

Published in final edited form as:

Bioorg Med Chem. 2009 October 1; 17(19): 7031–7041. doi:10.1016/j.bmc.2009.07.073.

IDENTIFICATION AND CHARACTERIZATION OF NOVEL SIRTUIN INHIBITOR SCAFFOLDS

Brandi D. Sanders^{1,2}, Brittany Jackson^{1,2}, Michael Brent^{1,2}, Alexander M. Taylor³, Stuart L. Schreiber³, Konrad Howitz⁴, and Ronen Marmorstein^{1,2}

¹The Wistar Institute, University of Pennsylvania, Philadelphia, PA, 19104 USA

²Department of Chemistry, University of Pennsylvania, Philadelphia, PA, 19104 USA

³Howard Hughes Medical Institute, Broad Institute of Harvard and MIT, Department of Chemistry and Chemical Biology, Harvard University, 12 Oxford Street, Cambridge, MA 02138

⁴ENZO Life Sciences, Inc., 5120 Butler Pike Plymouth Meeting, PA 19462

Abstract

The sirtuin proteins are broadly conserved NAD⁺-dependant deacetylases that are implicated in diverse biological processes including DNA recombination and repair, transcriptional silencing, longevity, apoptosis, axonal protection, insulin signaling and fat mobilization. Because of these associations, the identification of small molecule sirtuin modulators has been of significant interest. Here we report on high throughput screening against the yeast sirtuin, Hst2, leading to the identification of four unique inhibitor scaffolds that also inhibit the human sirtuins, SIRT1, SIRT2 and SIRT3. The identified inhibitor scaffolds range in potency from IC₅₀ values of 6.5–130 μM against Hst2. Each of the inhibitor scaffolds binds reversibly to the enzyme, and kinetic analysis reveals that each of the inhibitors is non-competitive with respect to both acetyl-lysine and NAD⁺ binding. Limited SAR analysis of the scaffolds also identifies which functional groups may be important for inhibition. These sirtuin inhibitors are low molecular weight and well-suited for lead molecule optimization, making them useful chemical probes to study the mechanism and biological roles of sirtuins and potential starting points for optimization into therapeutics.

Keywords

Sirtuin; Hst2; SIRT1; inhibitor

1. Introduction

The class III family of histone deacetylases (HDACs), or sirtuins, require NAD⁺ for the removal of an acetyl moiety from the ε-amino group of lysine residues within protein targets^{1, 2} to yield the deacetylated protein target, nicotinamide, and 2'-O-acetyl-ADP-ribose.^{3, 4} The founding member of this protein family, *Saccharomyces cerevisiae* Sir2p, was shown

© 2009 Elsevier Ltd. All rights reserved.

Address Correspondence to: Ronen Marmorstein, Ph.D., The Wistar Institute, 3601 Spruce Street, Philadelphia, PA 19104, Fax: 215-898-0381; marmor@wistar.org.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

to be a limiting factor in yeast aging, as deletion of the SIR2 gene resulted in reduced lifespan⁵, and additional copies of SIR2 resulted in increased yeast replicative lifespan.⁶ Furthermore, Sir2p proved to be required for the lifespan extension that results from restricting the caloric intake of yeast cells.⁷ Since the sirtuin protein family is broadly conserved⁸, it was also shown that increased expression of Sir2 led to increased lifespan in higher organisms such as worms,⁹ flies,¹⁰ and mice,¹¹ and increased longevity due to a calorie restricted diet has been shown in most of these animals to be Sir2 dependent.^{10, 12} Mammals have seven homologues of the yeast Sir2 protein (SIRT1-7),^{13, 14} and increased SIR2 copy number or Sir2 expression level provides several health benefits in mammals consistent with a reduction in age-related diseases (reviewed in¹⁵). The most closely related human Sir2p homologue, SIRT1, has been implicated to play a role in a number of age-related human diseases and biological functions such as cell survival, apoptosis, stress resistance, fat storage, insulin production, glucose homeostasis, and lipid homeostasis through direct deacetylation or regulation of its many known *in vivo* targets including p53, Ku70/Bax, FOXO, PPAR γ , PGC1 α , UCP2, LXR, and NF κ B (reviewed in^{15, 16}). Although the cellular mechanism by which increased Sir2 activity leads to increased lifespan and/or improvements in the biological functions listed above appears different in each organism, increased Sir2 activity seems to lead to an increase in mitochondrial biogenesis in all organisms, underlying the importance of the metabolic state of the cell for Sir2 activity levels.¹⁶

The catalytic mechanism by which sirtuin proteins couple NAD⁺ cleavage to deacetylation and the mechanism of nicotinamide inhibition have important implications for Sir2 regulation by the physiological regulators NAD⁺ and nicotinamide, and for development of synthetic regulators of sirtuin proteins. Nicotinamide (**1**), a reaction product and noncompetitive inhibitor of Sir2 proteins,^{2, 17} has also been shown to be a physiological regulator of this family of proteins.¹⁸ Yeast cells grown in the presence of nicotinamide show a dramatic reduction in silencing, an increase in rDNA recombination, and a shortening of replicative lifespan.¹⁷ Nicotinamide has also been shown to inhibit Sir2 deacetylation in a number of human cell lines.¹⁹ Nicotinamide exerts its inhibitory effect on deacetylation by reacting with a reaction intermediate to reform β -NAD⁺ at the expense of deacetylation.^{20, 21}

Since human sirtuin proteins are involved in a myriad of biological functions directly related to human aging and disease, and because several details of the catalytic mechanism of sirtuin proteins remain to be determined, this class of proteins is an active target for pharmacological small molecule effector design (Figure 1). In the case of human cancer, SIRT1 inhibitors may prevent the deacetylation of p53 and allow apoptosis in response to cellular damage;^{19, 22} inhibit silencing of tumor suppressor genes whose DNA is hypermethylated;²³ or increase H4-K16 and H3-K9 acetylation at endogenous promoters to induce gene re-expression in breast and colon cancer cells.²⁴ The first small molecule Sir2 inhibitors were identified from high throughput yeast phenotypic screens with a Ura3 reporter gene inserted into telomeric regions.^{25, 26} These inhibitors, sirtinol (**2**) and splitomycin (**3**), have moderate potency (low micromolar) toward Sir2, and while it is not clear exactly where they bind to the enzyme, splitomycin appears to be competitive with the acetyl-lysine substrate. Other sirtuin inhibitors have also been identified such as cambinol (**4**)²⁷ and the tenovins (**5**)²⁸ which also have low micromolar potency, decrease tumor cell growth, and are noncompetitive with respect to NAD⁺ binding. Cambinol is competitive with respect to acetyl-lysine binding while the tenovins are not. *In silico* screens against the known human SIRT2 structure^{29, 30} and a model of human SIRT1³¹ have also yielded inhibitors with mid to low micromolar potency. Several groups have taken more rational approaches to identifying sirtuin inhibitors. One group screened a library of known kinase inhibitors (ATP mimetics) for their effect on Sir2 activity and identified a potent (sub-micromolar) indole compound, Ro 31-8220 (**6**).³² Another group purified several molecules from natural sources that were known to have health benefits, but had no known mechanism of action.³³ Finally, a third group tested analogs of polyphenols

(putative sirtuin activators).³⁴ Both of the latter approaches yielded compounds with low micromolar potency. The most successful strategy to date for the identification of sirtuin inhibitors has been *in vitro* high throughput screening (HTS). Among the compounds identified in this way have been surfactin (**7**)³⁵ suramin (**8**)³⁶ and the most potent known Sir2 inhibitors, indole EX527 analogs (**9**, **10**).³⁷ Surfactin is a large cyclic lipopeptide that is thought to be competitive with NAD⁺ binding and may be an effective anti-malarial agent through its ability to inhibit *Plasmodium falciparum* Sir2. Suramin is very large, and although small molecule analogs of this compound have not been potent Sir2 inhibitors,³⁸ binding of the compound to human SIRT5 aided in crystallization of this Sir2 homologue.³⁹ The indoles are noncompetitive with both substrates, are postulated to bind after the release of nicotinamide, and have good ADME characteristics. Structure-activity relationship (SAR) studies of several of the identified inhibitor scaffolds have been performed in an attempt to identify compounds with increased potency and selectivity or more optimal drug-like characteristics with only modest success.^{38, 40-46}

In order to identify novel sirtuin effector scaffolds, we performed an *in vitro* high throughput screen of more than 50,000 small molecule compounds against *S. cerevisiae* Hst2. Here, we report four novel compound scaffolds (**11-14**) that inhibit deacetylase activity of several sirtuin homologues (Figure 1). To determine which functional groups of these scaffolds were important for deacetylase inhibition, limited SAR data on each of the identified scaffolds was collected. Finally, each of the inhibitor scaffolds was kinetically characterized to gain insight into the binding mode of the inhibitors. The identified inhibitor scaffolds have low micromolar potency and are non-competitive with both substrates. These inhibitor scaffolds may be used for lead molecule optimization to identify more potent and/or selective sirtuin inhibitors with possible therapeutic applications.

2. Results and discussion

2.1 High throughput screen (HTS) for modulators of Hst2

Approximately 50,000 compounds from several Broad Institute small molecule libraries were screened as potential effectors of the *Saccharomyces cerevisiae* sirtuin, Hst2. The *in vitro* fluorescently-based screening assay was developed to simultaneously screen for sirtuin inhibitors and activators. Nicotinamide, a reaction product and inhibitor of the Sir2 deacetylase reaction,² was added to each reaction well at its approximate IC₅₀ concentration in order to inhibit the deacetylase reaction by half. In this way, compounds that inhibited or activated deacetylation through K_m or V_{max} effects or increased the net turnover of the reaction through relief of nicotinamide inhibition could be detected.

The initial HTS identified 74 potential Hst2 effectors, a hit rate of approximately 0.14%. When retested, several of the compounds did not show reproducible effects, inhibited the trypsin assay developer or were autofluorescent at the wavelengths of the experiment (the putative Hst2 activators). These compounds were not pursued further. The remaining four scaffolds, 4-(4-Ethyl-phenoxy)-butyric acid 6-oxo-6H-benzo[c]chromen-3-yl ester (**11**), 3-(1-Oxo-1,3-dihydro-isoindol-2-yl)-propionic acid (**12**), hexachlorophene (**13**), 6-Methoxy-1-(3-methoxy-prop-1-ynyl)-2-methyl-1,2,3,4-tetrahydro-isoquinolin-7-ol or 6-Methoxy-2-(4-methoxy-benzyl)-1-(3-methoxy-prop-1-ynyl)-1,2,3,4,6,7-hexahydro-isoquinolin-7-ol (**14a** or **14b**, respectively) represented novel sirtuin inhibitor scaffolds (Figure 1). Each of these compounds also showed inhibition of deacetylase activity in an assay using radiolabeled NAD⁺ and an unlabeled acetyl-lysine containing peptide (Supplemental Figure 1), showing that the presence of a fluorescently labeled acetyl-lysine peptide is not required for inhibition. None of these compounds appear to be closely related analogs of either reaction substrate.

2.2 Effect of HTS hits on several sirtuin homologues

Each of the novel inhibitor scaffolds inhibited both Hst2 and the full length human sirtuin, SIRT1, in a dose-dependent manner. The IC_{50} value defines the concentration of inhibitor required to half-saturate the enzyme population under specific assay conditions and is commonly used as a measure of relative inhibitor potency among compounds. Because IC_{50} values are typically used to rank-order the potency of validated hits from HTS,⁴⁷ we determined the IC_{50} for each of the inhibitor scaffolds identified in our HTS for both Hst2 and human SIRT1 (Table 1). Several of the newly identified sirtuin inhibitors had low micromolar IC_{50} values (**11**, **12**, **13**) while the remaining inhibitors had IC_{50} values in the mid micromolar range (**14a** and **14b**). Because changes in solution conditions, such as pH, ionic strength, temperature and especially the concentration of substrates can alter the measured IC_{50} value, we determined the IC_{50} value of several previously identified sirtuin inhibitors (**1-10**) for comparison, using the same assays conditions that were used for the inhibitors identified in the HTS used in this study (Table 1). Significant inhibition for Hst2 or SIRT1 was not observed for several of these inhibitors, under our assay conditions, (**3-4**, **7**), indicating either that these inhibitors are specific for homologues other than the ones we tested or are not very potent inhibitors under our assay conditions. The previously identified indole compound (**9**)³⁷ was the most potent inhibitor for both enzymes. Interestingly, several of the scaffolds that we identified (**11-13**) were more potent than all previously identified inhibitors tested against Hst2, other than the indoles, suggesting that these scaffolds might be ideal lead molecules for the development of potent and selective sirtuin inhibitors.

Because each of the compounds identified by HTS inhibited both Hst2 and SIRT1, and these sirtuin proteins have very little sequence homology outside of the catalytic core region, these compounds are presumed to bind to the catalytic core region of the proteins and are thus expected to inhibit most sirtuin proteins. To see if this was the case, to determine to what extent other sirtuin homologues are inhibited, and to determine the specificity of the compounds for several sirtuin proteins, we tested the ability of the identified compounds to inhibit a panel of human sirtuin homologues. Scaffold **14** was a potent inhibitor of human SIRT2 and SIRT3, with an apparent IC_{50} value of less than 50 μ M. Interestingly, scaffold **11** was a potent inhibitor of SIRT2 but not SIRT3. Scaffolds **12** and **14a** had only weak inhibitory activity against human SIRT2 and SIRT3 (Supplemental Table 1). Human SIRT4 and SIRT7 do not possess *in vitro* deacetylase activity, and the deacetylase activity of SIRT5 and SIRT6 was too weak under the assay conditions described here to accurately determine the percent of residual enzyme activity. The fact that all of the compounds inhibited SIRT2 and SIRT3 to some extent indicates that these compounds indeed bind in the catalytic core region of sirtuin proteins to exert their inhibitory effects. The varying potencies of these compounds against different human sirtuin homologues suggests that modification of these lead compounds may increase the selectivity of these compounds for a specific homologue relative to the other human sirtuins.

2.3 Determining inhibitor scaffold binding reversibility

To aid in the determination of the mechanism of inhibition for each newly identified inhibitor scaffold and to assay reversibility of inhibitor binding to the enzyme, we tested whether or not deacetylase inhibition was rapidly reversible, slowly reversible or irreversible. To do this, 100 \times the normal assay concentration of enzyme was incubated with 10 \times the IC_{50} concentration of each inhibitor. Then, the enzyme was diluted 100-fold into reaction wells containing both substrates, and the amount of product formation over time was monitored (Figure 2). If the enzyme inhibitor is rapidly reversible, the progress curve should be linear with a slope equal to about 91% of the control sample since the final inhibitor concentration will be $0.1 \times IC_{50}$, or about 9% inhibited for a well-behaved concentration-response relationship. If the enzyme is irreversible or very slowly reversible on the time scale of the assay, then only about 9% of residual activity will be monitored after the dilution because the initial incubation of the

inhibitor was at a concentration of $10 \times IC_{50}$, or ~91% inhibition.⁴⁷ For each of the inhibitor scaffolds identified by HTS, the resulting slopes of the progress curves are all within 9% of the slope of the enzyme only sample, within the error of the experiment, and are not near the 9% slope indicative of irreversible inhibitors. Although in some specific cases irreversible inhibitors can be useful pharmacologic lead compounds, most lead molecules that function through enzyme inhibition do so through a simple, reversible binding mechanism.

2.4 Limited SAR data for each of the inhibitors identified in HTS

A series of structural analogs of the sirtuin scaffolds identified by HTS were evaluated as inhibitors of Hst2. Following the identification of two compounds with similar scaffolds, **14a** and **14b**, in the initial HTS as Hst2 inhibitors, several analogs of scaffold **14** were synthesized⁴⁸, and their effect as inhibitors of Hst2 was evaluated (Table 2). Compounds **14a** and **14b** differ only in their substitution at the isoquinoline nitrogen, and their structural similarity suggests that these small molecules interact with a similar binding site on the enzyme. The IC_{50} for scaffold **14a** was determined to be about 2-fold lower than that of scaffold **14b**, implying that the enzyme binding site of this scaffold can accommodate, but does not prefer, the larger 4-methoxybenzyl group of **14b**. Consequently, all of the structural analogs of this scaffold were prepared with a methyl rather than a methoxy-benzyl group on the nitrogen. Analysis of the activity of those analogs makes clear that both the alkyne and ether groups in the R^1 position are absolutely required for inhibitory activity. Varying the ligand in the R^3 position might be effective in the preparation of more potent related compounds, as these data suggest that substituents in this position can be accommodated by the enzyme active site and may impact the binding interaction with the enzyme.

For the remaining scaffolds identified by HTS, commercially available molecules with similar scaffolds were purchased and evaluated for their effect on Hst2 deacetylase activity. None of the analogs of scaffold **11** (Table 3) contained a phenyl group in the R^4 position and none of the analogs were as potent as compound **11**, suggesting that the phenyl group may be important for inhibitor activity especially since compounds 6802623, 7985301, and 6978945 had significantly reduced potency, although there are other differences between the analogs and the originally identified scaffold. The length of the carbon chain included in this scaffold (constituents A and B) may be flexible, as compound 5140108 showed some deacetylase inhibition.

Several analogs of scaffold **13** were also tested for their ability to inhibit deacetylation by Hst2 (Table 4). None of the compounds tested showed significant inhibitory activity against Hst2. The compound with the most inhibitory activity, 97252, had several methyl groups in place of the chloro groups. This seems to indicate that these groups are tolerated in the enzyme active site, but are not ideal for inhibition. The chloro and hydroxyl groups on the originally identified scaffold molecule probably make important interactions in the enzyme binding site and should be explored individually to see which are absolutely required for inhibitory effect and which may be changed in order to generate more potent or selective inhibitors.

Finally, several analogs of scaffold **12** were tested as potential Hst2 deacetylase inhibitors (Table 5). None of the compounds tested showed any deacetylase activity against Hst2. Generally, most of the analogs of this scaffold contained a second ketone functional group and a constituent in the R^1 position of varying carbon chain length. Because the presence of these groups completely abrogates inhibition, the identity of one or both of these groups in the original scaffold may be critical for Hst2 inhibition.

2.5 Kinetic analysis of inhibition of Hst2 by four inhibitor scaffolds identified in HTS

To determine the mechanism of inhibition of the scaffolds identified by HTS and to gain insight as to where these compounds may bind to the enzyme, inhibition modes were determined by varying each of the substrates with the other maintained at a fixed, saturating concentration (Figure 3, Table 6). With respect to NAD^+ , scaffolds **11-13** were best fit to a fully non-competitive inhibition model, with K_i equal to 1.2 ± 0.2 , 30 ± 6 and 3.9 ± 0.7 μM , respectively. Scaffold **14a** was best fit to a fully mixed inhibition model, with K_i equal to 42 ± 17 ($\alpha = 2.6$) μM , respectively. Both of these models imply that the scaffolds bind to the enzyme regardless of whether or not NAD^+ is bound, although with varying affinity in the case of scaffold **14a**, suggesting that the inhibitors bind to a site distinct from the NAD^+ binding site. With respect to the acetyl-lysine substrate, scaffolds **12**, and **14a** were best fit to a fully non-competitive inhibition model, with K_i equal to 39 ± 7 and 43 ± 8 μM , respectively. Scaffold **13** was best fit to a fully mixed inhibition model, with K_i equal to 2.5 ± 1.0 ($\alpha = 13.4$) μM . Finally, scaffold **11** was best fit to a partial non-competitive inhibition model, with K_i equal to 6.3 ± 1.7 μM . Again, each of these models suggests that the newly identified inhibitor scaffolds bind to both the enzyme alone and the enzyme plus acetyl-lysine complex, implying that they bind in a site other than the acetyl-lysine binding site.

3. Conclusion

The continued development of novel sirtuin inhibitor scaffolds is necessary for the successful development of potent and selective sirtuin probes for further study of sirtuin biology and for developing pharmaceuticals aimed at modulating sirtuin activity for possible therapeutic applications. To address this need, we focused our effector identification efforts on the *S. cerevisiae* Hst2 sirtuin target, an ideal model sirtuin homologue for its ease of overexpression and purification and robust deacetylase activity. Initial HTS hits tend to be general for protein homologues, while further lead molecule optimization can increase both potency and selectivity. Once suitable Hst2 inhibitor scaffolds are identified, they can be tested and potentially optimized using medicinal chemistry against other sirtuin proteins including the human sirtuin homologue, SIRT1.

In this study, we describe the identification of several novel inhibitors against Hst2 that also inhibit human SIRT1 through a process of *in vitro* high throughput screening, initial prioritization of hits by relative potency, characterization of inhibitor binding mode, SAR analysis to identify important functional moieties, and kinetic characterization of the mode of compound inhibition. Kinetic studies of the lead compound scaffolds identified here reveal that the mechanism of inhibition is noncompetitive or mixed type inhibition relative to both the acetyl-lysine and NAD^+ substrates. These competition models indicate that the compounds bind both to the free enzyme and to the enzyme/substrate complex, thus implying that the compounds bind to a site distinct from either substrate binding site to inhibit catalysis. Nonetheless, the observation that each of the compounds identified and further characterized here inhibit both yeast Hst2 and the human sirtuins SIRT1-3 implies that the target for these compounds are the conserved catalytic domain of these enzymes.

Noncompetitive inhibitors act independently of substrate concentration, which makes these inhibitors useful as lead compounds for development as pharmaceutical agents because the *in vivo* concentration of substrate may be unknown or may vary in diseased cells. Since the sirtuin inhibitors described here were identified through biochemical screens, they may show lower *in vivo* potency since these compounds have not been selected for their solubility, stability, permeability, localization to a cellular compartment, or accumulation to high concentration inside cells.²⁸ Similarly, many of the sirtuin inhibitors that have been previously identified are nonspecific,^{2, 36, 46} are of low *in vivo* potency, or have not been characterized for their *in vivo* effect on sirtuins. Other sirtuin inhibitors, however, have been shown to have direct *in*

vivo effects. Both sirtinol (**2**) and splitomycin (**3**) can inhibit telomeric silencing in yeast cells.^{25, 26} Inhibition of SIRT1 by cambinol (**4**) during genotoxic stress leads to hyperacetylation of stress response proteins and promotes cell cycle arrest. Further, treatment of BCL-6 expressing Burkitt lymphoma cells with cambinol (**4**) increased apoptosis and inhibited tumor growth in a Burkitt's lymphoma mouse model.²⁷ Tenovin-6 (**5**) kills BL2 Burkitt's lymphoma and ARN8 cells in culture and impairs the growth of BL2- and ARN8-derived tumors xenografts.²⁸ Ro-31-8220 (**6**) inhibits SIRT2 and increases α -tubulin acetylation in A529 cells.³² Several indole analogs (similar, but not identical to compounds **9** and **10**) reduce TNF- α levels and stimulate adipocyte differentiation.⁴⁹ Given that the compounds described here are more potent SIRT1 inhibitors *in vitro* than the compounds listed above, with the exception of the indole analogs, it is likely that they will show some of the same *in vivo* effects on SIRT1 that were described above. There are advantages to having several sirtuin inhibitors available. Observing similar effects with several structurally unrelated inhibitors may be an effective way to support the involvement of a sirtuin in a given biological process. In addition, structurally unrelated inhibitors may show benefit in a particular cell line or cancer type. With regard to extending the studies reported here towards therapy, our findings confirm the value of compound scaffolds **11-14** as bona-fide sirtuin inhibitors. The advancement of any of the candidate compounds presented here along a drug development path will require a significant investment in medicinal chemistry, preclinical and clinical studies. Nevertheless, these chemical scaffolds along with the limited SAR analysis reported here provide a starting point for the further development of molecules that might be useful for the modulation of aging and for the treatment of age associated disorders, most notably cancer, as inhibitors of the other HDAC families, which share many common protein targets as sirtuins, have potent antitumor activity⁵⁰⁻⁵².

4. Experimental

4.1 Protein expression and purification

The 64 residue C-terminal deletion construct of Hst2 (residues 1-294) was purified and expressed as previously described.⁵³ A plasmid containing full length human SIRT1 (FL SIRT1) was transformed into C41 (DE3) cells (Avidis), expressed overnight at 15°C by addition of 1 mM IPTG to cell cultures and yielded 6 \times histidine-tagged SIRT1. Cells were harvested and lysed by sonication in buffer containing 50 mM Tris, pH 7.5, 200 mM NaCl, 5 mM imidazole, 10 mM BME and 0.1 mg/mL PMSF. Soluble FL SIRT1 was purified by Ni-NTA (Qiagen) in a buffer containing 50 mM Tris, pH 7.5, 300 mM NaCl, 10 mM BME, 5% glycerol and 30-600 mM imidazole followed by Superdex 200 gel filtration in buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, and 10 mM BME. FL SIRT1 eluted between the 670 kDa and 158 kDa globular protein standards. Since SIRT1 is known to aggregate after several days at 4°C, the protein was aliquoted and frozen at -80°C for use in fluorogenic assays.

A construct of isoform II of SIRT2 with additional residues N-terminal to the SIRT2 gene in a pET30a expression vector was overexpressed as an N-terminal, thrombin-cleavable, His₆-tagged fusion protein in *Escherichia coli* BL21-Gold (DE3) cells, initially grown at 37°C to exponential phase and induced with 0.5 mM IPTG at 15°C overnight. Cells harboring SIRT2 were disrupted by sonication in 20 mM Tris, pH 8.5, 500 mM NaCl and 10 mM BME. Soluble SIRT2 was purified using a combination of Ni-NTA resin, followed by overnight thrombin cleavage, Q sepharose resin and Superdex-200 analytical gel filtration chromatography, where the protein eluted between the 128- and 44-kDa globular protein standards, in a buffer containing 20 mM Tris, pH 8.5, 150 mM NaCl and 10 mM BME. Human recombinant SIRT3 was purchased from Enzo Life Sciences.

4.2 High throughput screening (HTS)

A high throughput screening protocol based on the BIOMOL SIRT1 Fluorimetric Activity Assay/Drug Discovery Kit (AK-555) was developed. 30 μL of a master mix containing 0.4 μL of the 5mM Fluor de Lys-SIRT1 deacetylase substrate (KI-177, BIOMOL), 3.2 μL of 1 mM NAD^+ (N1636, Sigma), 0.052 μL of 130 mM nicotinamide (N3376, Sigma), and 26.348 μL of assay buffer (25 mM Tris/Cl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl_2) per well was added to each reaction well. 100 nL of either a DMSO control or a compound from the small molecule screening library was added to each well by pinning. To begin the reaction, 10 μL of an enzyme master mix containing 2 μM Hst2 (1-294) (MW = 34859.9 Da) in assay buffer was added to each reaction well. After four hours, a stop/developer solution containing 8 μL of 100 mM nicotinamide, 8 μL of 5 \times Fluor de Lys Developer II Concentrate (5 \times) (KI-176, BIOMOL), and 24 μL of assay buffer per well was added to each reaction well. After 45 minutes, each plate was read on a fluorescence plate reader at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. The nicotinamide and the relatively high concentration of substrates were added to facilitate detection of activators and inhibitors simultaneously. The high throughput screen was carried out at the Broad Institute Chemical Biology Platform's screening facility on ~ 50,000 small molecules from their ChemDiv3, PKO4, HSCI2, SPBio, and BCB03 libraries, which were selected because they contained commercially available compounds with drug-like structures and known activity, diversity-oriented organic synthesis derived skeletally and stereochemically diverse small molecule analogs of several synthetic pathways, commercially available compounds with known biological activity that are candidates for influencing stem-cell differentiation including COX, NO, adenylate cyclase, and protein kinase effectors, drug-like molecules from the Prestwick and Spectrum commercial libraries, or diverse compound scaffolds synthesized by Broad Chemists and collaborators, respectively. All compounds were screened in duplicate. The fluorescence signal from each well was normalized to the DMSO controls on each 384-well plate and compared to a DMSO control population based on the following equation:

$$Z = \frac{\chi - \mu}{\sigma}$$
where χ is the fluorescence signal of the well, μ is the mean of the control (DMSO) population and σ is the standard deviation of the control population. Generally, compounds that gave fluorescence signals higher than 3Z or lower than -3Z were considered hits. Approximately 74 compounds (0.14%) were identified as initial hits and requested as cherry picks. The cherry picked compounds were retested with careful controls to show reproducibility of effect, and rule out autofluorescence, developer inhibition and other assay artifacts.

4.3 IC₅₀ determination

The compounds determined to be reproducible and artifact free inhibitors of Hst2 were purchased (**11**, ChemDiv; **12**, TimTec; **13**, Spectrum Chemicals & Laboratory Products) or synthesized (**14**).⁴⁸ The purity of the compounds was verified by mass spectrometry (see Supplemental Figure 2). IC₅₀ values were then measured using the same fluorogenic assay described above for Hst2 (160 μM NAD^+ , 100 μM Fluor de Lys-SIRT1 deacetylase substrate, 1 μM Hst2, 15 min reaction time) and FL SIRT1 (240 μM NAD^+ , 200 μM Fluor de Lys-SIRT1 deacetylase substrate, 1 μM FL SIRT1, and 15 min reaction time). All compounds were solubilized in 25 mM DMSO and diluted for use in the fluorimetric assay of no more than 10% final DMSO concentration. The concentrations of the compounds in the IC₅₀ experiment spanned the range of enzyme activity from no inhibition to complete inhibition. The dose-response curves were then fit to one-site competition or sigmoidal-dose response curves as appropriate in GraphPad Prism (GraphPad Software, La Jolla, CA) and the IC₅₀ was determined. Three independent IC₅₀ measurements were performed for each compound and the average and standard deviation are reported. In order to directly compare the potency of these compounds to other inhibitors identified in the literature, we also purchased or obtained

several known sirtuin inhibitors (sirtinol, Alexis Biochemicals; splitomycin and suramin, BIOMOL International; tenovin-6, Cayman Chemicals; nicotinamide, Sigma; **9** and **10**, Interbioscreen, Ltd; Ro 31820, EMD Chemicals; surfactin, Sigma) and performed IC₅₀ experiments using the same assay conditions as described above. Several compounds (cambinol, surfactin and splitomycin) have been shown to be competitive with one of the reaction substrates. Additionally, the IC₅₀ values of these compounds also were determined in reaction conditions where the concentration of the competitive substrate was reduced to its approximate K_m value (10 and 45 μM of acetyl-lysine and 16 and 24 μM of NAD⁺ for Hst2 and FL SIRT1, respectively).

4.4 Reversibility assay

Each of the inhibitor scaffolds identified in the HTS was tested to determine whether they were reversible, slowly reversible or irreversible inhibitors of Hst2. The reversibility of each compound was identified using the fluorogenic assay described above. First, either the enzyme alone, the enzyme plus DMSO or the enzyme plus 10× the IC₅₀ concentration of an identified inhibitor was incubated with 100 μM Hst2 for 30 minutes. The enzyme, enzyme plus DMSO or enzyme plus inhibitor was then diluted 100-fold into reaction buffer containing the Fluor de Lys-SIRT1 deacetylase substrate and NAD⁺ at concentrations equal to their approximate K_m values (25 μM) to initiate the reaction. The reactions were quenched during the linear region of the reaction time course (1-30 min) with 10 mM nicotinamide. The progress curves were then plotted and compared to the appropriate enzyme control.⁴⁷

4.5 Structure activity relationship (SAR) analysis

We searched a number of commercially available small molecule databases and purchased analogs of the scaffolds identified in the HTS. Compounds 5140108, 6959933, 5237467, 7985301, 7988362, 6802623, 6836332, 6978945, and 5366302 were purchased from ChemBridge. Analogs of scaffold **14** were synthesized as previously described.⁴⁸ All other compounds used in the SAR analysis were purchased from Specs (R&D Chemicals). Each compound was tested in triplicate at 50 and 500 μM for its effect against Hst2 in the fluorogenic assay (1 μM Hst2, 100 μM Fluor de Lys-SIRT1 deacetylase substrate, and 160 μM NAD⁺, 15 minute reaction time). The resulting deacetylase activity for the three experiments was averaged and reported as a percentage relative to control wells containing no inhibitor. IC₅₀ values were then measured for compounds that showed significant inhibition of Hst2 as exhibited by no activity at a compound concentration of 500 μM by the protocol described above.

4.6 Competition assay

Each of the identified inhibitor scaffolds was characterized with regards to its ability to compete with the Fluor de Lys-SIRT1 deacetylase substrate and NAD⁺ for Hst2. The fluorogenic assay described above was used with a fixed enzyme concentration of 1 μM. When competition with NAD⁺ was being tested, the Fluor de Lys-SIRT1 deacetylase substrate was held at a constant concentration of 100 μM and the NAD⁺ was titrated from 1/3-5× K_m NAD⁺ (5-80 μM). When competition with the Fluor de Lys-SIRT1 deacetylase substrate was being tested, the NAD⁺ concentration was held constant at 160 μM and the Fluor de Lys-SIRT1 deacetylase substrate was titrated from 1/3-5× the K_m acetyl-lysine (3-50 μM). The inhibitors were titrated from ~1/2 to several times their estimated K_i as indicated in the figure legend. The reaction time was 15 minutes, and each condition was tested in duplicate. The K_m, k_{cat} and k_{cat}/K_m values were determined by direct fit of the data in SigmaPlot (Systat Software, Point Richmond, CA) to the Michaelis-Menten equation. The K_i and K_{is} (the slope and intercept inhibition constants, respectively) and the competition type were also determined by a direct fit in SigmaPlot to both partial and full competitive, noncompetitive, uncompetitive and mixed competition

models. Best-fit models were determined by several statistical methods including R^2 , AICs and Sy.x as well as empirical evaluation of α and β values.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The plasmid containing full length human SIRT1 (FL SIRT1) was a generous gift from Dr. David Sinclair (Harvard Medical School, Boston, MA), the plasmid containing isoform II of human SIRT2 was a generous gift from Danny Reinberg (Howard Hughes Medical Institute of NYU Medical School), and cambinol was a generous gift from Antonio Bedalov (Fred Hutchinson Cancer Center, Seattle, Washington). We would like to thank Nicola Toliday, Frank An, Stephanie Norton and Jason Burbank of the Broad Institute for help with HTS. We would like to thank Andre Isaacs for technical assistance and useful discussions. We would like to thank Tom Beer and the Wistar Institute Proteomics Facility for assistance with mass spectrometry. This work was supported by grants CA107107 and AG031862 from the National Institutes of Health to R.M and predoctoral fellowship T32-CA009171 from the National Institutes of Health to B.D.S. This project has been funded in part with Federal funds from the National Cancer Institute's Initiative for Chemical Genetics, National Institutes of Health, under Contract No. N01-CO-12400 and has been performed with the assistance of the Chemical Biology Platform of the Broad Institute of Harvard and MIT. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Service, nor does mention of trade names, commercial products or organizations imply endorsement by the U.S. Government.

The abbreviations used are

Sir2	silent information regulator number 2
Hst2	homologue of Sir two number 2
SIRT1	silent information regulator two number 1
HTS	high throughput screening
SAR	structure activity relationship
ADME	absorption distribution metabolism and excretion

References

1. Imai S, Armstrong CM, Kaerberlein M, Guarente L. Nature 2000;403:795. [PubMed: 10693811]
2. Landry J, Sutton A, Tafrov ST, Heller RC, Stebbins J, Pillus L, Sternglanz R. Proc Natl Acad Sci U S A 2000;97:5807. [PubMed: 10811920]
3. Jackson MD, Denu JM. J Biol Chem 2002;277:18535. [PubMed: 11893743]
4. Sauve AA, Celic I, Avalos J, Deng H, Boeke JD, Schramm VL. Biochemistry 2001;40:15456. [PubMed: 11747420]
5. Kim S, Benguria A, Lai CY, Jazwinski SM. Mol Biol Cell 1999;10:3125. [PubMed: 10512855]
6. Kaerberlein M, McVey M, Guarente L. Genes Dev 1999;13:2570. [PubMed: 10521401]
7. Lin SJ, Defossez PA, Guarente L. Science 2000;289:2126. [PubMed: 11000115]
8. Brachmann CB, Sherman JM, Devine SE, Cameron EE, Pillus L, Boeke JD. Genes Dev 1995;9:2888. [PubMed: 7498786]
9. Tissenbaum HA, Guarente L. Nature 2001;410:227. [PubMed: 11242085]
10. Rogina B, Helfand SL. Proc Natl Acad Sci U S A 2004;101:15998. [PubMed: 15520384]
11. Cohen HY, Miller C, Bitterman KJ, Wall NR, Hekking B, Kessler B, Howitz KT, Gorospe M, de Cabo R, Sinclair DA. Science 2004;305:390. [PubMed: 15205477]
12. Boily G, Seifert EL, Bevilacqua L, He XH, Sabourin G, Estey C, Moffat C, Crawford S, Saliba S, Jardine K, Xuan J, Evans M, Harper ME, McBurney MW. PLoS ONE 2008;3:e1759. [PubMed: 18335035]
13. Frye RA. Biochem Biophys Res Commun 1999;260:273. [PubMed: 10381378]

14. Frye RA. *Biochem Biophys Res Commun* 2000;273:793. [PubMed: 10873683]
15. Kim EJ, Um SJ. *BMB Rep* 2008;41:751. [PubMed: 19017485]
16. Guarente L. *Cold Spring Harb Symp Quant Biol* 2007;72:483. [PubMed: 18419308]
17. Bitterman KJ, Anderson RM, Cohen HY, Latorre-Esteves M, Sinclair DA. *J Biol Chem* 2002;277:45099. [PubMed: 12297502]
18. Schmidt MT, Smith BC, Jackson MD, Denu JM. *J Biol Chem* 2004;279:40122. [PubMed: 15269219]
19. Luo J, Nikolaev AY, Imai S, Chen D, Su F, Shiloh A, Guarente L, Gu W. *Cell* 2001;107:137. [PubMed: 11672522]
20. Jackson MD, Schmidt MT, Oppenheimer NJ, Denu JM. *J Biol Chem* 2003;278:50985. [PubMed: 14522996]
21. Sauve AA, Schramm VL. *Biochemistry* 2003;42:9249. [PubMed: 12899610]
22. Vaziri H, Dessain SK, Ng Eaton E, Imai SI, Frye RA, Pandita TK, Guarente L, Weinberg RA. *Cell* 2001;107:149. [PubMed: 11672523]
23. Jones PA, Baylin SB. *Nat Rev Genet* 2002;3:415. [PubMed: 12042769]
24. Pruitt K, Zinn RL, Ohm JE, McGarvey KM, Kang SH, Watkins DN, Herman JG, Baylin SB. *PLoS Genet* 2006;2:e40. [PubMed: 16596166]
25. Bedalov A, Gatabonton T, Irvine WP, Gottschling DE, Simon JA. *Proc Natl Acad Sci U S A* 2001;98:15113. [PubMed: 11752457]
26. Grozinger CM, Chao ED, Blackwell HE, Moazed D, Schreiber SL. *J Biol Chem* 2001;276:38837. [PubMed: 11483616]
27. Heltweg B, Gatabonton T, Schuler AD, Posakony J, Li H, Goehle S, Kollipara R, Depinho RA, Gu Y, Simon JA, Bedalov A. *Cancer Res* 2006;66:4368. [PubMed: 16618762]
28. Lain S, Hollick JJ, Campbell J, Staples OD, Higgins M, Aoubala M, McCarthy A, Appleyard V, Murray KE, Baker L, Thompson A, Mathers J, Holland SJ, Stark MJ, Pass G, Woods J, Lane DP, Westwood NJ. *Cancer Cell* 2008;13:454. [PubMed: 18455128]
29. Tervo AJ, Kyrylenko S, Niskanen P, Salminen A, Leppanen J, Nyronen TH, Jarvinen T, Poso A. *J Med Chem* 2004;47:6292. [PubMed: 15566299]
30. Tervo AJ, Suuronen T, Kyrylenko S, Kuusisto E, Kiviranta PH, Salminen A, Leppanen J, Poso A. *J Med Chem* 2006;49:7239. [PubMed: 17125277]
31. Huhtiniemi T, Suuronen T, Rinne VM, Wittekindt C, Lahtela-Kakkonen M, Jarho E, Wallen EA, Salminen A, Poso A, Leppanen J. *J Med Chem* 2008;51:4377. [PubMed: 18642893]
32. Trapp J, Jochum A, Meier R, Saunders L, Marshall B, Kunick C, Verdin E, Goekjian P, Sippl W, Jung M. *J Med Chem* 2006;49:7307. [PubMed: 17149860]
33. Gey C, Kyrylenko S, Hennig L, Nguyen LH, Buttner A, Pham HD, Giannis A. *Angew Chem Int Ed Engl* 2007;46:5219. [PubMed: 17516596]
34. Kahyo T, Ichikawa S, Hatanaka T, Yamada MK, Setou M. *J Pharmacol Sci* 2008;108:364. [PubMed: 19008647]
35. Chakrabarty SP, Saikumari YK, Bopanna MP, Balaram H. *Mol Biochem Parasitol* 2008;158:139. [PubMed: 18221799]
36. Howitz KT, Bitterman KJ, Cohen HY, Lamming DW, Lavu S, Wood JG, Zipkin RE, Chung P, Kisielewski A, Zhang LL, Scherer B, Sinclair DA. *Nature* 2003;425:191. [PubMed: 12939617]
37. Napper AD, Hixon J, McDonagh T, Keavey K, Pons JF, Barker J, Yau WT, Amouzegh P, Flegg A, Hamelin E, Thomas RJ, Kates M, Jones S, Navia MA, Saunders JO, DiStefano PS, Curtis R. *J Med Chem* 2005;48:8045. [PubMed: 16335928]
38. Trapp J, Meier R, Hongwiset D, Kassack MU, Sippl W, Jung M. *ChemMedChem* 2007;2:1419. [PubMed: 17628866]
39. Schuetz A, Min J, Antoshenko T, Wang CL, Allali-Hassani A, Dong A, Loppnau P, Vedadi M, Bochkarev A, Sternglanz R, Plotnikov AN. *Structure* 2007;15:377. [PubMed: 17355872]
40. Kiviranta PH, Leppanen J, Kyrylenko S, Salo HS, Lahtela-Kakkonen M, Tervo AJ, Wittekindt C, Suuronen T, Kuusisto E, Jarvinen T, Salminen A, Poso A, Wallen EA. *J Med Chem* 2006;49:7907. [PubMed: 17181175]

41. Kiviranta PH, Leppanen J, Rinne VM, Suuronen T, Kyrylenko O, Kyrylenko S, Kuusisto E, Tervo AJ, Jarvinen T, Salminen A, Poso A, Wallen EA. *Bioorg Med Chem Lett* 2007;17:2448. [PubMed: 17329104]
42. Hirao M, Posakony J, Nelson M, Hruby H, Jung M, Simon JA, Bedalov A. *J Biol Chem* 2003;278:52773. [PubMed: 14534292]
43. Lara E, Mai A, Calvanese V, Altucci L, Lopez-Nieva P, Martinez-Chantar ML, Varela-Rey M, Rotili D, Nebbioso A, Roperio S, Montoya G, Oyarzabal J, Velasco S, Serrano M, Witt M, Villar-Garea A, Inhof A, Mato JM, Esteller M, Fraga MF. *Oncogene* 2009;28:781. [PubMed: 19060927]
44. Mai A, Massa S, Lavu S, Pezzi R, Simeoni S, Ragno R, Mariotti FR, Chiani F, Camilloni G, Sinclair DA. *J Med Chem* 2005;48:7789. [PubMed: 16302818]
45. Neugebauer RC, Uchiechowska U, Meier R, Hruby H, Valkov V, Verdin E, Sippl W, Jung M. *J Med Chem* 2008;51:1203. [PubMed: 18269226]
46. Posakony J, Hirao M, Stevens S, Simon JA, Bedalov A. *J Med Chem* 2004;47:2635. [PubMed: 15115404]
47. Copeland, RA. *Evaluation of Enzyme Inhibitors in Drug Discovery*. John Wiley & Sons, Inc.; Hoboken: 2005.
48. Taylor AM, Schreiber SL. *Org Lett* 2006;8:143. [PubMed: 16381588]
49. Nayagam VM, Wang X, Tan YC, Poulsen A, Goh KC, Ng T, Wang H, Song HY, Ni B, Entzeroth M, Stunkel W. *J Biomol Screen* 2006;11:959. [PubMed: 17099246]
50. Marks PA, Richon VM, Breslow R, Rifkind RA. *Curr Opin Oncol* 2001;13:477. [PubMed: 11673688]
51. Piekarz RL, Robey R, Sandor V, Bakke S, Wilson WH, Dahmouch L, Kingma DM, Turner ML, Altemus R, Bates SE. *Blood* 2001;98:2865. [PubMed: 11675364]
52. Sandor V, Bakke S, Robey RW, Kang MH, Blagosklonny MV, Bender J, Brooks R, Piekarz RL, Tucker E, Figg WD, Chan KK, Goldspiel B, Fojo AT, Balcerzak SP, Bates SE. *Clin Cancer Res* 2002;8:718. [PubMed: 11895901]
53. Zhao K, Chai X, Clements A, Marmorstein R. *Nat Struct Biol* 2003;10:864. [PubMed: 14502267]
54. Landry J, Slama JT, Sternglanz R. *Biochem Biophys Res Commun* 2000;278:685. [PubMed: 11095969]
55. Guy CA, Fields GB. *Methods Enzymol* 1997;289:67. [PubMed: 9353718]
56. Wellings DA, Atherton E. *Methods Enzymol* 1997;289:44. [PubMed: 9353717]

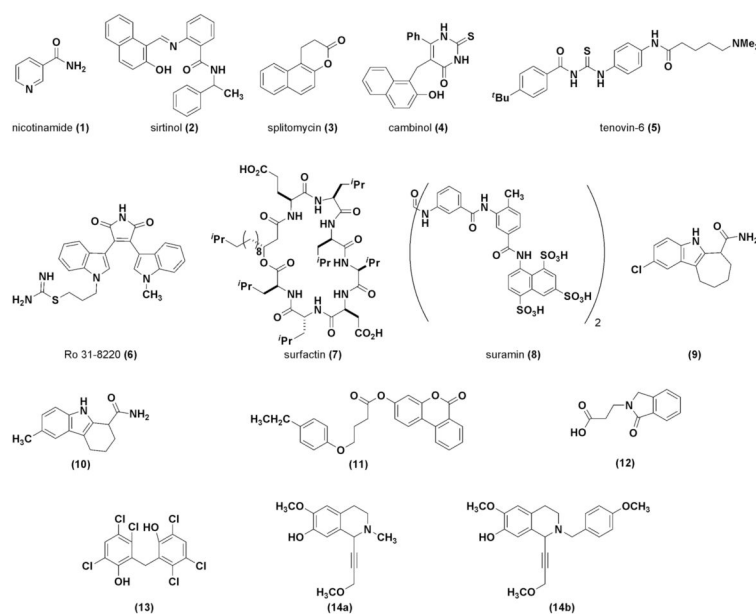


Figure 1.
Chemical structure of each of the previously described sirtuin inhibitors and the newly described sirtuin inhibitors identified by HTS.

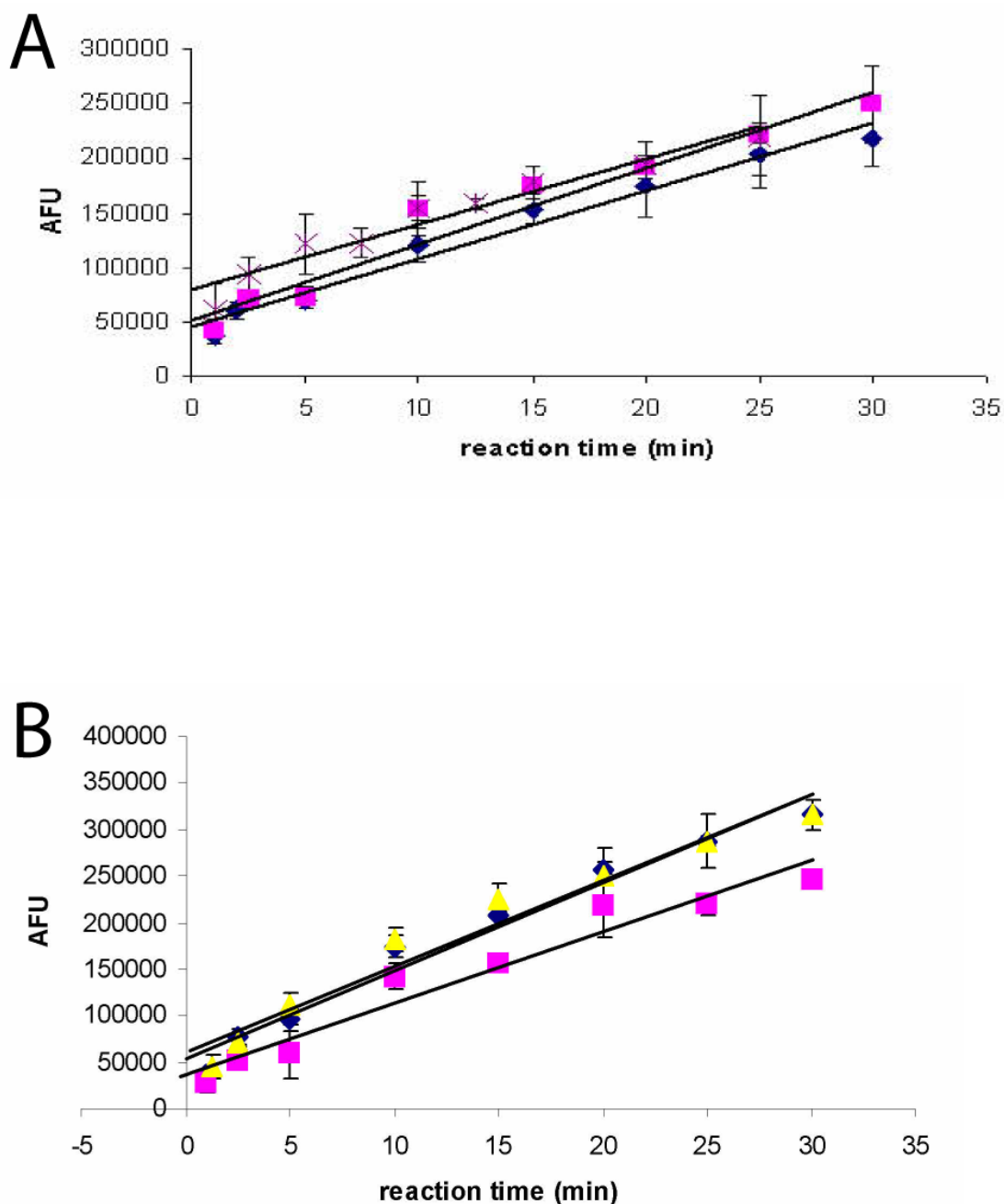


Figure 2.

All four HTS inhibitor scaffolds are reversible Hst2 inhibitors. A) Hst2 reaction time course experiment with both Hst2 alone (blue diamonds) and Hst2 incubated with 10 \times the IC_{50} concentration of the indicated inhibitor, after a 100-fold dilution. Compounds **14a** (slope 112% of enzyme only control, pink squares) and **13** (slope 85% of enzyme only control, purple asterisks) are reversible inhibitors of Hst2. B) Hst2 reaction time course experiment with both Hst2 + DMSO (blue diamonds) and Hst2 incubated with 10 \times the IC_{50} concentration of the indicated inhibitor, after a 100-fold dilution. Compounds **11** (slope 82% of enzyme plus DMSO control, pink squares) and **12** (slope 96% of enzyme plus DMSO control, yellow triangles) are reversible inhibitors of Hst2.

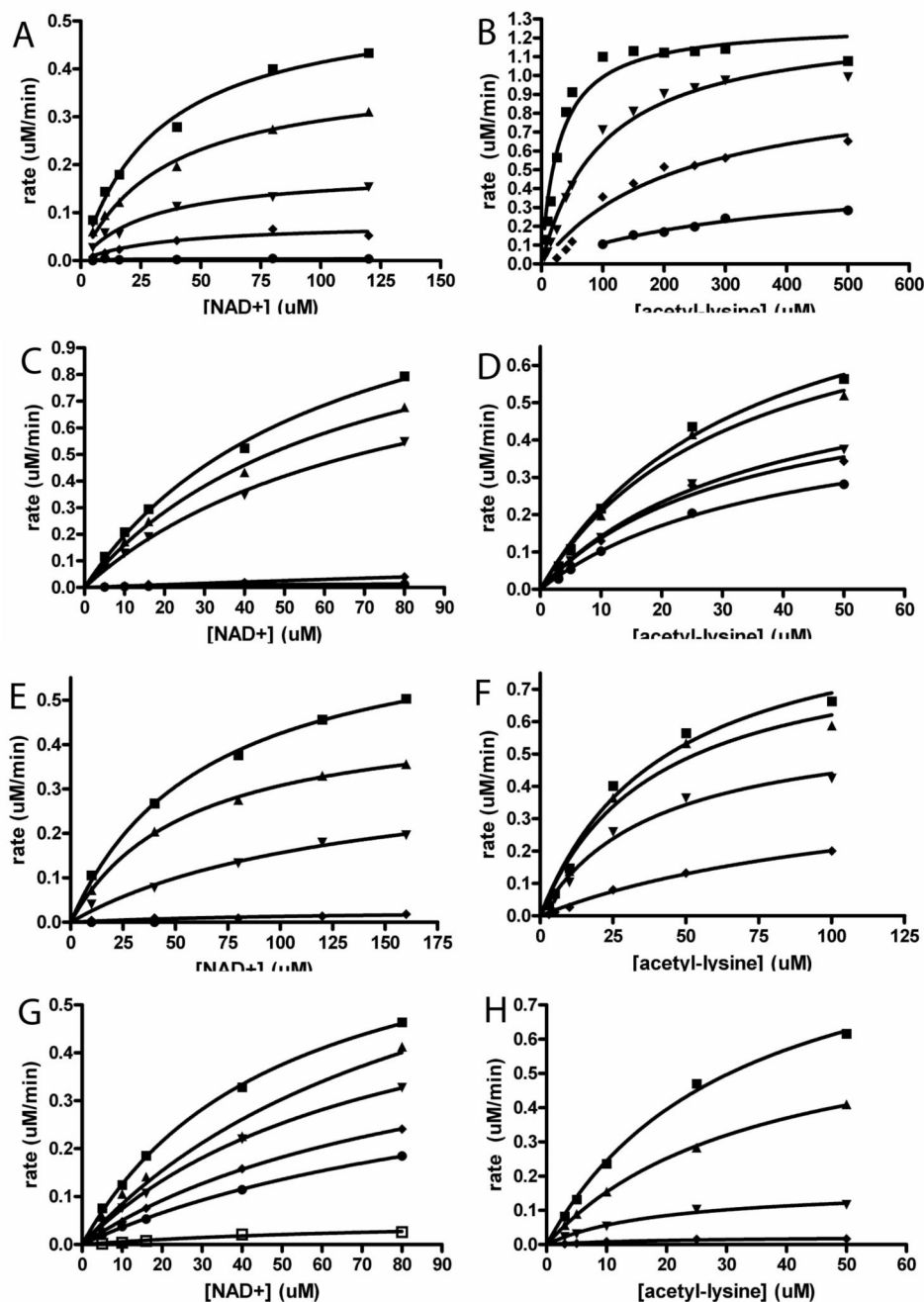


Figure 3.

Michaelis-Menten plots of Hst2 in the presence of identified inhibitor scaffolds. A) Hst2 versus **13** in the presence of varying concentrations of NAD⁺. 0 μ M **13** (squares), 5 μ M **13** (triangles), 7.5 μ M **13** (inverted triangles), 10 μ M **13** (diamonds), and 15 μ M **13** (circles). B) Hst2 versus **13** in the presence of varying concentrations of acetyl-lysine. 0 μ M **13** (squares), 10 μ M **13** (inverted triangles), 15 μ M **13** (diamonds), and 25 μ M **13** (circles). C) Hst2 versus **11** in the presence of varying concentrations of NAD⁺. 0 μ M **11** (squares), 0.5 μ M **11** (triangles), 1 μ M **11** (inverted triangles), 5 μ M **11** (diamonds), and 7 μ M **11** (circles). D) Hst2 versus **11** in the presence of varying concentrations of acetyl-lysine. 0 μ M **11** (squares), 1 μ M **11** (triangles), 5 μ M **11** (inverted triangles), 7.5 μ M **11** (diamonds), and 15 μ M **11** (circles). E) Hst2 versus

12 in the presence of varying concentrations of NAD^+ . 0 μM **12** (squares), 25 μM **12** (triangles), 50 μM **12** (inverted triangles), 100 μM **12** (diamonds), and 150 μM **12** (circles). F) Hst2 versus **12** in the presence of varying concentrations of acetyl-lysine. 0 μM **12** (squares), 15 μM **12** (triangles), 30 μM **12** (inverted triangles), and 60 μM **12** (diamonds). G) Hst2 versus **14a** in the presence of varying concentrations of NAD^+ . 0 μM **14a** (squares), 25 μM **14a** (triangles), 50 μM **14a** (inverted triangles), 75 μM **14a** (diamonds), 100 μM **14a** (circles), and 200 μM **14a** (empty squares). H) Hst2 versus **14a** in the presence of varying concentrations of acetyl-lysine. 0 μM **14a** (squares), 50 μM **14a** (triangles), 100 μM **14a** (inverted triangles), 200 μM **14a** (diamonds).

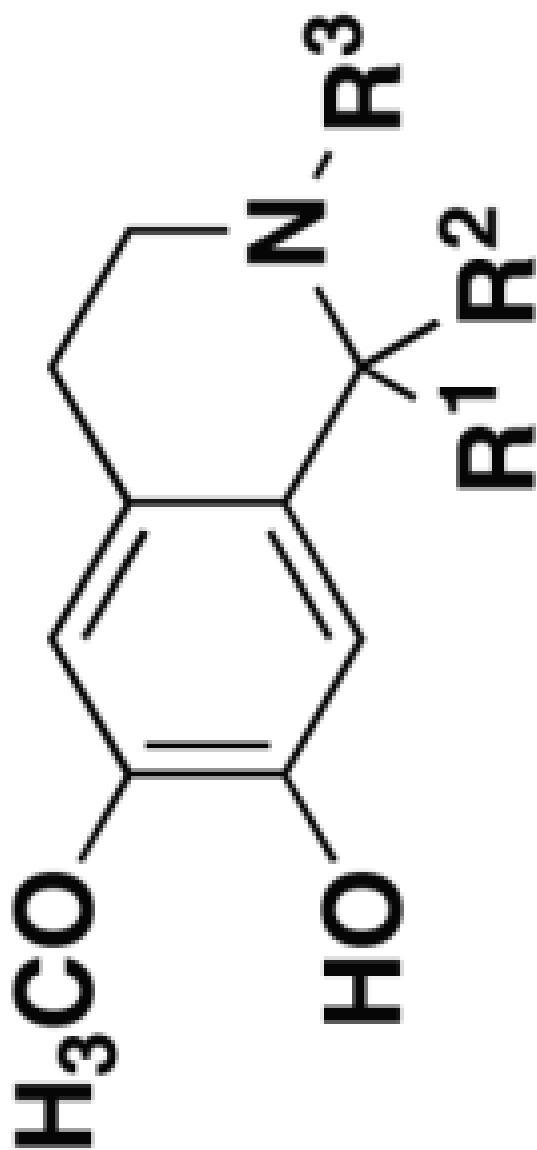
Table 1

In vitro IC₅₀ determination of sirtuin inhibitors identified by HTS and described in the literature for Hst2 and FL SIRT1.

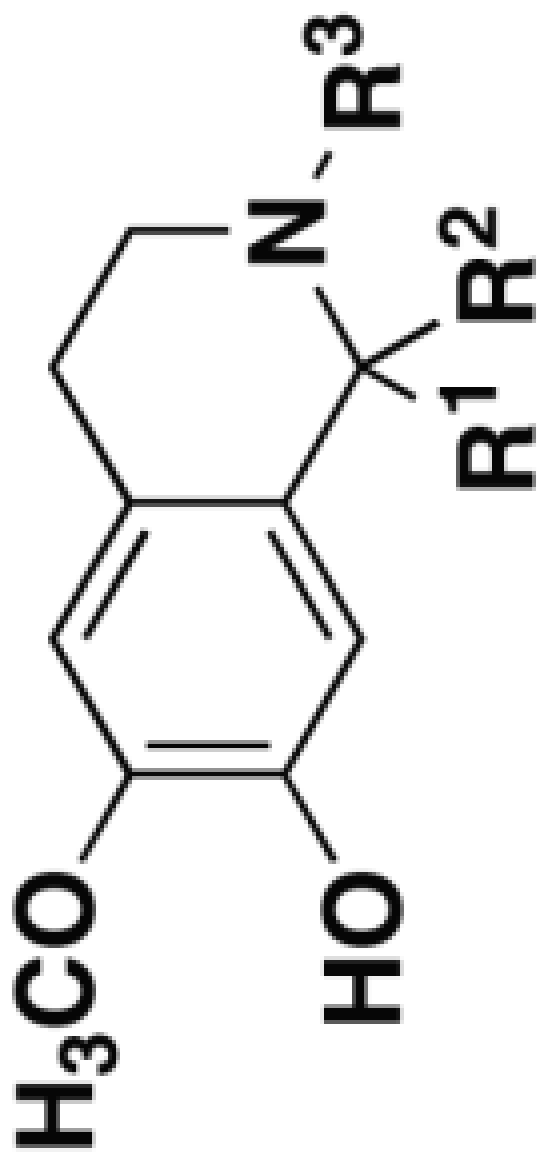
Compound	Hst2 IC ₅₀ (μM)	SIRT1 IC ₅₀ (μM)
Nicotinamide (1)	91 ± 7	250 ± 80
Sirtinol (2)	48 ± 11	120 ± 23
Splitomycin (3)	>600	>500
Cambinol (4)	>1000	>600
Tenovin-6 (5)	>100*	~100*
Ro 31-8220 (6)	20.0 ± 0.9	25 ± 7
Surfactin (7)	>700	>600
Suramin (8)	240 ± 70	0.6 ± 0.3
Indole 35 (9)	1.3 ± 0.1	0.18 ± 0.02
Indole 2 (10)	14.5 ± 0.6	0.64 ± 0.06
11	6.5 ± 1.3	6.0 ± 0.4
12	19.9 ± 0.6	80 ± 5
13	12.5 ± 0.6	34 ± 10
14a	130 ± 4	570 ± 200
14b	260 ± 20	nd

IC₅₀ data are reported as the mean and standard deviation of three independent determinations. IC₅₀ values that were not determined (nd) or are above the highest concentration tested (>) are indicated.

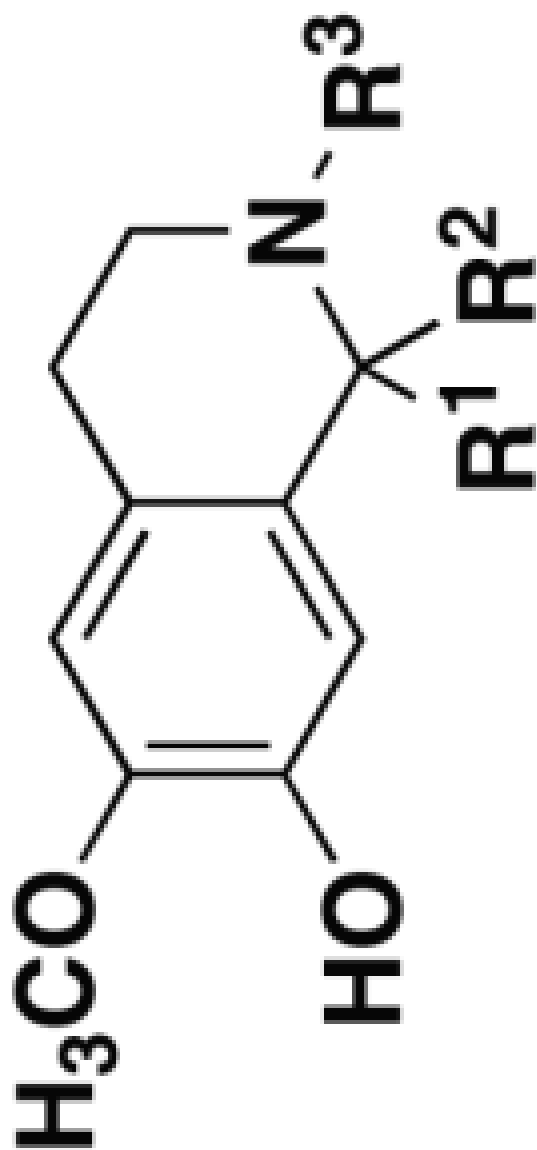
* IC₅₀ determination for this compound was limited by a solvent effect on the enzymatic assay although this compound did show inhibitory activity against both enzymes at the concentrations tested.



R^2	R^3	% Activity at 50 μM	% Activity at 500 μM
H		100	98



R ²	R ³	% Activity at 50 μ M	% Activity at 500 μ M
H		100	86



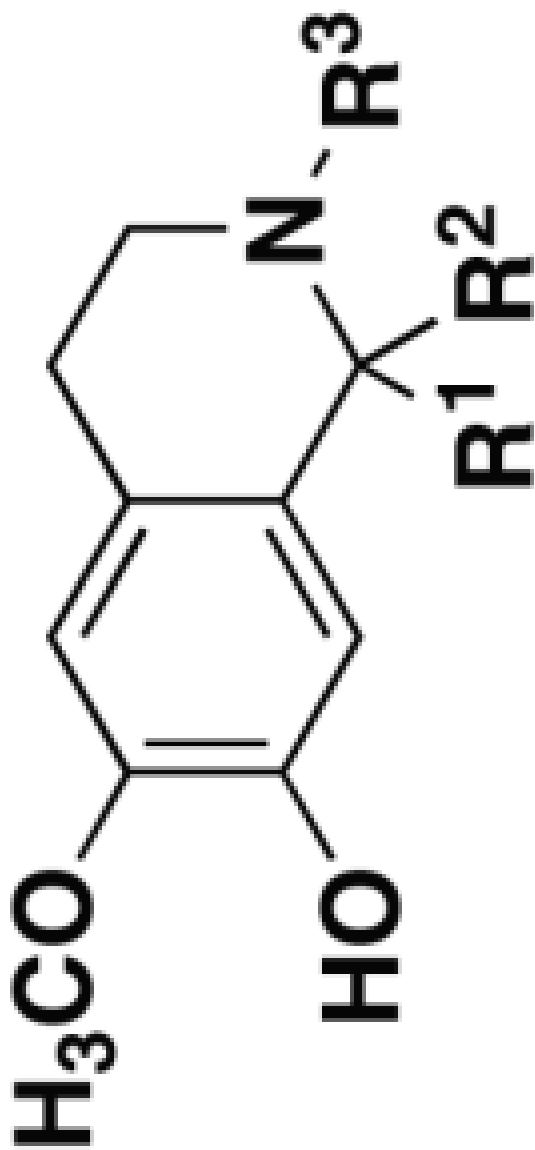
R ²	R ³	% Activity at 50 μ M	% Activity at 500 μ M
----------------	----------------	--------------------------	---------------------------



H

96

70



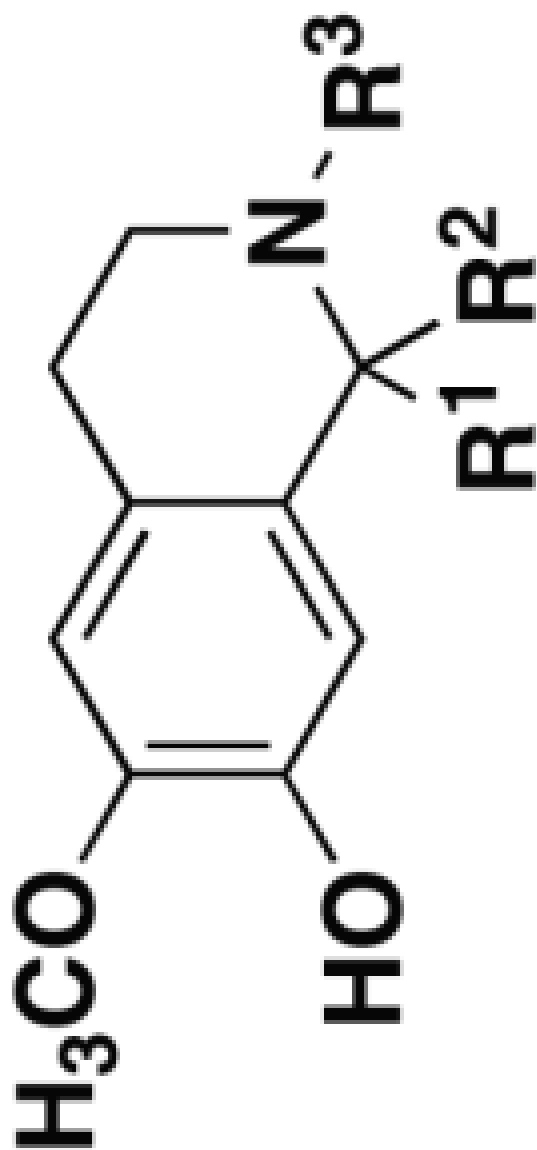
R ²	R ³	% Activity at 50 μ M	% Activity at 500 μ M
----------------	----------------	--------------------------	---------------------------



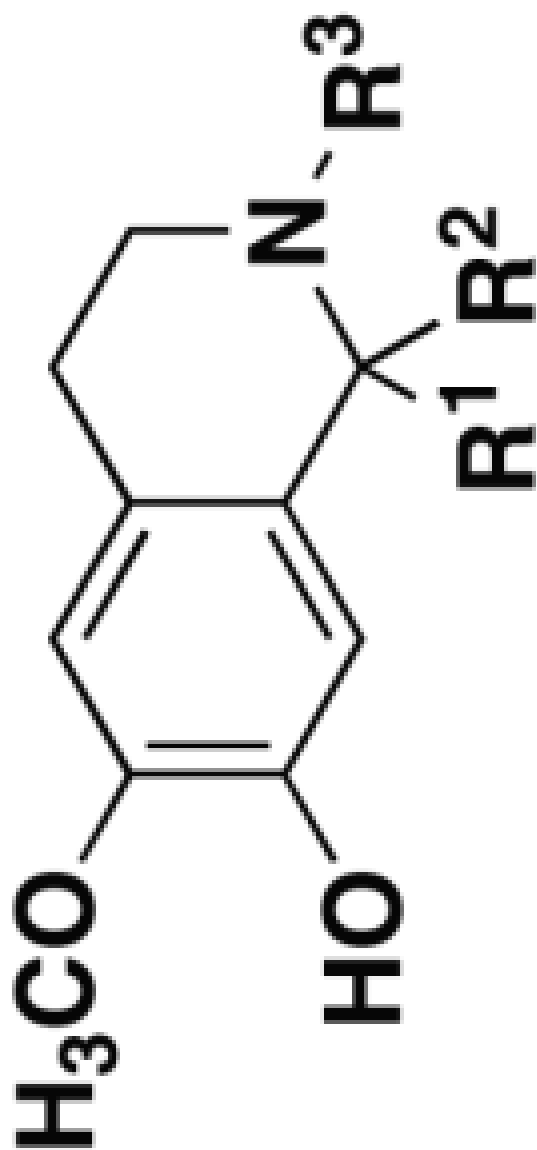
H

97

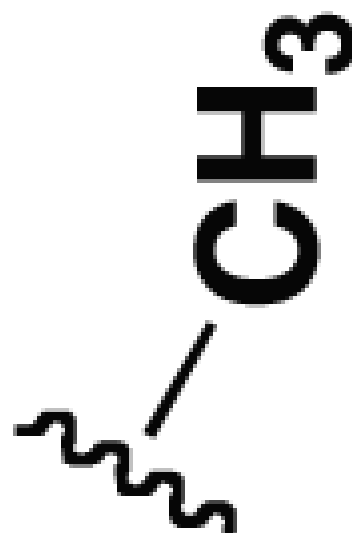
74



R^2	R^3	% Activity at 50 μM	% Activity at 500 μM
H		76	5



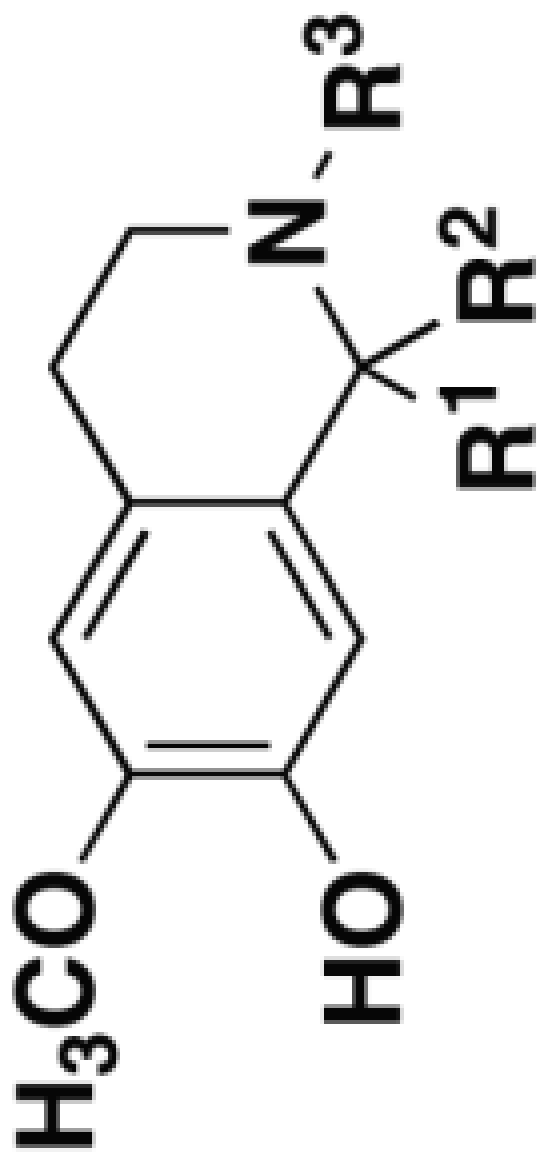
	R ²	R ³	% Activity at 50 μM	% Activity at 500 μM
--	----------------	----------------	---------------------	----------------------



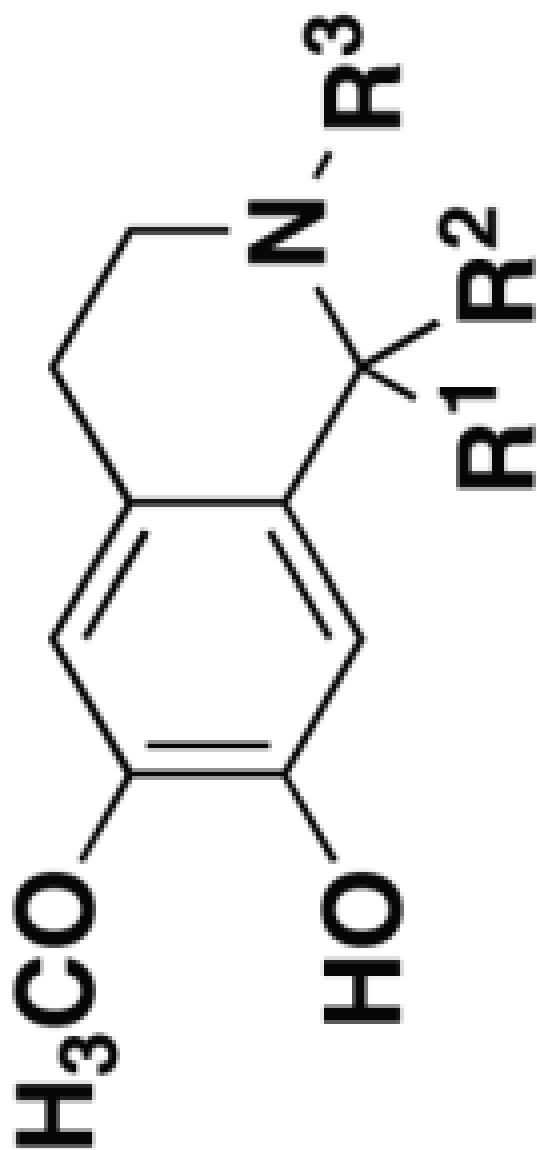
H

92

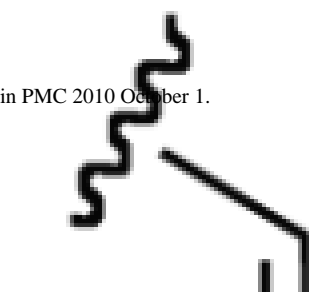
2



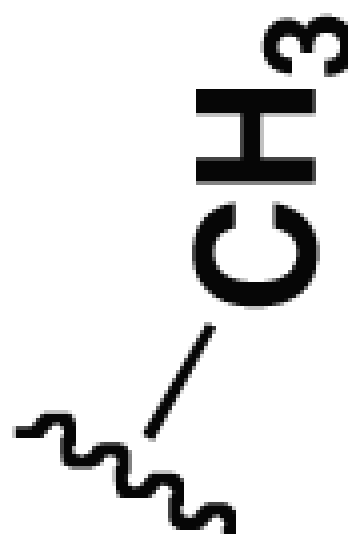
R ²	R ³	% Activity at 50 μ M	% Activity at 500 μ M
		99	92



R ²	R ³	% Activity at 50 μ M	% Activity at 500 μ M
----------------	----------------	--------------------------	---------------------------



H



97

94

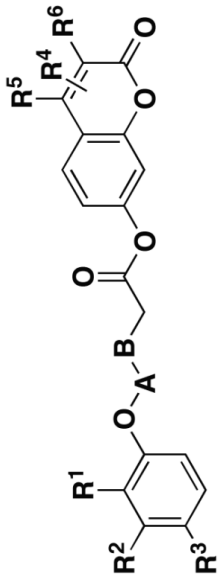


100

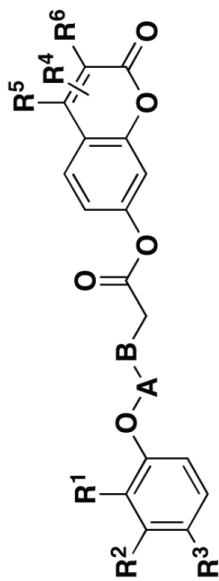
100

Data are reported as the percent of residual enzyme activity in the presence of 50 or 500 μ M compound relative to the control reaction with no added inhibitor. Data are reported as the average of three independent determinations, standard deviation of the average $\leq 10\%$.

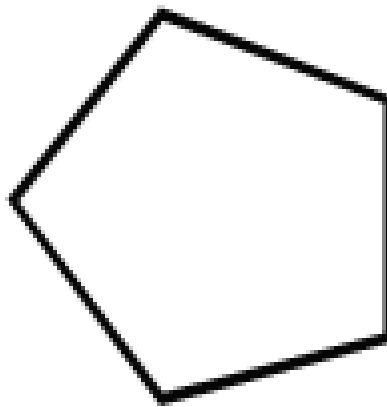
Table 3
SAR analysis of scaffold **11** by closely related commercial compounds.



Compound	A	B	R ¹	R ²	R ³	R ⁴	R ⁵	R ⁶	% Activity at 50 μM	% Activity at 500 μM
108	CH ₂	CH ₂	H	H	CH ₂ CH ₃	-	-	-	<1	<1
133	CH ₂	CH ₂	H	H	OCH ₃	-	-	-	13	35
167	CH ₂	CH ₂	H	H	H	-	-	-	91	45
201	CH ₂	CH ₂	CH ₃	H	CH ₃	-	-	-	93	49
262	CH ₂	CH ₂	CH ₃	H	CH ₃	-	-	-	90	51



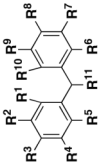
Compound	A	B	R ¹	R ²	R ³	R ⁴	R ⁵	R ⁶	% Activity at 50 μ M	% Activity at 500 μ M
23	CH ₂	CH ₂	H	H	CH ₂ CH ₃	-	CH ₃	H	86	53
32	CH ₂	CH ₂	H	H	CH ₃	-	CH ₃	H	99	57
45	CH ₂	CH ₂	H	H	OCH ₃	-	-	-	95	58
52	CH ₂	CH ₂	Cl	H	Cl	-	CH ₃	H	90	68
8	CONH	CH ₂	H	H	H	-	CH ₃	Cl	100	74

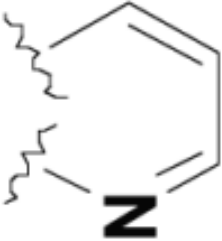


ported as the percent of residual enzyme activity in the presence of 50 or 500 μ M compound relative to the control reaction with no added inhibitor. Data are reported as the average of three independent ions, standard deviation of the average \leq 10%.

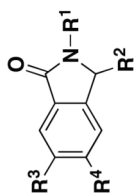
Table 4



ommercial compounds.

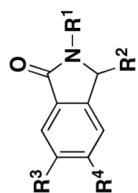


R ⁷	R ⁸	R ⁹	R ¹⁰	R ¹¹	% Activity at 50 μM	% Activity at 500 μM
Cl	H	Cl	OH	H	<1	<1
CH ₃	OH	CH ₃	H	H	89	43
H	H	H	H	OH	100	81
H	OH	H	H	H	100	88
NH ₂	H	H	H	H	100	100
						
		H	H	NHCOCH ₃	100	100

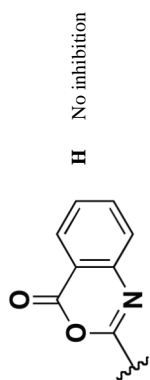
50 or 500 μM compound relative to the control reaction with no added inhibitor. Data are reported as the average of three independent



	R ²	R ³	R ⁴	IC ₅₀ (μM)
	H	H	H	19.9 ± 0.5
		Cl	Cl	No inhibition



R ²	R ³	R ⁴	IC ₅₀ (μM)
----------------	----------------	----------------	-----------------------



No inhibition

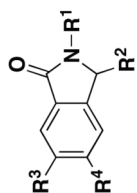
H


H

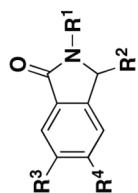
NH₂


No inhibition

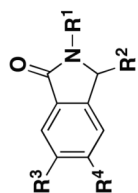
HO




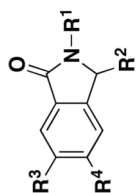
R ²	R ³	R ⁴	IC ₅₀ (μM)
	H	H	No inhibition



	R ³	R ⁴	IC ₅₀ (μM)
	H	H	No inhibition



R ²	R ³	R ⁴	IC ₅₀ (μM)
	H	H	No inhibition



R ²	R ³	R ⁴	IC ₅₀ (μM)
	H	H	No inhibition

Table 6

Kinetic properties of the four identified HTS inhibitor scaffolds for Hst2.

Scaffold	Substrate	Inhibition Mechanism	K_i (μ M)	α
11	NAD ⁺	NC (full)	1.2 ± 0.2	
11	Acetyl-lysine	NC (partial)	6.3 ± 1.7	
12	NAD ⁺	NC (full)	30 ± 6	
12	Acetyl-lysine	NC (full)	39 ± 7	
13	NAD ⁺	NC (full)	3.9 ± 0.7	
13	Acetyl-lysine	Mixed (full)	2.5 ± 1.0	13.4
14a	NAD ⁺	Mixed (full)	42 ± 17	2.6
14a	Acetyl-lysine	NC (full)	43 ± 8	

Data reported are the average of two independent determinations and the standard deviation of the average.