copper, iron, or zinc.

To determine whether the effect of **1a** in *S. cerevisiae* is also involved in its ability to activate the TGF-β-responsive regulatory sequence of the reporter in mammalian cells, we tested the effect of various metal ions on reporter activity in the presence of **1a** or **2**. Addition of 1 mM alkali or earth alkali metal (Na^+, K^+, Ca^{2+}, Mg^{2+}, and Ba^{2+}) did not affect reporter activation by **1a** or **2** (Table 1). However, 30 μM Fe^{3+} completely suppressed the activity of both **1a** and **2** (Table 1 and Figure S1), but had no effect on TGF-β-induced activation of the reporter (data not shown). In addition, 7.5 μM Zn^{2+}, 10 μM Cu^{2+}, and 20 μM Al^{3+} potently suppressed the activity of **1a** but failed to suppress the activity of **2** or TGF-β at these or higher concentrations (Table 1 and Figure S1).

Surprisingly, Cu^{2+} alone activated the reporter (Figures 3 and S1). Moreover, **2** synergized potently with Cu^{2+}, but not other metal ions (Figures 3 and S1). These observations are consistent

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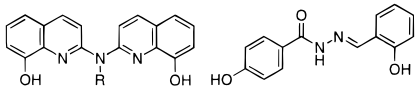
10.1021/ja9921221 CCC: $18.00
Published on Web 10/30/1999



1a R = Me
1b R = H
1c R = nBu

2

Figure 1. Small molecule activators of a TGF-β-responsive reporter gene.

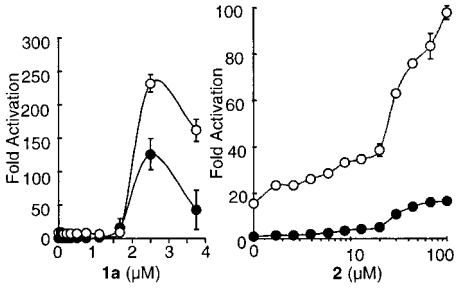


Figure 2. Dose-responses of **1a** and **2** for activating a TGF-β-responsive reporter gene. 20000 6F mink lung cells[11] were seeded in 384 well plates, allowed to attach for 16 h in 10% mink medium, and treated with the indicated concentrations of **1a** or **2** in the absence (black circles) or presence (white circles) of 400 pM TGF-β1. Luciferase activity was measured as described in the Supporting Information.

Table 1. Metal Binding by **1a** and **2** Correlates with Sensitivity to Metal Antagonism

metal[a]	EC_{50} UV **1a** (μM)[b]	EC_{50} Su **1a** (μM)[c]	EC_{50} UV **2** (μM)[d]	EC_{50} Su **2** (μM)[c]
Zn^{2+}	<7.5	0.5	500	>300
Fe^{3+}	<7.5	2.0	<10	9
Cu^{2+}	10	1.0	<10	*
Al^{3+}	30	1.0	200	>300
Mn^{2+}	80	200	> 1000	>300
Co^{2+}	100	10	50	30
Ni^{2+}	280	10	500	200
Mg^{2+}	>1000	>1000	>1000	>1000
K^{+}	>1000	>1000	>1000	>1000
Ca^{2+}	>1000	>1000	>1000	>1000
Na^{+}	>1000	>1000	>1000	>1000
Ba^{2+}	>1000	>1000	>1000	>1000

[a] All metals were used in the form of the chloride salts, except for $NiSO_4$ and $Ba(OAc)_2$. [b] Concentration at which the metal ion induces a 50% shift in the λ_{max} of a shoulder in the UV spectrum of **1a** ([**1a**] = 15 μM) from 292 to 273 nm. [c] Concentration at which metal ion inhibits 50% of the activation of p3TPLux by **1a** or **2** in 6F mink lung cells ([**1a**] = 3 μM, [**2**] = 64 μM). An asterisk (*) indicates that synergy, rather than suppression, was observed. [d] Concentration at which the metal ion induces a 50% shift in the λ_{max} of a shoulder in the UV spectrum of **2** from 297 to 310 nm ([**2**] = 20 μM). Values are accurate to within ±50% of the number shown.

with a model in which **2** acts as a copper transporter. In addition, the known copper chelator diethyldithiocarbamate (DDC)[15] partially suppressed the activity of **2**, TGF-β, and copper, although DDC had no effect on the activity of **1a** (data not shown).

Fe^{3+} may suppress the activity of **2** by binding to **2** and thereby preventing copper from binding to **2**. To measure the ability of various metal ions to bind directly to **1a** and **2**, we measured ultraviolet light absorbance of each compound from 240 to 320 nm in the absence or presence of metal ions. We found a reproducible shift in the λ_{max} of a shoulder in the UV spectrum of **1a** from 292 to 273 nm and of **2** from 297 to 310 nm, in the presence of certain metal ions, likely due to binding of the metals (data not shown). Alkali and earth alkali metals (Na^{+}, K^{+}, Ca^{2+}, Mg^{2+}, and Ba^{2+}) did not cause this perturbation in the UV spectrum of **1a** or **2**, at concentrations up to 1 mM (Table 1). Furthermore, the metal ions that suppressed **1a** and **2** also induced this perturbation at metal concentrations in the micromolar range

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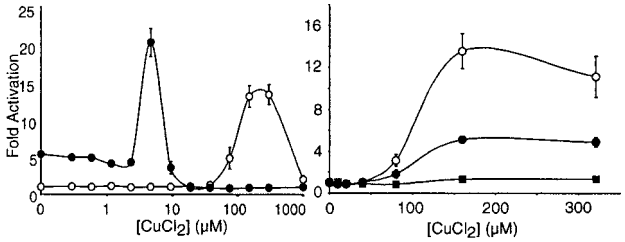

Figure 3. Copper-induced activation of a TGF-β-responsive reporter gene. The luciferase assay described in Figure 2 was performed. White circles represent addition of $CuCl_2$ only. Left panel: dark circles represent activation in the presence of 64 μM **2**. Right panel: dark circles and squares represent activation in the presence of 10 and 100 μM, respectively, $ZnCl_2$.

(Table 1), resulting in a correlation between the binding affinity and suppression ability of metal ions in the two assays. Interestingly, **1a** and **2** had different metal-binding specificity (Table 1). Most notably, **1a** binds Zn^{2+} with high affinity while **2** binds Zn^{2+} poorly. In addition, we found that Zn^{2+} suppressed the activity of Cu^{2+} (Zn^{2+} EC_{50} = 10 μM, Figure 3) while other metal ions did not (data not shown).

Compound **1a** is a bis(8-quinolinol) (Figure 1). Monomeric 8-quinolinol itself had no activity in the reporter gene assay, neither activating on its own nor suppressing or enhancing the activity of **1a** (data not shown). 2,2′-Dipyridylamine, which is a partial structural mimic of **1a**, had no effect in the reporter gene assay (data not shown). Thus, simple structural analogues of **1a** do not mimic the properties of this compound.

We determined that **1a**, **2**, and Cu^{2+} do not activate the reporter by affecting free radical formation or upregulating TGF-β itself, since H_2O_2, 2,2′-azobis(2-methylpropinonitrile) (AIBN), and a neutralizing antibody to TGF-β did not affect the activity of **1a**, **2**, or Cu^{2+} (data not shown). Activation of the reporter by **1a**, **2**, and Cu^{2+} is selective, since none of these reagents activated a stably transfected NFAT-lacZ reporter gene (data not shown). However, we have not yet determined whether **1a**, **2**, and Cu^{2+} activate a component of the TGF-β signaling pathway, or whether the reporter gene that we used contains a previously unidentified copper-responsive element.

Remarkably, all of the active compounds identified in our screen, including **1a**, bind to metal ions. This property of **1a** is consistent with the observation that all three of the characterized genes induced by **1a** in yeast are likely to be involved in metal ion homeostasis. Thus, if **1a** depletes Zn^{2+}, Fe^{3+}, and Cu^{2+} in vivo, then induction of a zinc transporter (*ZRT1*), a copper-dependent iron oxidase required for iron transport (*FET3*), and an osmotic stress response (*HSP26*) is a reasonable response of yeast to **1a**. The genomic approach has the added benefit of revealing potential functions for uncharacterized genes, such as *YDR534C* and *YOL155C*. This strategy of using small molecules, cell-based screens, and transcriptional profiling is complementary to the classical bioinorganic approach of studying a single metal−protein interaction, and furthermore may reveal new and interesting roles for metal ions in biology.

Acknowledgment. We thank Jeremiah Scharf for helpful discussions. The Harvard Institute of Chemistry and Cell Biology is supported by grants from Merck and Co., Inc. and the National Cancer Institute (1 P01 CA78048-01). B.R.S. is a Howard Hughes Medical Institute predoctoral fellow. S.L.S. is an investigator at the Howard Hughes Medical Institute.

Supporting Information Available: Detailed experimental procedures and an additional figure (Figure S1) showing metal ion suppression of compound-induced reporter gene activity (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA9921221