

Identification of a Class of Small Molecule Inhibitors of the Sirtuin Family of NAD-dependent Deacetylases by Phenotypic Screening*

Received for publication, July 18, 2001, and in revised form, July 31, 2001
Published, JBC Papers in Press, August 1, 2001, DOI 10.1074/jbc.M106779200

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The yeast transcriptional repressor Sir2p silences gene expression from the telomeric, rDNA, and silent mating-type loci and may play a role in higher order processes such as aging. Sir2p is the founding member of a large family of NAD-dependent deacetylase enzymes, named the sirtuins. These proteins are conserved from prokaryotes to eukaryotes, but most remain uncharacterized, including all seven human sirtuins. A reverse chemical genetic approach would be useful in identifying the biological function of sirtuins in a wide variety of experimental systems, but no cell-permeable small molecule inhibitors of sirtuins have been reported previously. Herein we describe a high throughput, phenotypic screen in cells that led to the discovery of a class of sirtuin inhibitors. All three compounds inhibited yeast Sir2p transcriptional silencing activity *in vivo*, and yeast Sir2p and human SIRT2 deacetylase activity *in vitro*. Such specific results demonstrate the utility and robustness of this screening methodology. Structure-activity relationship analysis of the compounds identified a key hydroxy-naphthaldehyde moiety that is necessary and sufficient for inhibitory activity. Preliminary studies using one of these compounds suggest that inhibition of sirtuins interferes with body axis formation in *Arabidopsis*.

In yeast, the Sir2 family of proteins functions in transcriptional regulation, cell cycle progression, DNA-damage repair, and aging (1, 2). Sir2p deacetylates histones in an NAD-dependent manner (reviewed in Ref. 3), which is thought to account for both its transcriptional silencing activity at the telomeric, rDNA, and silent mating-type loci (reviewed in Ref. 1), as well as its ability to regulate lifespan in response to metabolic rates (reviewed in Ref. 2). Deacetylation of histones is a known mechanism for causing chromatin condensation and transcriptional silencing; indeed, the loci regulated by Sir2p are hypoacetylated (4), and deletion of Sir2 results in a dra-

matic increase in the acetylation of the histones at these sites (5). Furthermore, it is believed that Sir2p regulates aging in yeast by forming condensed chromatin structures at the rDNA locus. This decreases recombination rates, thereby decreasing the formation of toxic extrachromosomal rDNA circles, which shorten lifespan (reviewed in 2). Therefore, according to this hypothesis, increasing Sir2p activity or overexpressing Sir2p leads to an increased lifespan in yeast. Interestingly, lifespan can also be extended by glucose deprivation, and this is postulated to be mediated by the NAD-dependence of Sir2p. Decreasing glucose intake drops metabolic rates, resulting in the oxidation of NADH to NAD. Increasing the amount of available NAD increases Sir2p activity, thereby preventing the formation of toxic extrachromosomal rDNA circles. The critical role of NAD levels in this process is evident by the fact that mutations in an NAD biosynthetic pathway in yeast leads to a decrease in lifespan that is not rescued by mutation in the glucose-sensing pathways or overexpression of Sir2p (6). Thus, the metabolic and oxidative state of the cell may be linked directly to transcriptional regulation of certain genes via Sir2p, which in turn may regulate lifespan.

Sir2p represents the founding member of a large family of NAD-dependent deacetylases, termed the sirtuins (7, 8). These enzymes are highly conserved from prokaryotes to eukaryotes, with five sirtuins in yeast and seven in humans. The sirtuins contain a conserved catalytic domain of ~275 amino acids, and mutation of the highly conserved residues in this domain abrogates enzymatic activity (7, 9). Although Sir2p and yeast HST1p appear to be involved directly in transcriptional silencing, the biological functions of the other sirtuins remain unknown, though recently a Sir2p homolog in *Caenorhabditis elegans* was shown to regulate lifespan in this organism, as well, albeit via a different mechanism (10). Several yeast and human sirtuins are not localized to the nucleus (11–13), suggesting that these proteins may have diverse roles in the cell aside from transcriptional regulation. Indeed, one *Salmonella* sirtuin was found to be competent to function in the cobalamin biosynthetic pathway (14), and thus some of these enzymes may have small molecules, rather than proteins, as substrates.

One approach to dissecting the biological function of sirtuins in a variety of biological systems is reverse chemical genetics. Rather than deleting or mutating the sirtuin genes in a particular system, a cell-permeable small molecule inhibitor of sirtuins can be used to block sirtuin activity. Such an inhibitor would permit fine temporal regulation of sirtuin inhibition, and thus the function of sirtuins in essential or developmental processes could be studied. Furthermore, these inhibitors should be easily transferable to model organisms or systems in which it is extremely difficult to specifically delete genes. In-

* This work was supported in part by NIGMS, National Institutes of Health Grant GM38627 (to S. L. S.). Research at the Harvard Institute of Chemistry and Chemical Biology is supported by the NCI, the Keck Foundation, Merck KGOA and Merck & Co. S. L. S. is an Investigator at the Howard Hughes Medical Institute. H. E. B. is supported by a post-doctoral fellowship from the Jane Coffin Childs Memorial Fund for Medical Research (sponsored by Merck & Co.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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deed, inhibitors of the family of histone deacetylases, HDACs,¹ have greatly accelerated the characterization of the cellular function of these proteins. For example, inhibition of HDAC activity by the nanomolar inhibitor trichostatin A (TSA) produces a dramatic morphological change in tumor cell lines, which subsequently was shown to be caused by an HDAC-dependent transcriptional regulation of gelsolin, an actin-binding protein (15). Thus, a general sirtuin inhibitor could be used to refine our understanding of yeast sirtuins and initiate investigations into the function of insect, vertebrate, and human sirtuins. Thus far, however, no cell-permeable sirtuin inhibitors have been reported. A non-hydrolyzable NAD analog (carbanicotamide adenine dinucleotide) does inhibit sirtuin activity *in vitro* (16), but such molecules are not cell-permeable and undoubtedly inhibit other NAD-dependent enzymes, as well. Thus, these NAD analogs would not be suitable for *in vivo* studies.

Herein we describe the identification of a class of cell-permeable small molecule inhibitors of sirtuin NAD-dependent deacetylase activity from a high throughput cell-based screen of 1600 unbiased compounds. The primary screen was for inhibitors of Sir2p-mediated silencing of a URA3 reporter gene integrated into a telomeric locus. This yeast strain can grow in the presence of 5-fluoroorotic acid (5-FOA), but the addition of an inhibitor of Sir2p will result in expression of the URA3 gene and death in the presence of FUra. Three compounds of 1600 scored positively in this screen, and two of these possessed substructures derived from 2-hydroxy-1-naphthaldehyde. All three compounds inhibit Sir2p transcriptional silencing *in vivo*, in the context of different Sir2p complexes and at different chromosomal domains. These compounds inhibit yeast Sir2p, as well as human SIRT2 activity *in vitro*, demonstrating that they inhibit enzymatic activity directly and function as general inhibitors of sirtuins. Interestingly, 2-hydroxy-1-naphthaldehyde and other compounds containing this moiety also inhibit Sir2p activity *in vivo* and *in vitro*, suggesting that these small molecules represent a new class of inhibitors of sirtuins. Preliminary studies with one of these inhibitors (sirtinol; Sir two inhibitor napthol) suggest that sirtuins do not regulate global histone acetylation levels in mammalian cells and that they may be involved in body-axis formation during plant development.

EXPERIMENTAL PROCEDURES

Yeast Strains

The genotype for the TEL::URA3 strain (UCC1001) is as follows: *MAT α ura3-52 lys2-801 ade2-101 trp Δ 63 his3 Δ 200 leu2- Δ 1 TEL adh4::URA3*. The genotype for the HML::URA3 strain (UCC3515) is as follows: *MAT α ura3-52 lys2-801 ade2-101 trp Δ 63 his3 Δ 200 leu2- Δ 1 HML α ::URA3*. The genotype for non-TEL::URA3 strain (UCC1003), where the URA3 reporter is inserted in an internal chromosomal domain, is as follows: *MAT α ura3-52 lys2-801 ade2-101 trp Δ 63 his3 Δ 200 leu2- Δ 1 adh4::URA3*. These strains were obtained from D. Gottshling (Fred Hutchinson Cancer Research Center, Seattle, WA). The genotype for the rDNA::URA3 strain is as follows: *MAT α his3 Δ 200 leu2- Δ ura3-167 rDNA::Ty1-URA3*. The control strain for this is non-rDNA::URA3, where the URA3 gene is inserted into an unknown site outside of the rDNA locus, has the following genotype: *MAT α his3 Δ 200 leu2- Δ ura3-167 ???::Ty1-URA3*. These strains were obtained from J. D. Boeke (Johns Hopkins University).

Small Molecules

The compounds that were screened were derived from two libraries. The first was purchased from ChemBridge Corporation (San Diego, CA), and the second, an Institute of Chemistry and Cell Biology diver-

sity set, consisted of compounds synthesized at the Harvard Institute of Chemistry and Cell Biology (ICCB; Harvard University) using diversity-oriented synthesis (17). Compound A3 (8,9-dihydroxy-6H-(1)benzofuro[3,2-c]chromen-6-one) was synthesized by Hua Miao (ICCB). Compounds M15 (1-[(4-methoxy-2-nitro-phenylimino)-methyl]-naphthalene-2-ol) and Sirtinol (2-[(2-hydroxy-naphthalen-1-ylmethylene)-amino]-N-(1-phenyl-ethyl)-benzamide) were purchased directly from ChemBridge. 2-Hydroxy-1-naphthaldehyde, 2-hydroxy-1-naphthoic acid, 4-methoxy-2-nitroaniline, and trichostatin A were purchased from Sigma, whereas 2-amino-N-(1-phenyl-ethyl)-benzamide was synthesized by Travis Dunn (Harvard University).

In Vivo Yeast Screen

General Procedures—Several freshly streaked colonies of the desired yeast strain were resuspended in 1 ml of YPDA medium (YPD/0.003% adenine hemisulfate) by vortexing, diluted in 50 ml of YPDA, and vortexed again. A stock solution of 15% 5-FOA in Me₂SO was used to produce 0.4–0.8% 5-FOA solution for the TEL::URA3, rDNA::URA3, and HML::URA3 strains. An equivalent percentage of Me₂SO was used for the untreated samples to account for cytotoxicity. A multidropper apparatus (Labsystems) was used to add yeast to clear bottom 96-well or 394-well plates in volumes of 40–100 μ l. Although this density of yeast in the well was not visible initially, a wild-type strain typically saturated the bottom of the well after 24–48 h of growth at room temperature. To test the dose response on the three strains, 100 μ l of the various yeast strain suspensions were transferred to a 96-well plate, and 0.5 μ l of the compounds or Me₂SO were added. Growth was monitored by visual inspection or by the A₆₀₀ of the cultures using a Wallac spectrometer.

ICCB Diversity Set Compound Library

A collection of 400 compounds, called an ICCB diversity set of compounds, was screened for activity. These compounds were contributed by members of the Harvard Institute of Chemistry and Chemical Biology (ICCB; Harvard University), and were aliquoted into 96-well plates at dilutions between 10 and 20 mM. For this screen, 100 μ l of the TEL::URA3 yeast suspensions \pm 5-FOA were transferred to 96-well plates. 100 nl of 10–20 mM compound stocks were manually pin-transferred to these plates, and thus the compounds were tested at concentrations of 10–20 μ M. Growth was monitored by visual inspection and scored after 1, 2, and 3 days.

ChemBridge Library

The screens tested 1200 compounds of plates from the ICCB ChemBridge Library (plates 1–3). An automated robot pin-transferred 40 nl of compound to each well of the plates. For this screen, 40 μ l of the yeast suspensions, \pm 5-FOA, were transferred to 384-well plates. 40 nl of 5 mg/ml stocks of compounds were pin-transferred to the plates, and thus these were tested at a concentration of \sim 10 μ M. Growth was monitored by visual inspection and scored after 1, 2, and 3 days.

DNA Constructs and Mutagenesis

The bacterial expression construct for GST-SIRT2 (amino acids 18–340) in the pGex4T3 vector (Amersham Pharmacia Biotech) was generously provided by M. Finnin and N. Pavletich (Memorial Sloan-Kettering Cancer Research Center, New York).

Protein Expression and Purification

BL21-(DE3)LysS cells were transformed with the GST-SIRT2 (amino acids 18–340)/pGex4T3 construct. Cells were grown to an A₆₀₀ of 0.3 in NCZYM medium (Life Technologies, Inc.) and induced for 5 h with 0.5 mM isopropyl-1-thio- β -D-galactopyranoside (Sigma) at room temperature. Cells were lysed in 50 mM Tris-HCl, pH 8, 200 mM NaCl, 5 mM dithiothreitol, and 1 mg/ml lysozyme (Sigma) with a freeze-thaw cycle. 40 units of DNase I (Promega) were added to the solution and incubated for 20 min on ice. The lysate was clarified by centrifugation at 14,000 rpm for 15 min, and the incubated was clarified with 1.5 ml of glutathione-agarose beads (Amersham Pharmacia Biotech) at 4 $^{\circ}$ C for 1 h. The beads were washed twice with phosphate-buffered saline, and the protein was eluted by incubation with 10 mM glutathione (Sigma) in JLB (50 mM Tris-HCl, pH 8, 150 mM NaCl, 10% glycerol, 0.5% Triton X-100) for 1 h at 4 $^{\circ}$ C and twice with 10 mM glutathione, JLB for 30 min. This solution was then dialyzed overnight against JLB and stored at -70 $^{\circ}$ C. Expression and purification of recombinant yeast Sir2 protein has been described previously (18).

¹ The abbreviations used are: HDAC, histone deacetylase; TSA, trichostatin A; GST, glutathione S-transferase; 5-FOA, 5-fluoroorotic acid; ICCB, Harvard Institute of Chemistry and Cell Biology; HM, homoallic mating-type locus; JLB, Jurkat lysis buffer.

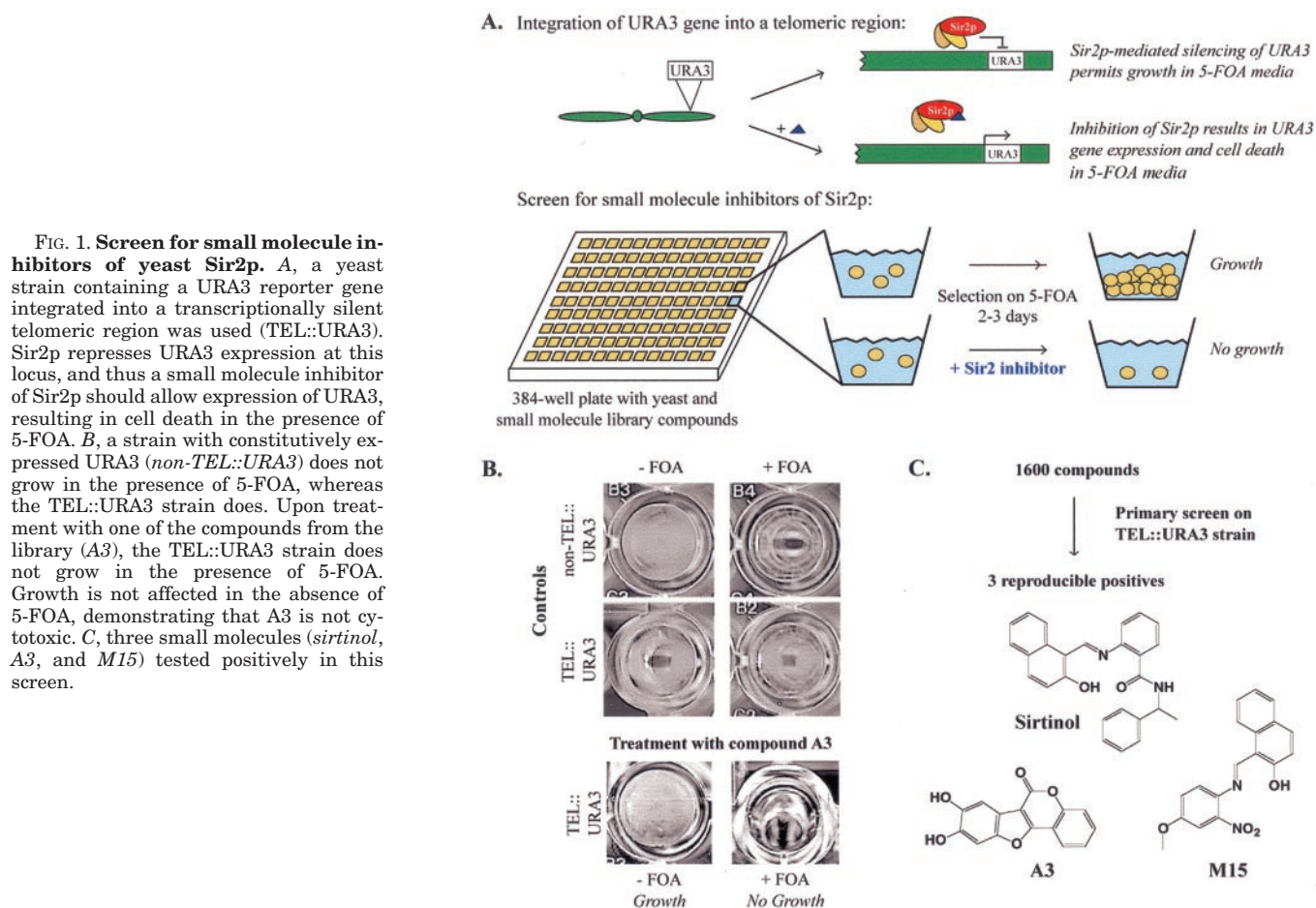


FIG. 1. Screen for small molecule inhibitors of yeast Sir2p. A, a yeast strain containing a URA3 reporter gene integrated into a transcriptionally silent telomeric region was used (TEL::URA3). Sir2p represses URA3 expression at this locus, and thus a small molecule inhibitor of Sir2p should allow expression of URA3, resulting in cell death in the presence of 5-FOA. B, a strain with constitutively expressed URA3 (*non-TEL::URA3*) does not grow in the presence of 5-FOA, whereas the TEL::URA3 strain does. Upon treatment with one of the compounds from the library (A3), the TEL::URA3 strain does not grow in the presence of 5-FOA, demonstrating that A3 is not cytotoxic. C, three small molecules (*sirtinol*, A3, and M15) tested positively in this screen.

Immunoprecipitation of HDAC1 from HeLa Cells

HeLa cells were lysed in JLB containing a complete protease inhibitor mixture (Roche Molecular Biochemicals). Lysis proceeded for 15 min at 4 °C, after which the cellular debris was pelleted by centrifugation at 14,000 rpm for 5 min. Endogenous HDAC1 was purified from the supernatant by incubation with anti-HDAC1 antibody (Sigma) for 30 min and then precipitation with protein G beads (Life Technologies, Inc.) for 45 min at 4 °C. The beads were washed three times with JLB at 4 °C and resuspended in HD buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 10% glycerol) for the histone deacetylase assays.

In Vitro Histone Deacetylation Assays

1.5 μ g of recombinant human GST-SIRT2 (amino acids 18–340) or 0.5 μ g of recombinant yeast Sir2p were incubated for 2 h at 30 °C in 50 μ l of assay buffer (50 mM Tris-HCl, pH 8.8, 4 mM MgCl₂, 0.2 mM dithiothreitol) (9), with or without 50 μ M NAD and acetylated HeLa histones (1000 cpm), purified by acid extraction. HDAC activity was determined by scintillation counting of the ethyl acetate-soluble [³H]acetic acid (19).

Antibodies and Western blot analysis

Antibodies to tetra-acetylated histones H3 and H4 were purchased from Upstate Biotechnology (Lake Placid, NY), and antibodies to acetylated α -tubulin were purchased from Sigma. 1.2×10^6 primary foreskin fibroblast cells were plated in 6 ml of DMEM/10% FBS media, and treated with 0.5% Me₂SO, 10 μ M sirtinol, 50 μ M sirtinol, or 1 μ M TSA for 24 h. Cells were trypsinized, harvested, and lysed in 75 μ l of JLB. The protein concentrations of the lysates were determined by a Bradford assay, and a normalized amount of protein for each sample was separated by SDS-polyacrylamide gel electrophoresis and analyzed by Western blot.

Analysis of Cell Morphology

100×10^5 HeLa cells were plated onto gelatin-coated coverslips and treated with 1% Me₂SO, 10 μ M sirtinol, 25 μ M sirtinol, or 1 μ M TSA for 24 h. Cells were fixed in 4% formaldehyde and viewed with a light microscope.

Analysis of Effect of E8 on Arabidopsis Development

Seeds (*Arabidopsis thaliana* ecotype *Lansberg erecta* (*Le*)) were surface-sterilized by treatment with 70% ethanol for 1 min and then 50% Clorox/0.1% Tween 20 for 8 min and washed 6 times with sterile water. The seeds were air-dried on sterile filter paper in a laminar flow hood for 3 h. Murashige and Skoog (MS) medium (pH 5.7; Sigma) (20) containing 0.8% PhytoAgar (Life Technologies, Inc.) was pipetted into 96-well, flat bottom microtiter plates. Me₂SO (99.9%; EM Science) or sirtinol solutions in Me₂SO were pipetted into the warm, liquid agar and thoroughly mixed. The agar was allowed to set for 30 min prior to manually arraying the seeds into each well with a moistened, sterile pipette tip (4–6 seeds per well). Plates were sealed and incubated for 3 days in the dark at 5 °C to break dormancy (stratification). Plates were incubated thereafter at 25 °C under continuous white fluorescent light, and seedling development was measured in terms of days of growth after transfer to 25 °C.

For unstained whole-mount preparations, plants were placed in FluorSave reagent (CalBiochem) on glass slides and sealed with a coverslip. For staining of the vasculature, plants were fixed in acetic acid: 95% ethanol (3:1) for 1 h, incubated in 25% formaldehyde for 2 h, dehydrated for 1 h in 95% ethanol, stained for 1 min in 1% safranin-O (Sigma) in 95% ethanol, hydrated through an ethanol series to water, and mounted on slides with FluorSave (21).

RESULTS

Screening for Compounds That Derepress Telomeric Genes—A high throughput, cell-based URA3 reporter-based screen was used to identify candidate compounds that interrupt the Sir2p silencing pathway at the telomeres (1). To assess telomeric silencing, the yeast strain (TEL::URA3) containing a URA3 reporter gene inserted near the telomeres was used. Addition of a small molecule inhibitor of Sir2p should derepress the URA3 gene, resulting in death in the presence of 5-FOA. A parallel screen was conducted in the absence of 5-FOA to identify small molecules that were simply cytotoxic (Fig. 1). As a control, a

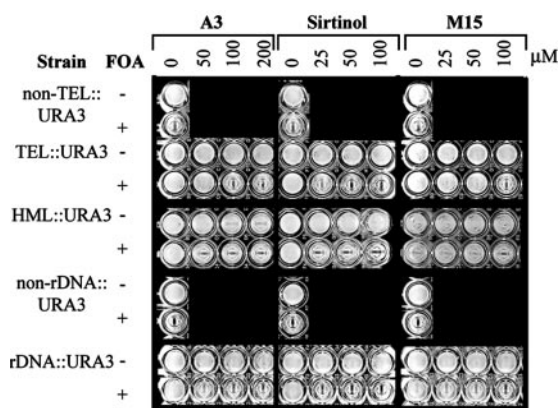


FIG. 2. Sirtinol, A3, and M15 inhibit Sir2p transcriptional silencing at three loci in yeast. The three positives from the primary screen were tested for their ability to inhibit Sir2p-mediated transcriptional silencing at the telomeric locus (*TEL::URA3*), silent mating-type locus (*HML::URA3*), and rDNA locus (*rDNA::URA3*). Cultures were visually inspected for growth, and growth was quantitated by taking the A_{600} of the wells (data not shown). A3 and M15 inhibited growth in the three strains for at least 3 days at 100 μM , whereas sirtinol was effective at 25 μM .

strain with a URA3 reporter gene integrated into a transcriptionally active internal chromosomal region (non-*TEL::URA3*) was tested for growth in the presence of 5-FOA (see Fig. 1B; note that in the absence of growth, the media in the well is clear, whereas growth results in a layer of yeast covering the bottom of the well, and thus the well appears opaque).

Two libraries, with a total of 1600 compounds, were screened for small molecules that derepress telomeric silencing. Compounds were pin-transferred to a 96- or 384-well clear bottom plate containing yeast in YPDA media, with or without 0.4% 5-FOA. This method transferred 40 or 100 nl of the 10 to 20 mM stock solutions into a volume of 40 or 100 μl . Thus, this screen was conducted at a concentration of 10 to 20 μM . An example of a positive from this screen is shown in Fig. 1B. Twelve hits were identified in the initial screen, three of which were verified as reproducible positives in a subsequent retest of these compounds in the same assay. The structures of these compounds are shown in Fig. 1C. Note that all of these compounds are planar and aromatic, similar in structure to the adenine and nicotinamide moieties of NAD. Furthermore, two of the three compounds (sirtinol and M15) contain substructures derived from 2-hydroxy-1-naphthaldehyde.

Dose Response for *in Vivo* Inhibition of Sir2p-mediated Silencing at Three Loci—Sir2p can repress transcription at the telomeric, silent mating-type, and rDNA loci, as a member of the Sir2-3-4 complex at the first two loci and as a member of the Net1-cdc14-Sir2 complex for the rDNA locus (reviewed in Ref. 1). To ensure that the three compounds specifically targeted Sir2p and did not simply disrupt its interactions with other proteins, the compounds were resynthesized or purchased, and the new stocks were tested for depression of URA3 reporter genes at the telomeric, HM, and rDNA loci. All three compounds inhibited growth in the three strains of yeast in the presence of 5-FOA, even after more than 3 days of growth (Fig. 2). A3 and M15 inhibited growth at 100 μM , whereas sirtinol inhibited growth at 25 μM . Thus, these compounds appear to inhibit Sir2p transcriptional silencing activity directly.

Inhibition of Yeast Sir2p and Human SIRT2—The ability of these compounds to abrogate silencing at several chromosomal domains controlled by Sir2p suggested that they directly inhibit Sir2p deacetylase activity. To confirm this, the three compounds were tested for their ability to inhibit NAD-dependent histone deacetylase activity of purified recombinant yeast

TABLE I
Inhibition in vitro of yeast Sir2p and human SIRT2 activity by A3, sirtinol, and M15

Purified, recombinant yeast Sir2p and human hGST-SIRT2 (amino acids 18–340) were incubated at 30 °C for 2 h with different concentrations of the three compounds, 50 μM NAD, and tritiated acetylated histones. Enzymatic activity was monitored by amount of released tritiated acetate. Each experiment was performed in triplicate. Note that M15 precipitated out at concentrations higher than 50 μM .

Inhibitor	% Sir2p activity remaining at 50 μM	% SIRT2 activity remaining at 50 μM	IC ₅₀ (μM) of Sir2p	IC ₅₀ (μM) of SIRT2
A3	62 ± 4	45 ± 4	66 ± 4	45 ± 3
Sirtinol	81 ± 6	31 ± 4	68 ± 3	38 ± 2
M15	60 ± 3	75 ± 5		

Sir2p *in vitro*. Furthermore, these compounds were assessed for their ability to generally inhibit sirtuin deacetylase activity by testing them with the catalytic domain of human SIRT2 (amino acids 18–340). A3 inhibited yeast Sir2p and human SIRT2 at IC₅₀ values of ~70 and 45 μM , respectively (Table I), whereas sirtinol was slightly more potent against the human sirtuin, with IC₅₀ values of 70 and 40 μM . M15 did not inhibit either enzyme particularly well. Unfortunately, M15 was not very soluble in aqueous media and precipitated out at concentration higher than 50 μM . Thus, it is possible that M15 can inhibit these enzymes but at concentrations higher than solubility allows. Alternatively, M15 may act on a common unidentified component in the Sir2p complexes.

Structure-Activity Relationship Analysis—Sirtinol and M15 are synthesized by coupling 2-hydroxy-1-naphthaldehyde to either 2-amino-*N*-(1-phenyl-ethyl)-benzamide or 4-methoxy-2-nitroaniline, respectively (Fig. 3A). Thus, it is possible that the 2-hydroxy-1-naphthaldehyde moiety is primarily responsible for the inhibitory activity of these compounds. To test this hypothesis, the individual fragments of sirtinol and M15 (2-hydroxy-1-naphthaldehyde, 2-amino-*N*-(1-phenyl-ethyl)-benzamide and 4-methoxy-2-nitroaniline) were tested for their ability to inhibit SIRT2 activity *in vitro* at 75 μM (Fig. 3B). Sirtinol potently inhibited SIRT2 at 75 μM , whereas 2-hydroxy-1-naphthaldehyde partially inhibited activity. The amine monomers did not significantly reduce activity. TSA, a specific nanomolar inhibitor of HDACs, did not inhibit sirtuin activity. Thus, 2-hydroxy-1-naphthaldehyde did inhibit sirtuin deacetylase activity *in vitro* but at much higher concentrations than required for sirtinol.

2-Hydroxy-1-naphthaldehyde was also tested for its ability to inhibit Sir2p transcriptional silencing in yeast, at the telomeric, HM, and rDNA loci (Fig. 3C). This compound was able to activate transcription of the URA3 reporter at 50 μM in all cases but became cytotoxic at 200 μM .

Inhibition of HDAC1 Activity—A3, sirtinol, and 2-hydroxy-1-naphthaldehyde were tested for their ability to inhibit human HDAC1 (Table II) to assess their specificity for sirtuin deacetylases. A3 is the only compound that significantly inhibits HDAC activity at 50 μM . This is somewhat surprising, because it does not resemble any known HDAC inhibitors (22). Sirtinol and 2-hydroxy-1-naphthaldehyde did not affect HDAC1 activity at 50 and 100 μM , respectively, suggesting that they specifically inhibit sirtuin deacetylase activity.

Effect of Sirtinol Treatment on Mammalian Cells—Treatment of mammalian cells with the potent HDAC inhibitor TSA causes acetylation of histones (15) and α -tubulin, as well as morphological changes resulting from the rearrangement of the actin cytoskeleton produced by increased expression of the actin-binding protein gelsolin (23). To assess the effect of sirtinol treatment on cell morphology, HeLa cells were treated for

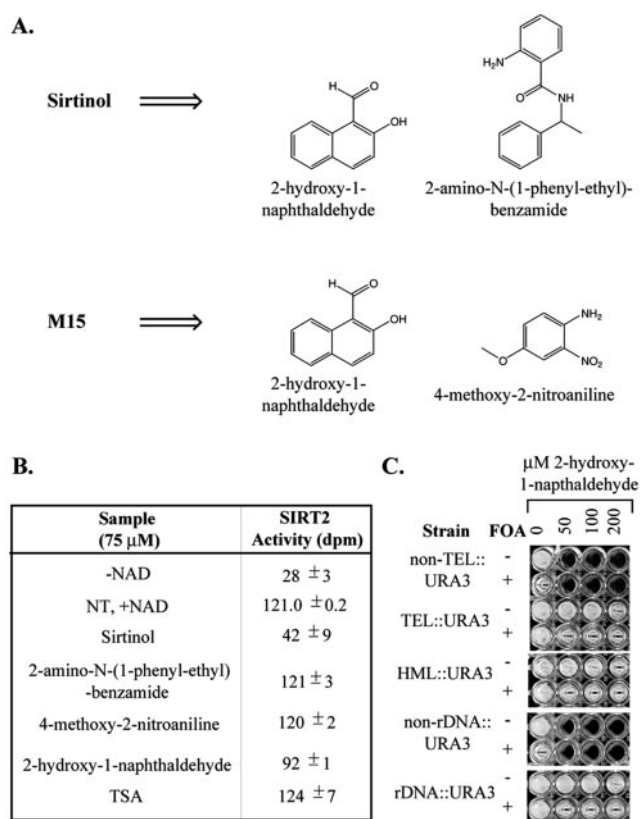


FIG. 3. Structure-activity relationship analysis. A, sirtinol and M15 are synthesized by coupling 2-hydroxy-1-naphthaldehyde with an amine. B, assay for inhibition of SIRT2 activity *in vitro*. Sirtinol, 2-hydroxy-1-naphthaldehyde, and the amine moieties of sirtinol and M15 were incubated with purified, recombinant human GST-SIRT2 (amino acids 18–340), in the presence of 50 μM NAD and tritiated acetylated histones. Enzymatic activity was monitored by determining the amount of released tritiated acetic acid by scintillation counting. Each experiment was performed in duplicate. Sirtinol potentially inhibited SIRT2 activity, whereas 2-hydroxy-1-naphthaldehyde moderately inhibited activity at this concentration. C, 2-hydroxy-1-naphthaldehyde was tested for its ability to inhibit Sir2p-mediated transcriptional silencing at the telomeric locus (*TEL::URA3*), mating locus (*HML::URA3*), and rDNA locus (*rDNA::URA3*). 2-Hydroxy-1-naphthaldehyde inhibited growth in the presence of 5-FOA at 50 μM .

TABLE II
Effect of A3, sirtinol, and 2-hydroxy-1-naphthaldehyde on HDAC1 activity

HDAC1 was immunoprecipitated from HeLa cell lysate and was incubated with the indicated compounds in the presence of tritiated acetylated histones. Enzymatic activity was monitored by determining the amount of released tritiated acetate by scintillation counting. Each experiment was performed in triplicate. At 50 μM , A3 inhibits activity weakly, whereas sirtinol has no effect. 2-Hydroxy-1-naphthaldehyde does not significantly inhibit HDAC1 activity at 100 μM .

Sample	HDAC activity
	dpm
Mock immunoprecipitation	76 \pm 1
No treatment	208 \pm 15
A3 (50 μM)	165 \pm 19
Sirtinol (50 μM)	225 \pm 14
2-Hydroxy-1-naphthaldehyde (100 μM)	187 \pm 8
TSA (500 nM)	48 \pm 5

24 h with 10 and 50 μM sirtinol, as well as 1 μM TSA, and visualized under a light microscope. Treatment with TSA caused HeLa cells to change from a rounded morphology to a flattened morphology (Fig. 4B), whereas treatment with sirtinol did not produce this effect. These studies provide further evidence that sirtinol does not affect HDAC activity and sug-

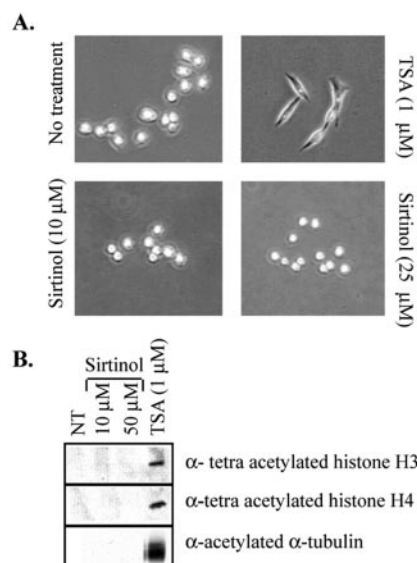


FIG. 4. Inhibition of HDACs and sirtuins does not produce the same effect in mammalian cells. A, inhibition of sirtuins does not result in a morphological change in HeLa cells. HeLa cells were treated with 10 μM sirtinol, 25 μM sirtinol, or 1 μM TSA for 24 h. Cells were fixed in 4% formaldehyde and viewed with a light microscope. Treatment with TSA resulted in a dramatic morphological change, in which cells spread from their normally rounded phenotype. Treatment with sirtinol did not produce this effect. B, inhibition of sirtuins does not result in global acetylation of histones or tubulin. Primary foreskin fibroblasts were treated with 10 μM sirtinol, 50 μM sirtinol, or 1 μM TSA for 24 h. Cells were harvested and lysed, and the total protein content was normalized. The amount of acetylated histones H3 and H4 and α -tubulin in each sample was assessed by Western blot analysis using antibodies to tetra-acetylated histone H3 and H4 (Upstate Biotechnology) and acetylated α -tubulin (Sigma). TSA caused robust acetylation of all three proteins, whereas sirtinol had no effect. NT, not treated.

gests that sirtuins are involved in processes distinct from those of HDACs in mammalian cells.

To determine whether sirtuins are involved in the regulation of the global acetylation state of histones or tubulin, primary fibroblast cells were treated with 10 and 50 μM sirtinol, as well as 1 μM TSA, for 24 h (Fig. 4A). The cells were harvested and lysed, and equal amounts of total cellular protein were separated by SDS-polyacrylamide gel electrophoresis and analyzed for acetylation levels of histones and α -tubulin by Western blot. Treatment with 1 μM TSA caused robust acetylation of histones H3 and H4, as well as α -tubulin, whereas treatment with sirtinol did not produce any of these effects.

Effect of Sirtinol Treatment on Arabidopsis Development—*Arabidopsis* seeds were germinated and grown for 4 days on agar containing 1% Me_2SO , 25, 50, and 100 μM sirtinol. Plants were visually inspected under a dissecting light microscope. Seedlings grown in the presence of Me_2SO alone developed normally, with a long hypocotyl (seedling stem) and primary root apparent by 3 days of growth. In the case of plants treated with sirtinol, the hypocotyl was significantly shorter and thicker, and the primary root was completely absent (Fig. 5A). This phenotype was apparent at all sirtinol concentrations, though was most severe (shortest hypocotyls and smallest cotyledons) at 100 μM . All plants that germinated in sirtinol exhibited this phenotype in two independent experiments.

Similar phenotypic alterations in the formation of the apical-basal body axis (which collectively refers to the hypocotyl and primary root structures) have been observed in the *Arabidopsis* mutant *MONOPTEROS* (24, 25). In this case, vascularization of the seedling is also severely affected, and thus only the central vascular strands are apparent. To assess the state of the vascular system in the seedlings treated with sirtinol,

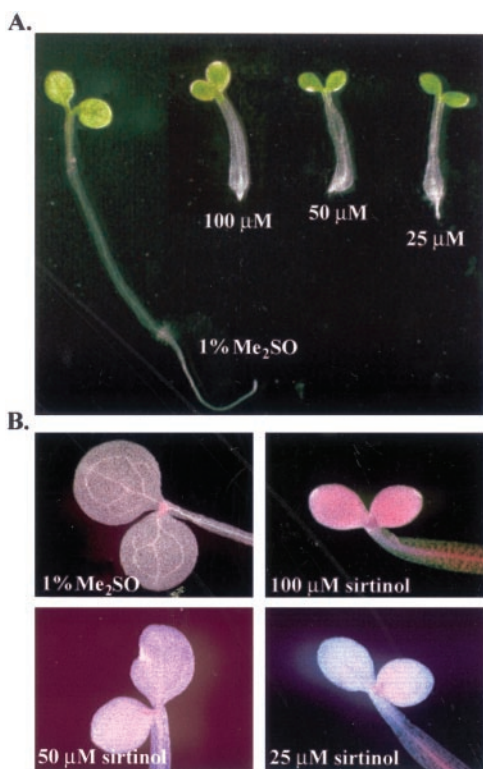


FIG. 5. Treatment with sirtinol inhibits body-axis formation and vascularization in *Arabidopsis*. *Arabidopsis* seedlings were germinated in the presence of 1% Me₂SO, 25, 50, and 100 μM sirtinol (A). After 4 days of growth in the presence of sirtinol, the seedlings lack primary roots and have shortened and thickened hypocotyls. Staining of seedlings after 5 days of growth revealed that sirtinol treatment also blocks the formation of the vascular system in the cotyledons these plants (B).

plants were stained and visualized under a light microscope. Although Me₂SO-treated plants have distinct veins apparent in the hypocotyl and cotyledons after 5 days of growth, sirtinol-treated plants have distinct veins only apparent in the hypocotyl (Fig. 5B). Note, however, that the cotyledons of the treated plants are somewhat smaller and might develop normal veins at a much slower rate than untreated seedling. Thus, sirtinol inhibits body axis formation and vascularization in growing plants.

DISCUSSION

Herein we described a high-throughput, phenotypic screen in cells using 1600 unbiased small molecules. The assay was aimed to uncover cell-permeable inhibitors of yeast and human sirtuins; indeed it led to the identification of a class of molecules that specifically inhibit yeast Sir2p and human SIRT2. The initial screen identified inhibitors of Sir2p silencing activity in yeast, and this was followed by several subsequent secondary assays. First, the three compounds identified in the primary screen were tested for their ability to inhibit Sir2p transcriptional silencing in the context of different Sir2p complexes and at different chromosomal domains. All three compounds scored positively in this assay, suggesting that they directly inhibited Sir2p transcriptional silencing activity. Second, these compounds were tested for their ability to inhibit yeast Sir2p and human SIRT2 activity *in vitro*. Two of the three (A3 and sirtinol) are inhibitors of both enzymes, with IC₅₀ values ranging from 40 to 70 μM. Third, these two compounds were tested for their ability to inhibit HDAC1 histone deacetylase activity *in vitro*. Although sirtinol had no effect on HDAC1 activity, A3 does weakly inhibit it, suggesting that it may not specifically inhibit sirtuin deacetylase activity. Thus, sirtinol is

the first reported cell-permeable inhibitor of the sirtuin class of deacetylases. Furthermore, because it is competent to inhibit sirtuin enzymes from two different species, it is likely to be a general sirtuin inhibitor and thus transferable between a number of systems.

Screening Methodology—This yeast-based reporter gene assay provides a platform for screening for small molecule modulators of Sir2p and possibly of other transcriptional regulators. The screen is high throughput, requires minimal labor, is easy to score for positives, and does not require specialized equipment. Furthermore, the screen gave remarkably specific results, as is evident by the fact that all three hits functioned in secondary assays. It is interesting to note that though the screen was for inhibitors of yeast Sir2p, both A3 and sirtinol were more potent inhibitors of human SIRT2, thus further demonstrating the versatility of this screening methodology. Finally, because it is a cell-based screen, it is selective for compounds that are cell-permeable and therefore more useful *in vivo*.

Identification of a Class of Sirtuin Inhibitors—Two of the compounds (sirtinol and M15) identified in the primary screen were derived by coupling 2-hydroxy-1-naphthaldehyde to an amine. 2-Hydroxy-1-naphthaldehyde alone also inhibited human SIRT2 activity, though not as well as sirtinol. Subsequent testing of other 2-hydroxy-1-naphthaldehyde derivatives from the ChemBridge library revealed that a subset of these inhibited SIRT2 *in vitro* deacetylase activity at approximate IC₅₀ values of 50–70 μM, as well (data not shown). Thus, it appears that the naphthaldehyde group makes important contacts with the enzyme active site and is primarily responsible for the inhibitory activity of these molecules. It may be possible to produce more potent inhibitors by making analogs of these 2-hydroxy-1-naphthaldehyde derivatives. An even more exciting possibility is that it will be possible to generate specific inhibitors of the different sirtuin homologs by modifying this basic structure.

Preliminary Studies of Effects of Sirtinol Treatment—This general inhibitor of sirtuin enzymes can be applied to the study of the biological function of sirtuins in a variety of systems and species. Thus far, the *in vivo* function of sirtuins has only been well understood in yeast, and even in this case there are several sirtuins that have not been characterized. Unfortunately, because of the lack of characterization of sirtuins in mammals, there is no direct method to test sirtinol for its ability to inhibit sirtuins in human cells. Attempts to create an artificial system in which a reporter gene was repressed by the catalytic domain SIRT2 were unsuccessful, perhaps because SIRT2 does not function in transcriptional regulation, or the recombinant protein was unable to recruit essential associated proteins. However, given that sirtinol can penetrate the yeast cell, it is expected that it can enter human cells, as well. Furthermore, because sirtinol inhibits both yeast Sir2p and human SIRT2 *in vitro*, it may be competent to inhibit all sirtuin enzymes, given the high degree of conservation of the catalytic domains. Unlike TSA, treatment of human primary fibroblasts with sirtinol did not cause global changes in acetylation of histones and tubulin, nor did it induce a morphological changes in the HeLa tumor cell line. This provides further evidence that sirtinol is selective for sirtuin deacetylases and does not affect HDACs and suggests that sirtuins perform very different functions than HDACs *in vivo*.

Preliminary work with sirtinol on *Arabidopsis*, which contain at least two sirtuins (GenBank™ accession numbers 2656026 and 9955507), suggests that these proteins play a critical role in apical-basal body axis development and vascularization. Seedlings grown in sirtinol have thick, short hypocotyls and no pri-

mary roots, and the vein system was abnormal in the cotyledons. This morphology is very similar to that observed in *MONOPTEROS* mutants (24, 25). The *MONOPTEROS* protein is a member of a family of transcriptional regulators, several of which have been shown to regulate expression of auxin-responsive genes (24, 26). Auxin is a plant hormone that is involved in vascularization and elongation of the hypocotyl and primary root. Inhibition of auxin transport through the plant by certain compounds results in seedlings with shortened hypocotyls and roots or lacking in the primary root structure altogether (27, 28). Interestingly, seedlings treated with these auxin transport inhibitors still possess normal vein development in the cotyledons but not in the first true leaves (28). Thus, the mutation of *MONOPTEROS* and the phenotype produced by treatment with sirtinol do not match exactly that observed by auxin transport inhibition but do suggest that these operate on a related or the same pathway. At this stage, it is unclear whether the observed phenotype is due solely to the inhibition of sirtuin activity, though it does provide an interesting avenue to explore with respect to the transcriptional regulation of basal-apical body axis formation in plants. Sirtuins may control the synthesis of auxin or auxin-transporting proteins, or they may regulate the cellular transcriptional response to auxin.

Future Directions—Reverse chemical genetics using small molecule modulators of proteins can be used to probe the cellular functions of proteins in different systems. Such inhibitors can be used in a temporally defined manner and are applicable in a variety of experimental systems. Fine temporal control has not been possible with even the best characterized sirtuin, yeast Sir2p, because no temperature-sensitive alleles have been reported. Thus, inhibitors of sirtuins will allow us to refine our understanding of, for example, yeast Sir2p, by comparing the immediate effects of a loss of Sir2p function with those steady-state cellular changes observed in yeast strains in which Sir2 has been deleted. A particularly powerful approach for assessing the resulting effect of sirtuin inhibition, by analogy to studies of HDACs, is global transcriptional profiling (29). Furthermore, these small molecules can be used to initiate studies of sirtuin function in a wide variety of experimental systems, ranging from yeast to human cell cultures, to organisms such as zebrafish and *Arabidopsis*.

Sirtuin proteins in yeast and bacteria have diverse biological functions, including the regulation of transcription, aging, the

control of the cell cycle, DNA-damage repair, and metabolism (reviewed in Ref. 1) (14). With seven human sirtuins (8), it is expected that the roles of these proteins in mammalian cells are equally diverse, but thus far these proteins have not been characterized. The identification of a class of small molecule inhibitors of the sirtuin family of deacetylases will now allow for a broad investigation of the functions of these proteins and the cellular pathways in which they are involved.

Acknowledgments—We thank Mary Kay Pflum and Christian Hassig for critical reading of the manuscript.

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