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SIRT1 Modulation as a Novel Approach to the Treatment of Diseases of Aging

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Introduction

SIRT1 is a member of the silent information regulator 2 (Sir2[a]) protein family of enzymes that is ubiquitous in terrestrial life. Following the discovery of the yeast Sir2 protein, mammalian homologues were subsequently identified and were designated sirtuins.[1,2] In humans, these enzymes possess either NAD^+ dependent deacetylase activity or protein ADP-ribosyltransferase activity.

As shown in Figure 1, all seven human sirtuins share a conserved catalytic core domain (designated in yellow), although overall the proteins vary significantly in length. The sirtuins also differ in their subcellular localization and substrate specificity and have distinct downstream effects.[3]

SIRT1, which is the focus of this review, is by far the most studied of all the seven human sirtuins. Interest in SIRT1 has grown since the initial reports that the *S. cerevisae* and *C. elegans* orthologues mediate the effects of calorie restriction (CR) to extend the lifespan of these organisms.[4] In mammals, SIRT1 transgenic mice show phenotypes and gene expression profiles that resemble those observed in mice on CR,[5] and further studies have provided evidence that SIRT1 plays a central role in regulating metabolic responses to nutrient conditions.[6−11] SIRT1 knockout mice, however, have been harder to assess, as they have poor viability and developmental deficits on most genetic backgrounds.[12] Nonetheless, SIRT1 overexpression and knockdown in a variety of cell systems and in mice have supported the notion that SIRT1 regulates not only metabolic responses but also circadian rhythm,[13−16] DNA repair,[17−19] and cell survival, senescence, death, and differentiation.[20−22] Furthermore, use of the cell systems as well as tissue-specific SIRT1 knockout mice has uncovered potential roles for SIRT1 in disease settings such as diabetes and cardiovascular disease,[6,9,23,24] inflammation,[9,25−27] neurodegeneration,[28,29] and cancer.[18,30]

The report that SIRT1 is a target of resveratrol, the polyphenol found in grapes and red wine that has been associated with health benefits,[31] has fueled many subsequent studies showing that resveratrol in fact improves metabolism and glucose tolerance caused by a high-fat diet,[32,33] protects endothelial cell function,[34] attenuates inflammation,[25,34,35] inhibits tumorigenesis,[36,37] and prevents neurodegeneration in cellular and animal models.[38−40] The effects of resveratrol have shown that it is possible to amplify SIRT1 activity and, in so doing, mimic the beneficial effects of CR.[41−43] However, besides modulating SIRT1 activity, resveratrol has been found to act on other targets shown to contribute to the metabolic effects of CR including AMPK and S6K,[44−47] and since polyphenols do not represent a particularly good starting point for medicinal chemistry, attention has turned to finding more potent and selective pharmacological activators of SIRT1.

Several groups have published on SIRT1 inhibitors and activators, which will be reviewed here. Such agents could offer important clues to the specific role of SIRT1 in biological and disease mechanisms. While the preponderance of genetic data indicates that increasing SIRT1 levels or its activity has beneficial physiological effects, reports are sometimes conflicting. Mice deficient in SIRT1 specifically in the liver have been shown to have less body weight gain, better glucose tolerance, and lower accumulation of fat in adipose and liver tissue when put on a high-fat diet,[48] while another study also looking at liver-specific SIRT1 KO mice on a high-fat diet showed the exact opposite.[49] The ages of the mice in these two studies differed, which could contribute to the contrasting observations. Mice expressing moderately elevated levels of SIRT1 in heart are protected from oxidative stress in the heart, but higher SIRT1 expression enhances oxidative stress and induces cardiac hypertrophy.[50] In circadian rhythm studies, the amplitude of Per2 expression in livers of SIRT1 deficient mice was elevated[14] while SIRT1-null MEFs show depressed amplitude of Per2.[13] In cancer, the APC min/+ mice when crossed to mice transgenic for SIRT1 specifically in the gut villi show a significantly decreased level of tumor incidence, and overexpression of SIRT1 by transfection lowered the β-catenin-driven proliferation of tumor cell lines.[30] Consistent with the role of SIRT1 being protective against tumors, a more recent report describes SIRT1 shRNA-mediated knockdown

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[a] Abbreviations: Sir2, silent information regulator 2; NAD^+, nicotinamide adenine dinucleotide; AMPK, 5′ adenosine monophosphate activated protein kinase; CR, calorie restriction; MEF, mouse embryonic fibroblast; Per2, period circadian protein homologue 2; APC, adenomatosis polyposis coli protein/gene; RNA, ribonucleic acid; shRNA, short hairpin RNA; siRNA, small interfering RNA; ADP, adenosine diphosphate; FOXO1, forkhead box O1; HIVtat, human immunodeficiency virus trans-activator of transcription; PGC-1α, peroxisome proliferator activated receptor-γ coactivator-1α; PCAF, P300/CBP-associated factor; MyoD, myogenic differentiation protein; 5-FU, 5-fluorouracil; CADD, computer aided drug design; ACS2, acetyl coenzyme A synthetase 2; PABPN1, polyadenylate-binding protein nuclear 1; TAMRA, tetramethyl-6-carboxyrhodamine; AMC, 7-amino-4-methyl coumarin; STAC, SIRT1-activating compound; DIO, diet-induced obesity; FAO, fatty acid oxidation; MCP-1, monocyte chemotactic protein-1; TNFα, tumor necrosis factor α; JNK, c-jun N-terminal kinase; IL-6, interleukin 6; LPS, lipopolysaccharide; IKK, IκB kinase; NF-κB, nuclear factor κ light-chain-enhancer of activated B cells; iNOS, inducible nitric oxide synthase; TORC2, transducer of regulated CREB activity 2; NAFLD, nonalcoholic fatty liver disease; UUO, unilateral ureteral obstruction.

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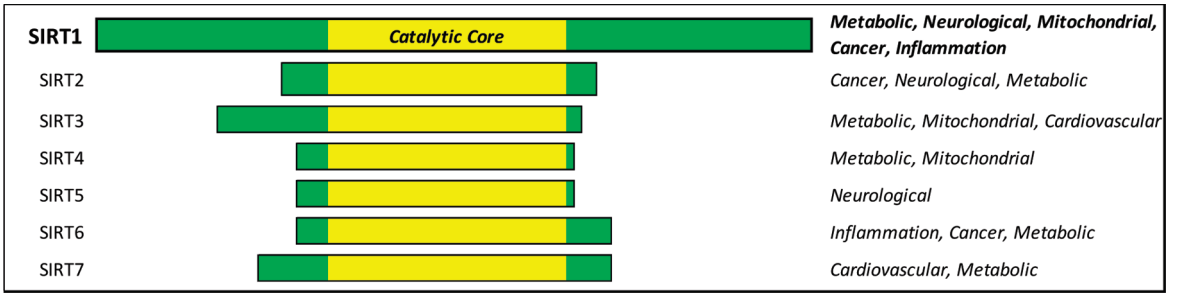


Figure 1. The seven human sirtuins.

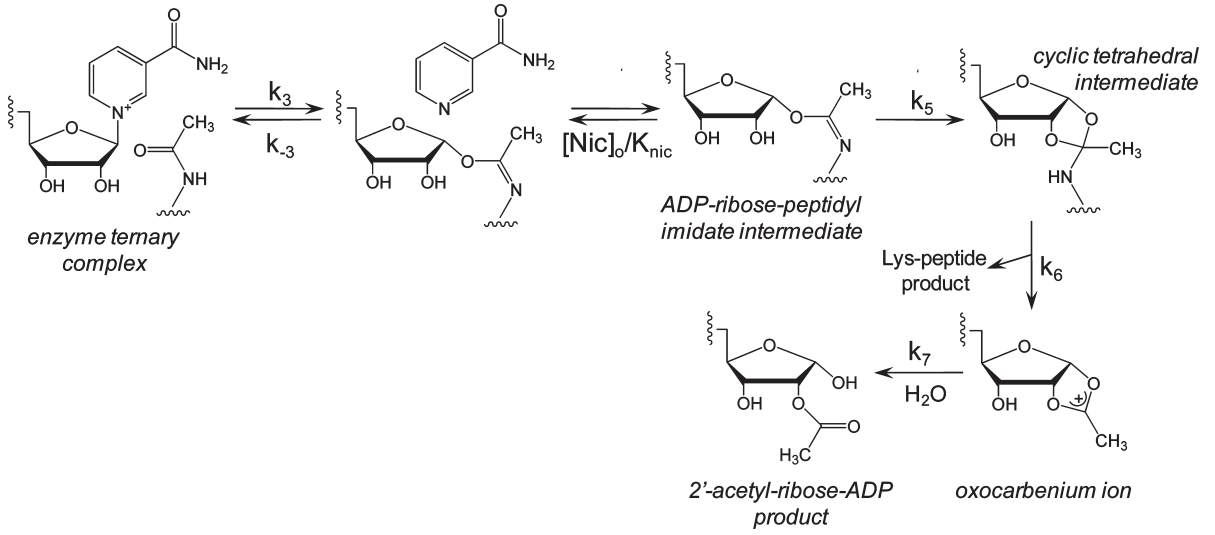


Figure 2. Chemical mechanism for SIRT1-catalyzed deacetylation.

accelerated growth of xenograft HCT116 tumors. However, the same report showed that SIRT1 knockdown and inhibition also sensitized tumor cells to 5-FU-induced apoptosis.[51] In tumor samples, heterogeneous SIRT1 levels have been observed in subsets of tumors,[52−54] and therefore, the stage of the tumor, the type of tumor, and the varying cellular environments and signals might all contribute to whether SIRT1 has tumor suppressor or oncogenic functions. This theme of SIRT1's functional effects being tissue- and context-dependent might underlie the discrepancies observed in the examples described above. These studies illustrate the limitations of genetic manipulations and highlight the need for potent and selective inhibitors and activators. Pharmacological tools would help clarify whether inhibition or activation of SIRT1 would lead to the desired therapeutic outcome.

Inhibition of enzymes has been a standard line of attack in drug discovery programs for many years, and the approach has resulted in the discovery of a number of safe and effective marketed drugs. By way of contrast, only a few examples of small molecule enzyme activators exist to date.[55] In the case of SIRT1 activators, the exact molecular mechanism by which these act on SIRT1 in vitro and in cells remains to be defined and has been under debate. Adding to the complexity is the fact that over two dozen substrates have been identified for SIRT1.[56] While this might not be surprising in light of all the pathways in which SIRT1 has been implicated, it raises further questions of how to unravel the effects of small molecule modulators in the context of a cell or organism. This article will review what is known about the enzymatic parameters of SIRT1 and describe the current status of SIRT1 modulators, including their potential mechanisms of action and their pharmacological effects in vivo.

SIRT1 Enzymology

Chemical Mechanism. Perhaps the most striking feature of sirtuin-catalyzed deacetylation is the requirement for NAD^+. In a series of elegant experiments, Schramm and colleagues were able to elucidate the chemical mechanism by which this NAD^+-dependent reaction occurs (see Figure 2).[57−59] Specifically, the ternary complex of the enzyme, NAD^+, and acetylated peptide reacts followed by expulsion of nicotinamide to form an ADP-ribose-peptidyl imidate intermediate. This is a key intermediate in sirtuin-catalyzed reactions, which partitions between forward reaction steps leading to production of deacetylated peptide and *O*-acetyl-ADP-ribose and reverse reaction steps with reincorporation of nicotinamide and synthesis of substrates. The existence and partitioning of this intermediate account for the inhibition of SIRT1 activity by excess nicotinamide, as well as the activation of sirtuins by isonicotinamide, which occurs through a mechanism involving the release of nicotinamide inhibition. It has been suggested that this latter phenomenon provides a mechanism for the identification of sirtuin activators.[60]

Kinetic Mechanism. To date, only one paper has been published that addresses the kinetic mechanism of a sirtuin. Elucidating the kinetic mechanism of a two-substrate enzyme involves determining the order of substrate addition and product release and estimating the rate and equilibrium constants for these processes. In 2004, Denu and co-workers

reported that SIRT2-catalyzed deacetylation of histone H3 related peptide KSTGG**K**^{Ac}APRKQ follows an ordered kinetic mechanism in which peptide binds to enzyme prior to NAD^+. The resultant ternary complex of enzyme, NAD^+, and peptide substrate then undergoes reaction by a process that is likely rate-limited by a reaction step subsequent to formation of imidate, such as product release.

It is still unknown if all sirtuins follow an ordered mechanism. Indeed, there are crystal structures of several sirtuins complexed with either NAD^+ or ADP-ribose, which indicates that NAD^+ can bind to free enzyme.[59,61−64] However, it is not clear if these E/NAD^+ complexes are productive.

Perhaps the most compelling data in support of a mechanism in which peptide substrate must bind prior to NAD^+ are calorimetric and equilibrium dialysis studies that demonstrate that while peptide substrates can bind to free enzyme, NAD^+ binds very poorly if at all.[65,66]

Substrate Selectivity of SIRT1. In vivo and in cell-based systems, SIRT1 is known to deacetylate a large number of protein substrates, including histones H1, H3, and H4, p53, p300, FOXOs 1, 3a, and 4, p65, HIVTat, PGC-1α, PCAF, MyoD, and Ku70.[59] However, for none of these proteins have enzyme kinetic data been determined, save for the SIRT1-catalyzed deacetylation of acetyl-CoA synthetase (see below). Quantitative studies of this kind would clearly define SIRT1's selectivity for protein substrates and thus add greatly to the understanding of what really counts as a SIRT1 substrate. In addition, if the results of these studies were then compared with results from similar studies for other sirtuins, the specificity of a protein for a particular sirtuin would provide clues about which sirtuin is responsible for a given protein's deacetylation.

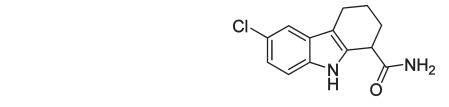
Despite the heavy reliance on peptide substrates in mechanistic studies as well as reliance on screens for inhibitors and activators, no systematic study of SIRT1's substrate selectivity has been reported. What is known is summarized below.

Two peptide library approaches intended to define SIRT1's substrate specificity came to different conclusions. In one study,[67] a peptide library containing the sequences Fmoc-MAXXXX-Lys(Ac)-XXXXXAEEE, where X is all amino acids except Lys and Cys, was constructed and tested as a substrate for SIRT1. Mass spectral analysis of products allowed determination of kinetically preferred amino acids at the variable positions. It was found that there is no amino acid preference at any of the variable positions, leading to the conclusion that substrate recognition by SIRT1 does not depend on the amino acid sequence proximate to the Lys(Ac). In the other study, a peptide array method was used to generate 10^5 unique SIRT1 substrate sequences based on the 5mer XX-Lys(Ac)-XX.[68] While these studies demonstrated a preference for QI or QF immediately C-terminal of Lys(Ac), differences in k_c/K_m among the most reactive peptide substrates were not significantly different. For example, k_c/K_m for the best substrate, QP-Lys(Ac)-QI, is 27 mM^{-1} s^{-1} while k_c/K_m for WH-Lys(Ac)-FQ, the "worst of the best", is 3 mM^{-1} s^{-1}, only 10-fold less.

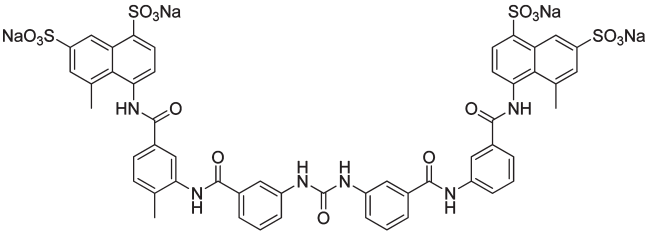
In one case, the deacetylation of active-site-acetylated acetyl-CoA synthetase, the kinetics of SIRT1's action on a protein substrate was reported.[69] The kinetic parameters for this reaction, along with those for the two-amino acid substrate Fmoc-Lys-Lys(Ac)-NH_2 and the one-amino acid substrate Z-Lys(Ac)-NH_2 [Stein, R.; Kustigian, L. unpublished data], are summarized in Table 1. From these studies and those discussed above, it seems clear that SIRT1 is relatively nondiscriminating for amino acid sequences out of the immediate vicinity of the Lys(Ac) residue.

Table 1. Steady-State Kinetic Parameters for SIRT1-Catalyzed Deacetylation

substrate	k_c (s^{-1})	K_m (μM)	k_c/K_m (mM^{-1} s^{-1})
Ac-CoA synthetase	0.08	12	7
Fmoc-Lys-Lys^{Ac}-NH_2	0.60	17	36
Z-Lys^{Ac}-NH_2	0.12	54	2



2 (EX-527)



3 (Suramin)

Figure 3. Potent SIRT1 inhibitors.

SIRT1 Medicinal Chemistry

The following describes SIRT1 inhibitors and activators from the recent literature. Several reviews are available that cover SIRT modulators appearing before 2008.[56,70−73]

SIRT1 Inhibitors

Over the past several years, a number of SIRT1 inhibitors have been reported. At best, they are moderately selective over SIRT2 and SIRT3, with single-digit micromolar IC_{50} values against SIRT1. Notable exceptions include indole-based inhibitor **2** (EX-527) (Figure 3) (SIRT1 IC_{50} = 0.098 μM; SIRT2 IC_{50} = 20 μM)[74] and **3** (suramin) (Figure 3) (SIRT1 IC_{50} = 0.3 μM; SIRT2 IC_{50} = 1.2 μM)[75] and their derivatives. Compound **2** was shown to be orally bioavailable in mice with a serum half-life of 194 min. Compound **3** is important as a tool compound for studying sirtuins and has been successfully cocrystallized with SIRT5 to give the first ligand-bound X-ray crystal structure of a human sirtuin enzyme.[76] However, its highly anionic structure limits its utility as a small molecule drug discovery lead. In order to fully evaluate the potential of SIRT1 inhibition as a mechanism suitable for a drug discovery program, SIRT1 inhibitors that are more potent and selective and that may also serve as viable leads are needed. Recent efforts in search of novel SIRT1 inhibitors have not yet resulted in a more potent and selective SIRT1 inhibitor than these benchmark compounds, but optimization progress has been reported for known SIRT1 inhibitor scaffolds, often with the use of focused library screening or the use of computer aided drug design (CADD) as more sirtuin crystal structures are solved.

Mechanism-Based Inhibitors. Following the determination of the SIRT5-suramin cocrystal structure, a ligand-bound X-ray crystal structure of SIRT3 was also generated, this time using a mechanism-based inhibitor.[66] As an acetyl lysine mimic, thioacetyl lysine has been incorporated into peptide fragments based on various sirtuin substrates

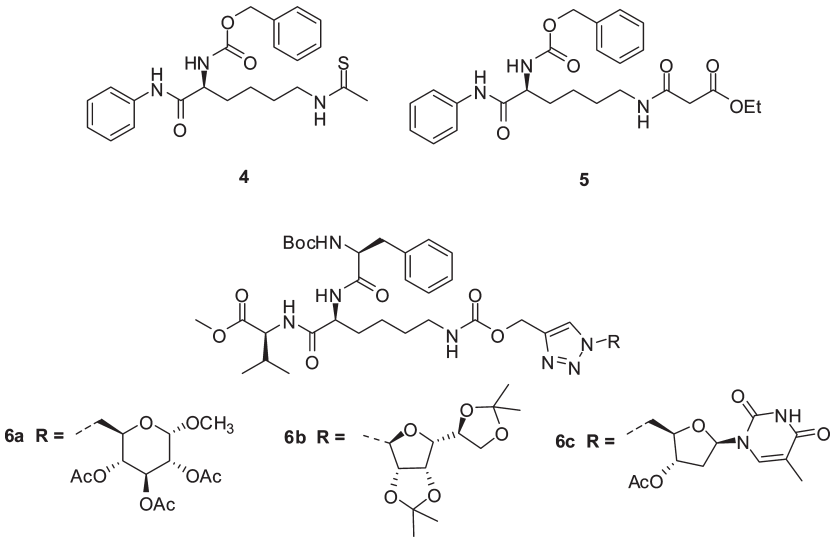


Figure 4. Acetyl lysine mimics that inhibit SIRT1.

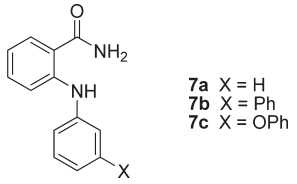


Figure 5. 2-Anilinobenzamides that inhibit SIRT1.

including p53[77,78] and ACS2.[66,79] Soaking of a SIRT3 crystal with an ACS2-derived thioacetyl peptide fragment and NAD^+ resulted in a cocrystal of SIRT3 and a stable thioacetyl lysine-ADP ribose conjugate. Recently, small molecule versions of these peptide inhibitors were described that retained inhibitory activity. It was shown that truncation of the peptide fragment substrates down to a single amino acid analogue containing acetyl lysine was still recognized by SIRT1 as a substrate.[80] Thioacetyl modification of the lysine analogue resulted in compound **4**, which inhibited SIRT1 ($IC_{50} = 2.7\ \mu M$) selectively over SIRT2 ($IC_{50} = 23\ \mu M$) and SIRT3 ($IC_{50} > 100\ \mu M$) (Figure 4). Compound **4** increased p53 acetylation in human colon cancer HCT116 cells in a dose-dependent manner.[81] By introduction of an anion-stabilizing group such as the ethyl ester adjacent to the acetyl group in compound **5**, the nucleophilicity of the α-carbon is expected to be enhanced for a preferential attack on the NAD^+ over the usual attack by the oxygen of the acetamide on NAD^+, leading to a carbon−carbon linked acetyl lysine-ADP ribose conjugate (Figure 4). Indeed, the acetyl lysine analogue **5** inhibited SIRT1 ($IC_{50} = 4\ \mu M$) selectively over SIRT2 ($IC_{50} = 65\ \mu M$) and SIRT3 ($IC_{50} > 300\ \mu M$) and increased p53 acetylation in HCT116 cells.[82] Chakrabarty et al. designed bisubstrate mimics **6a**−**c** as sirtuin inhibitors with features of an acetyl lysine and NAD^+ mimic using sugar/nucleoside modifications (Figure 4).[83] Although kinetic studies showed that these sugar and nucleoside containing tripeptides did not compete for the NAD^+ binding site, α-D-methyl glucopyranoside-derived **6a** showed 50% inhibition of SIRT1 at 20 μM. Both D-mannose-derived **6b** and thymidine-derived **6c** showed 40% inhibition of SIRT1 at 20 μM.

Nicotinamide Derivatives. Anilinobenzamide **7a** was originally identified from a targeted library of nicotinamide and benzamide analogues designed to yield inhibitors that occupy the nicotinamide subpocket of the NAD^+ binding site (Figure 5). However, it was shown that while SIRT1 inhibition by **7a** ($IC_{50} = 17\ \mu M$) was noncompetitive with NAD^+, it was competitive with the acetylated lysine substrate.[84] Analogues **7b** and **7c**, with substitution in the meta position, had SIRT1 enzyme activities comparable to that of **7a** and in addition showed growth inhibitory activity on human colon HCT116 cancer and Burkitt's lymphoma Daudi cells.[85] No data were reported for compounds **7a**−**c** against any of the other human sirtuins.

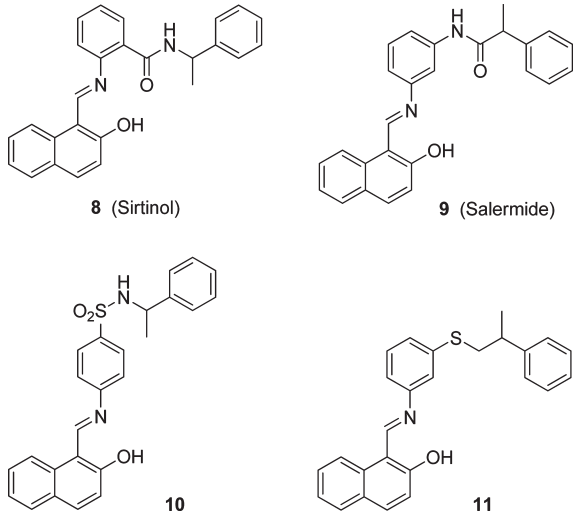


Figure 6. Salermide and other sirtinol analogues that inhibit SIRT1.

Sirtinol Derivatives. Compound **9** (salermide) (Figure 6), an analogue of **8** (sirtinol), was rationally designed to improve on the SIRT1 activity of **8**.[86] When the amide is reversed and moved from the 2′ to the 3′ position of the phenyl ring, the amide oxygen in salermide is oriented to better hydrogen-bond with a key glutamine in the NAD^+ binding pocket. When tested under the same assay conditions, **9** was moderately more potent than **8** (SIRT1 and SIRT2 IC_{50} of 43 and 25 μM vs 123 and 46 μM, respectively). It was further shown that **9** was active against cancer cells, especially MOLT4 and KG1A leukemia and Raji lymphoma

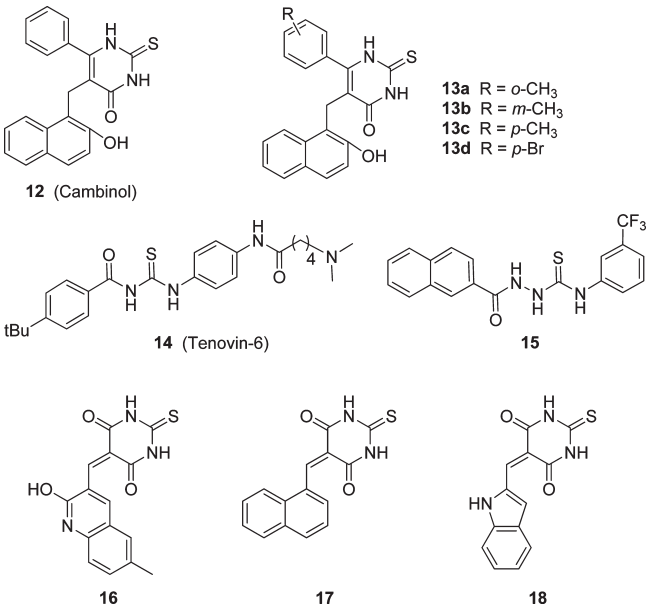


Figure 7. Cambinol and tenovin derivatives that inhibit SIRT1.

cells, by inducing apoptosis and was tolerated in mice at the active concentration, although no in vivo efficacy was reported. The antitumor effects were shown to be most likely SIRT1-mediated, in part by reactivation of proapoptotic genes that were repressed in cancer cells by SIRT1-mediated K16H4 deacetylation. The structural modifications in para sulfonamide analogue **10** and meta thioether analogue **11** were tolerated for SIRT1 and SIRT2, with IC_{50} values similar to that of **9** (Figure 6). All three inhibitors were probed for potential cell protection effects in muscular dystrophy using *C. elegans* transgenics that coexpress nuclear GFP and mutant PABPN1 in muscle cells. Compounds **9** and **10** were found to be protective against PABPN1 toxicity, while the effect of compound **11** could not be evaluated because it did not reach the muscle cells.[87]

Cambinol and Tenovin Derivatives. Compounds **12** (cambinol) (Figure 7) and **14** (tenovin-6) are two sirtuin inhibitors that show antitumor activity in preclinical models.[88,89] Together with the correlation observed between these inhibitors' activities in biochemical and whole-cell assays, these promising in vivo results stimulated efforts to optimize **12** (SIRT1 IC_{50} = 56 μM and SIRT2 IC_{50} = 59 μM) for SIRT1 potency and selectivity. It was found that selectivity for either SIRT1 or SIRT2 could be achieved with modifications on specific parts of the scaffold of **12**. Substitution on the phenyl ring produced modest improvements in SIRT1 selectivity (**13a**−**d**, SIRT1 IC_{50} = 13−45 μM, SIRT2 IC_{50} > 60 μM, Figure 7).[90] Whereas methyl substitution was tolerated at all positions on the phenyl ring (**13a**−**c**), of the three possible bromo substitutions, only the para-substituted analogue exhibited SIRT1 inhibition. The *p*-bromo analogue **13d** had the highest SIRT1 potency with an IC_{50} of 13 μM and increased levels of acetylated p53 in MCF-7 breast adenocarcinoma cells. N-Alkylation α to the phenyl group conferred SIRT2 selectivity. Tenovin derivative **15** was optimized from a virtual screen hit using a comparative model of SIRT1 and a binding model for **2** (Figure 3) analogues[91] and was selective for SIRT1 (IC_{50} = 13 μM) over SIRT2 (IC_{50} = 113 μM) (Figure 7).[92]

A set of thiobarbiturates was identified as sirtuin inhibitors from a virtual screen using the X-ray crystal structure of SIRT2 and docking studies in the putative nicotinamide subpocket. Nonselective sirtuin inhibitors were found showing both SIRT1 and SIRT2 activities, with the most potent being thiobarbiturates **16** (SIRT1 IC_{50} = 13 μM and SIRT2 IC_{50} = 11 μM) and **17** (SIRT1 IC_{50} = 13 μM and SIRT2 IC_{50} = 9 μM) (Figure 7).[93] Free-energy calculations indicated a favorable hydrophobic interaction above the naphthalene of docked **17**, leading to the design of compound **18** which showed a 3-fold selectivity for SIRT1 (SIRT1 IC_{50} = 6 μM and SIRT2 IC_{50} = 20 μM) (Figure 7).

Other SIRT1 Inhibitors. Four scaffolds with sirtuin inhibitory activity were found from a high-throughput screen of 50 000 compounds, two of which had good to moderate nonselective activity against SIRT1. Compound **20**, a structural relative of **19** (splitomicin), was potent against SIRT1 (IC_{50} = 6 μM; SIRT2 IC_{50} = 2 μM; SIRT3 IC_{50} = 89 μM), compared to **19** which showed no SIRT1 inhibition up to 500 μM in the same assay (Figure 8).[94] Compound **21** had more moderate SIRT1 activity with an IC_{50} of 34 μM (SIRT2 IC_{50} = 19 μM; SIRT3 IC_{50} = 40 μM) (Figure 8).

Combination of the scaffold of a kinase inhibitor that demonstrated SIRT1 inhibitory activity[95] with the features from the antiproliferative and antiviral natural product β-carboline resulted in the sirtuin inhibitor **22** (Figure 8).[96] Oxindole **22** inhibited SIRT1 with an IC_{50} of 40 μM but was a better inhibitor for SIRT2 and SIRT3 (IC_{50} = 28 and 24 μM, respectively) and showed SIRT2-dependent tubulin hyperacetylation in MCF-7 breast adenocarcinoma and Hep G2 liver carcinoma cells. Bis-naphthalimidopropylalkyldiamine **23** was found to be a moderate SIRT1 inhibitor (IC_{50} = 43 μM) and was not tested against other human sirtuins (Figure 8).[97]

SIRT1 Activators

Naturally Occurring Small-Molecule SIRT1 Activators Related to Resveratrol. Resveratrol (**1**, Figure 15), a polyphenolic flavonoid with potent antioxidant properties, is the first reported small molecule SIRT1 activator. For an overview of resveratrol's biological targets and functions, see the excellent review by Pervaize and Holme.[98] Although the mechanisms through which resveratrol asserts its beneficial physiological effects are unclear, its capacity to modulate SIRT1 activity is currently a topic of much interest and controversy.[99] As a consequence, other naturally occurring phenolic plant constituents have been investigated in order to better understand the nature of their positive effects on health. Recently, several isoflavones were characterized with regard to their ability to promote mitochondrial biogenesis,[100] a process known to be linked to SIRT1 activity via the transcriptional coactivator PGC-1α.[33] Indeed, an increase in mitochondrial biogenesis was observed for flavones that either activated SIRT1 (**25**, **28**) or increased SIRT1 expression (**29**, **30**, **31**, **32**) or did both (**26**, **27**) (Figure 9). Compounds **25**, **26**, **27**, and **28** were found to activate recombinant SIRT1 in a concentration-dependent manner (10−100 μM), as measured by deacetylation of lysine residues on a human fluorogenic p53 peptide fragment. The data suggest that the 5-hydroxy group on **29** (genistein) and **30** (biochanin A) can eliminate the SIRT1 activating properties of these compounds. The SAR also suggests that the 7-hydroxy group is an important feature for imparting SIRT1 activation, since methylation of the hydroxyl group led to the inactive

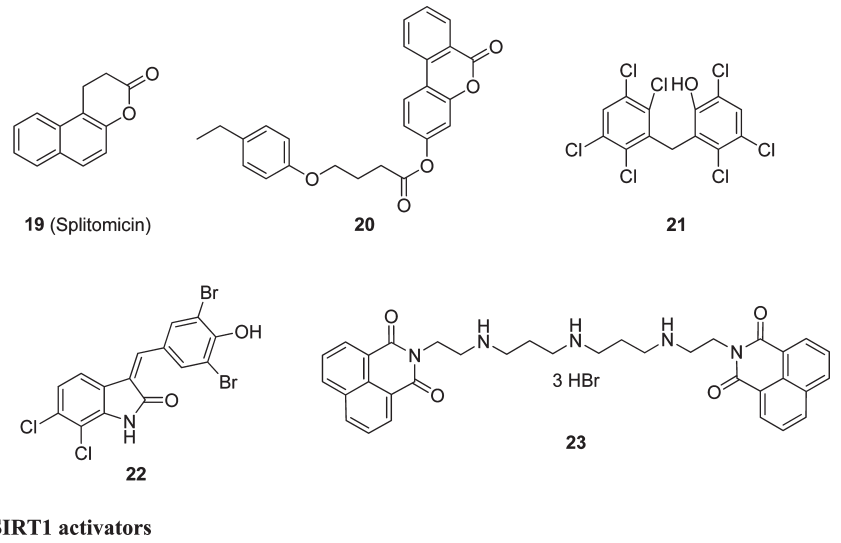


Figure 8. Other SIRT1 inhibitors.

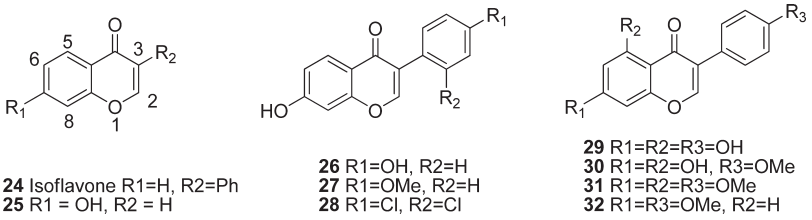
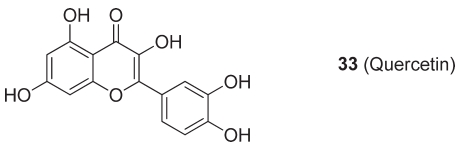


Figure 9. Isoflavones that activate or increase expression of SIRT1.

analogue **32**. Taken together, this identifies structure **25** as the basic isoflavone pharmacophoric unit needed to promote SIRT1 deacetylase activity. The authors show that the mitochondrial biogenesis derived from these compounds is independent of the estrogen receptor and suggest a dependence on a SIRT1-PGC-1α pathway.

Shortly after the work on isoflavones appeared, another group reported additional findings on a structurally related polyphenolic flavonoid, **33** (quercetin).[101] Specifically, the investigators were interested in assessing whether **33** would increase mitochondrial biogenesis in muscle and brain and whether this would be reflected in a change in exercise tolerance. Following 7 days of dosing of mice with **33**, SIRT1 expression (RT-PCR) increased by 200% in the soleus muscle (12.5 and 25 (mg/kg)/day) and by 50% and 100% in the brain (12.5 and 25 (mg/kg)/day, respectively). These same doses were associated with an increase in endurance running of these mice. SIRT1 protein levels were not measured, although changes in SIRT1 mRNA are often reflected in changes in protein levels and activity.[33] Mitochondrial biogenesis, which normally tracks with increased SIRT1 and PGC-1α levels, was also elevated as determined via mitochondrial DNA and cytochrome *c* enzyme levels. The authors note that **33** is an antagonist at the adenosine A1 receptor, and this complicates the interpretation of the endurance data.



SIRT1 Activators Based on Nicotinamide/Isonicotinamide. On the basis of reports that both nicotinamide and isonicotinamide can act as a sirtuin modulators,[102,103] Mai et al. prepared a series of 12 1,4-dihydropyridines (Figure 10).[104] These compounds were tested at 50 μM against human SIRT1, -2, and -3 using a biochemical assay utilizing a fluorogenic peptide substrate based on p53. The sirtuin modulating effects of compounds **34**−**36** were highly dependent on substitution at R and X (Figure 10), and benzylic substitution at R was found to be critical in order to impart SIRT activating properties, irrespective of substitution at X. Compounds wherein R = cyclopropyl, phenyl, or phenethyl were inhibitors of SIRT1 and SIRT2 and inactive for SIRT3. Compounds **34c**, **35c**, and **36c** exhibited some SIRT1 selectivity, activating with EC_{150} values (concentration able to increase enzyme activity to 150%) of ~1, ~1, and 36 μM, respectively. Less potent activation was observed for SIRT2 (**34c** EC_{150} = 25 μM; **35c** EC_{150} = 15 μM), while SIRT3 activation was minimal (**34**−**36c** $EC_{150} \geq 50$ μM). In a human primary mesenchimal stem cell line (hMSC), activators **34**−**36c** (50 μM) were shown to reduce the number of senescent cells by up to 40% using senescence-associated

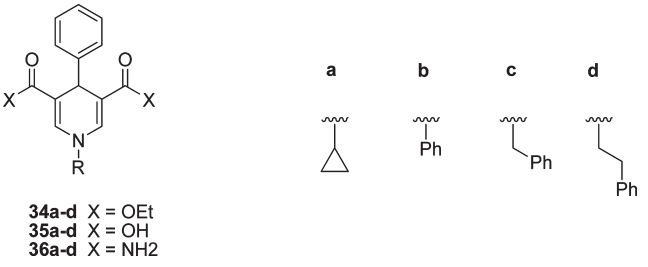


Figure 10. 1,4-Dihydropyridines that modulate SIRT1, SIRT2, and SIRT3.

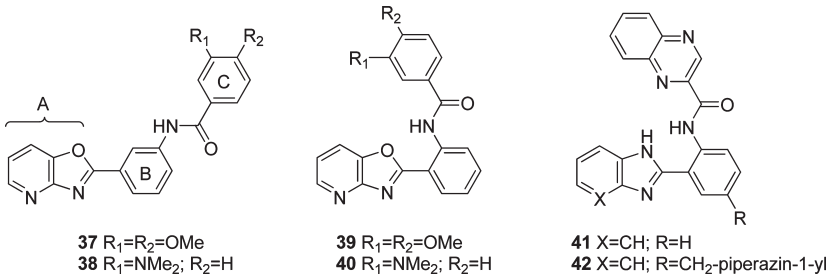


Figure 11. Oxazolo[4,5-*b*]pyridines that activate SIRT1.

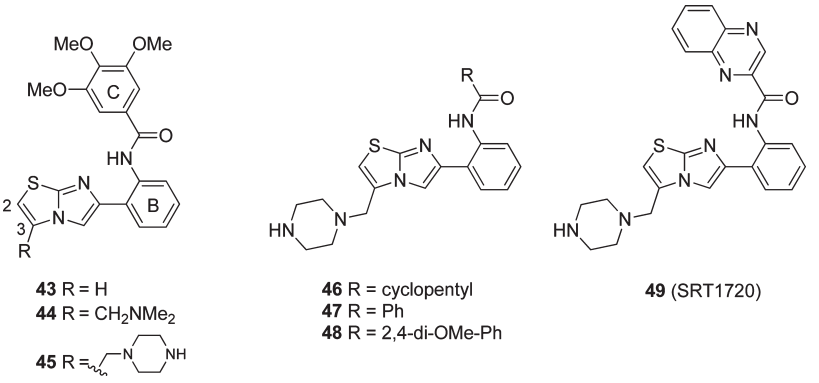


Figure 12. Imidazo[1,2-*b*]thiazoles that activate SIRT1.

β-galactosidase (SA-β-gal) as a biomarker. In a mechanism that was shown to be PGC-1α dependent, activator **34c** exhibited a concentration-dependent increase in mitochondrial function when tested in murine C2C12 myoblasts. No data were reported regarding off-target activities of these SIRT modulators.

Non-Natural Product Derived SIRT1 Activators. In 2007, the structures of three small-molecule SIRT1 activators containing an imidazothiazole scaffold were described by Sirtris Pharmaceuticals.[105] As discussed in Preclinical below, a number of reports have been published exploring the pharmacology of one of these compounds, **49** (SRT1720) (Figure 12).

Within the past year, two papers were published that describe the genesis of this imidazothiazole scaffold exemplified by the SIRT1 activator **49**. The first of these describes the discovery of a series of oxazolo[4,5-*b*]pyridines from high-throughput screening of a compound library.[106] The initial hits (**37** and **38**, Figure 11) were discovered using a fluorescence polarization assay, while subsequent optimization utilized a high-throughput mass spectrometry assay, both of which use a fluorophore (TAMRA) tagged peptide substrate as previously described.[105] Activation of SIRT1 was measured as the concentration of compound required to increase enzyme activity by 50% ($EC_{1.5}$) and also by the percentage maximum activation (max act.) achieved at the highest doses tested. The kinetics and relevance of this assay in elucidating SIRT1 activators are discussed below in the section Mechanisms of SIRT1 Inhibition and Activation.

Although **37** and **38** were moderate activators of SIRT1 ($EC_{1.5}$ = 6 μM, max act. of 240% and $EC_{1.5}$ = 25 μM, max act. of 207%, respectively), shifting the point of attachment of the amide functionality from the meta- to the ortho-position to give compounds **39** and **40** (Figure 11) led to a 5- to 6-fold improvement in the $EC_{1.5}$ values ($EC_{1.5}$ = 0.9 μM, 170% max act. and $EC_{1.5}$ = 4.4 μM, 209% max act., respectively). Additional exploratory SAR revealed that replacement of the oxazolopyridine core with benzimidazole was well tolerated as in analogue **41** ($EC_{1.5}$ = 0.3 μM, 253% max act.). Installation of basic groups off the B-ring was tried as a means of improving physiochemical properties, and this also led to potent SIRT1 activators (e.g., compound **42**, $EC_{1.5}$ = 0.4 μM, 820% max act.). Aza-benzimidazole bicycles (wherein X = N) also exhibited enzyme activation. No selectivity data or additional pharmacological data were reported for these compounds.

In a more recent publication, the SAR of this series was extended further and it was demonstrated that the SIRT1 activation properties of these compounds could be retained using an imidazo[1,2-*b*]thiazole bicyclic ring, as exemplified by compound **43** ($EC_{1.5}$ = 2.5 μM, 272% max act.) in Figure 12.[107] Analogues incorporating a basic side chain at either the 2- or 3-position of this bicycle also retained SIRT1 activity (e.g., $EC_{1.5}$ = 1.9 μM, 270% max act. and $EC_{1.5}$ = 1.8 μM, 271% max act. for **44** and **45**, respectively) while giving a significant boost in water solubility (< 0.1 mg/mL vs 10 mg/mL for **43** vs **45**, respectively). With the piperazinylmethyl group at the 3-position kept constant, carboxamide (C-ring) variants were prepared with the aim of enhancing SIRT1 potency. The SAR suggests that saturated rings (**46**, $EC_{1.5}$ > 50 μM) and unsubstituted phenyl rings (**47**, $EC_{1.5}$ = 25 μM) are not well tolerated. Alkoxy substitution is well tolerated at the meta- and para-positions (as in **43**−**45**), while *o*-methoxy groups are not (**48**, $EC_{1.5}$ = 31 μM). A number of bicyclic C-rings, for example, 2-quinolyl, 3-quinolyl, 2-benzofuranyl, and 5-benzofuranyl, also displayed SIRT1 activity ($EC_{1.5}$ of 0.99, 0.68, 0.61, and 6.9 μM, respectively). This SAR exploration culminated in the discovery of the potent SIRT1 activator **49** ($EC_{1.5}$ = 0.16 μM; 781% max act.) which incorporates a 2-quinoxalyl C-ring. For a description of the pharmacology of **49** see the section Preclinical below.

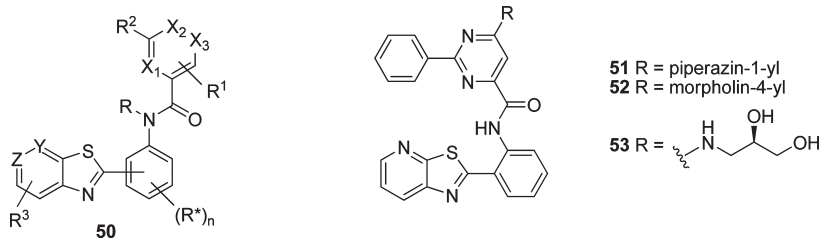


Figure 13. Thiazolopyridines that activate SIRT1.

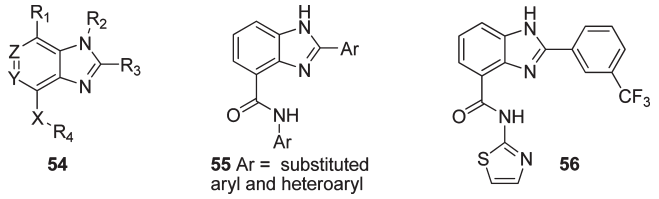


Figure 14. Benzimidazoles that activate SIRT1.

Two recent patent applications from Sirtris disclose SIRT1 modulators from two distinct chemical classes, thiazolopyridines[108,109] and benzimidazoles.[110,111] The thiazolopyridines, broadly characterized as structure **50** (Figure 13), were characterized as SIRT1 activators on the basis of their ability to promote deacetylation of a peptide substrate as detected by mass spectrometry. Exemplified compounds with $EC_{1.5} < 250$ nM include those in which R contains a basic amine (**51**), a neutral group (e.g., **52**), or hydrophilic groups (e.g., **53**).

The benzimidazoles can be broadly characterized as shown in structure **54** (Figure 14). Of the more than 600 compounds exemplified, the vast majority displayed the motif shown in structure **55** wherein each Ar is a substituted aryl or heteroaryl ring (Figure 14). The first example claimed to have $EC_{1.5} < 1$ μM and a fold activation of $>200\%$ is compound **56**.

Mechanisms of SIRT1 Inhibition and Activation

The chemical and kinetic mechanisms by which SIRT1 catalyzes deacetylation were discussed above. An understanding of an enzyme's mechanism is a necessary prerequisite for an accurate interpretation of results from experiments aimed at elucidating the mechanisms by which the enzyme is inhibited and activated. In this section, we will review what is known of these mechanisms for SIRT1 modulators.

Inhibitors of SIRT1

Inhibitors Originating from High-Throughput Screens. The SIRT1 inhibitor **2** (Figure 3) is an indole-based compound that was identified in a high-throughput screen.[74] The authors performed experiments aimed at elucidating the mechanism of inhibition in which reaction velocities were measured as a function of acetylated-peptide substrate and NAD^+ concentrations, at six concentrations of **2**. The results of these experiments indicate mixed inhibition of both substrates which were interpreted to suggest that **2** binds to a form of the enzyme that exists after both substrates have bound, thereby blocking product release. On the basis of inhibition of nicotinamide exchange ($IC_{50} = 1.5$ μM), the authors further speculated that the site to which **2** binds is close to or coincident with the nicotinamide binding site. However, mixed inhibition simply means that **2** binds to forms of the enzyme that exist both before and after substrate addition. The fact that mixed inhibition is seen for both substrates means that inhibitor binds to all forms of the enzyme and perhaps some form subsequent to the ternary complex of enzyme, NAD^+, and peptide. Unfortunately, mechanistic options are not restricted by the results of the inhibition of the nicotinamide exchange experiment, since this experiment alone cannot differentiate between **2** binding to the ternary complex or other forms of the enzyme. To probe this more fully, nicotinamide exchange kinetics would need to be performed as a function of both nicotinamide and **2** concentrations. At this point, all that can be concluded is that **2** binds to forms of the enzyme prior and subsequent to substrate addition of both peptide substrate and NAD^+.

In 2009, investigators at the Wistar Institute reported the results of an HTS campaign to identify inhibitors of SIRT1.[94] They conducted mechanism of inhibition experiments on one member of each of four classes of inhibitors that were identified (structures for **11**, **12**, **13**, and **14a** in ref 94). From an analysis of the data from these experiments, the authors concluded that all four compounds are mixed inhibitors of both substrates. Unfortunately, no details of the data analysis were given, so it is difficult to ascertain how the authors came to their mechanistic conclusion.

The 2-anilinobenzamides were also identified via HTS for SIRT1 inhibitors.[84] A kinetic analysis was performed with the unsubstituted parent compound **7a** (Figure 5) and produced data that indicate mixed inhibition vs NAD^+ and competitive inhibition vs peptide substrate Ac-His-Arg-Lys-Lys(Ac)-AMC (**61**). Interpreting the competitive inhibition to indicate that the inhibitors bind in the peptide binding pocket of SIRT1, the investigators docked 2-anilinobenzamide into that site on Sir2. The SAR for this series of 2-anilinobenzamides could be reasonably rationalized using this model.

Mechanism-Based Inhibitors. In 2006, Zheng and co-workers reported peptide substrates for SIRT1 in which the acetyl-Lys residue had been replaced with a thioacetyl-Lys.[77] The investigators found that the thioacetyl-Lys substrate was turned over by SIRT1 400 times slower than the acetyl-Lys substrate. Given the slow rate with which SIRT1 turns over the thioacetyl-Lys substrate ($k_c \approx 0.0004$ s^{-1}), the authors characterized this compound as a mechanism-based inhibitor. Mechanistic studies by Smith and Denu demonstrated that the origin of the slow turnover of thioacetyl-Lys containing peptides is the slow conversion of the thioimidate intermediate into the cyclic tetrahedral intermediate analogous to k_5 of Figure 2.[78] Finally, Suzuki et al. found that the thioacetyl-Lys need not be a residue within a peptide to afford an inhibitor but that Z-Lys(thioAc)-anilide (**4**, Figure 4) is also an inhibitor of SIRT1.[81]

In the course of their work, Suzuki et al. demonstrated that Z-Lys(X)-anilide is a general motif for SIRT1 inhibitor design that can be expanded to include a variety of X groups.[112] The most potent compound of those reported is Z-Lys($^{\varepsilon}$NH-CO-CH_2-COOEt)-anilide (**5**, Figure 4), which has an IC_{50} of 4 μM. Its potency is likely underestimated given that the compound is a slow-binding inhibitor. Mechanistic experiments demonstrate that the compound inhibits by forming a covalent adduct with NAD^+ in which nicotinamide is displaced through nucleophilic attack by the α-carbon of the enol form of the compound. An analysis of the time dependence of the inhibition, which would have afforded a K_i estimate of this in situ formed bisubstrate analogue, was not conducted.

Dependence of Inhibitor Potency on Substrate Potency. In at least two cases, it has been reported that IC_{50} values for certain inhibitors depend on the structure of the substrate. While the IC_{50} of **2** is 0.1 μM when Ac-His-Arg-Lys-Lys(Ac)-AMC (**61**) is the substrate, it increases by an order of magnitude to 1.3 μM when a p53-based 18mer is used as a substrate.[74] Similarly, IC_{50} values for several macrocyclic bis(indolyl)maleimides are increased by factors of at least 5-fold when the substrate is changed from the minimal substrate Z-Lys(Ac)-AMC (**61**) to a 25mer based on the N-terminus of histone H4.[95] In both cases, inhibitors show mixed-type inhibition, and thus bind to a form of enzyme in which peptide substrate is bound. Therefore, it is not unexpected that structural features of the substrate will influence inhibitor potency. This phenomenon is discussed in more detail below.

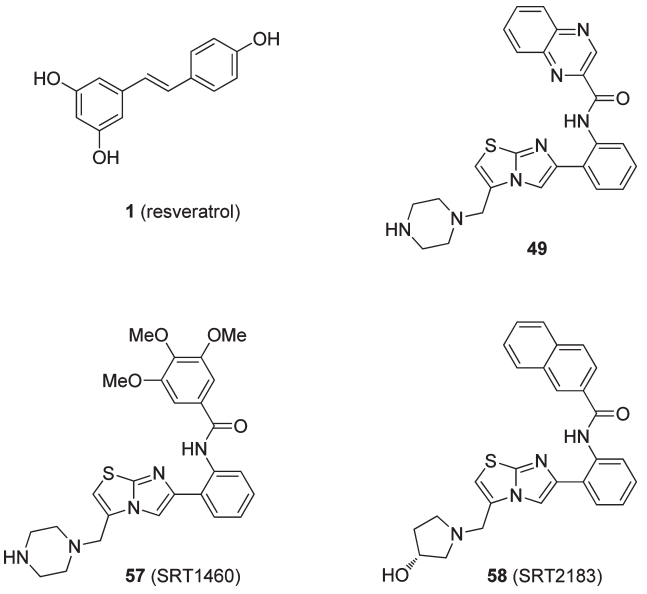


Figure 15. SIRT1 activators for which mechanistic data have been reported.

Activators of SIRT1

To date, there have been a limited number of publications directly addressing the mechanism of action of SIRT1 activating compounds (STACs) focusing primarily on the dependence of activation on structural features of peptide substrates.

This aspect of SIRT1 activation first came to light in 2005, when two papers appeared reporting that resveratrol (**1**)[31] can activate the SIRT1-catalyzed deacetylation of Ac-Arg-His-Lys-Lys^{Ac}-AMC but not the corresponding peptide lacking the AMC group, Ac-Arg-His-Lys-Lys^{Ac}-NH_2.[113,114] In 2009, these results were confirmed by Beher et al.[115] who concluded that **1** activation of SIRT1 is artifactual. This view was not entirely shared by the authors of the former two papers, who leave open the possibility that the ability of **1** to activate SIRT1 toward Ac-Arg-His-Lys-Lys^{Ac}-AMC may be telling us something significant about sirtuin biology. Kaeberlein et al. explain that it is "possible resveratrol causes sirtuins to deacetylate substrates in vivo that are normally low affinity targets."[114] And Borra et al. conclude their paper by commenting that "resveratrol may serve as a mimic to endogenous activators".[113]

These studies, which centered around **1** and Ac-Arg-His-Lys-Lys^{Ac}-AMC, were extended by Pacholec et al.[116] to include the three SIRT1 activators **49**, **57**, and **58** (see Figure 15) and the TAMRA-labeled 20mer substrate (**59**, Figure 16) that were originally described by Milne et al.[105] In addition, Pacholec et al. studied the effect of these activator compounds for the first time using three macromolecular substrates.

The primary in vitro observations of this study were the following: (i) the presence of the TAMRA-label is necessary for activation by **57**, **49**, and **58**; (ii) **49** and **57** can bind directly to TAMRA-peptide; (iii) there was no activation observed using three protein substrates of SIRT1 by any of these activator compounds. These observations led the authors to conclude that SIRT1 activation by STACs must be through an "indirect" mechanism involving the formation of a complex between activator and TAMRA-peptide substrate. Although Pacholec et al. did not advance a specific mechanistic hypothesis, activation of SIRT1 presumably resulted from favorable kinetics of the SIRT1-catalyzed turnover of this complex. However, it would be expected that unstable complexes between the substrate and activator with high K_d values should not activate SIRT1, since activation is presumably driven by formation of this complex. Despite the fact that both **57** and **49** were shown by Pacholec et al. to bind very weakly to TAMRA-peptide (K_d > 100 μM), the authors offer no explanation for how it is that such highly unstable activator–substrate complexes can lead to SIRT1 activation. The lack of a hypothesis explaining the activation of SIRT1 using the TAMRA-modified peptide by Pacholec et al. leaves several open questions on the molecular mechanism responsible for

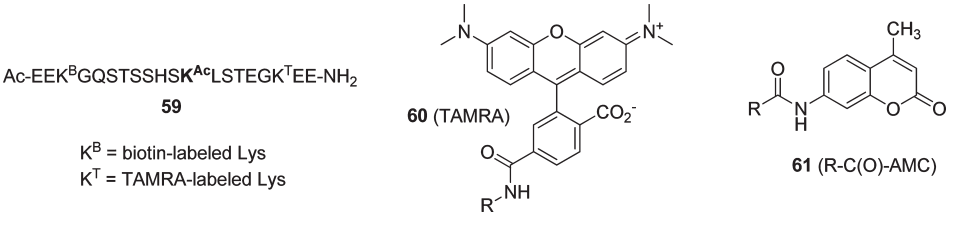


Figure 16. SIRT1 substrates.

this observation and whether it could be functionally related to the SIRT1-dependent activation by several of these compounds in cells and in vivo (see below). In contrast to Pacholec et al., Borra et al.[113] and Suave[60] have separately advanced theories regarding the mechanism of SIRT1 activation. In their paper, Borra et al. observe that resveratrol can activate the deacetylation of Ac-Arg-His-Lys-Lys^{Ac}-X, where X is AMC or rhodamine. They argue that for these substrates the fluorophore impedes binding of the substrate. Therefore, activation by **1** occurs through a mechanism in which it binds to an allosteric site and induces a conformational change in SIRT1 that allows the enzyme to better accommodate fluorophore moieties and thus bring about tighter binding of the substrate. In this mechanism, resveratrol promotes binding of otherwise poorly binding substrates.

In a recent review,[60] Sauve proposes that the apparent dependence of activation by **1** and STACs on the presence of a fluorophore covalently attached to the substrate can be explained by an allosteric mechanism in which substrate binding opens a site for activator binding, which then stabilizes substrate binding. This is essentially identical to nonessential activation described by Segel.[117] Like nonessential activation, Sauve's mechanism does not provide an explanation for the substrate structural-dependence of SIRT1 activation by STACs.

While it is clear that there is currently no mechanistic understanding that can explain the activation of SIRT1 by STACs in vitro and how this correlates with the unambiguous SIRT1-dependent effects of these compounds in cells and in vivo (see Preclinical below), there are several potential avenues that have yet to be explored. One area that is an extension of the previous studies is to understand the importance of substrate structure in the activation of SIRT1 by small molecules. As discussed above, the studies to date have investigated only a limited number of substrates in vitro. However, these data are consistent with a hypothesis that the in vitro activation of SIRT1 may be strictly dependent on substrate structure whether it be artificial or physiological based on the well-validated mechanism of allosteric modulation observed in other enzyme systems.[118−122] Evaluating this and other mechanistic possibilities will be the focus of additional future studies, since reconciling the biochemical and physiological effects of small molecule SIRT1 activators will be critical in optimizing these compounds as a new class of therapeutic agents.[123]

Preclinical

There is no clinical data available for the selective SIRT1 activators, although SRT2104 (structure not disclosed) is being dosed in multiple trials including trials in type 2 diabetes and in an acute inflammation study involving LPS challenge of healthy volunteers. In addition, a first time in human trial is underway for SRT2379 (structure not disclosed). As mentioned above, **49** has been the most extensively tested compound with preclinical data available from many laboratories in several disease models, including models of diabetes,[105,124,27] inflammation,[125] and fatty liver disease.[126]

In the original paper on **49**,[105] the compound was tested in three different models of type 2 diabetes at a dose of 100 mg/kg. In the diet-induced obesity (DIO) model, **49** significantly improved most metabolic parameters, including a reduction in blood glucose that was maintained over 10 weeks of dosing, a reduction in the hyperinsulinemia, and a reduction in glucose excursion in the intraperitoneal glucose tolerance test that was comparable to rosiglitazone. Compound **49** also increased mitochondrial biogenesis in DIO mice as measured by an increase in citrate synthase levels in the gastrocnemius muscle. In the leptin deficient ob/ob mouse, **49** significantly decreased blood glucose after 1 week of treatment with no effect on body weight. In the Zucker fa/fa model of diabetes, **49** improved insulin sensitivity as evidenced by a reduction in glucose and insulin levels during an oral glucose tolerance test. In a hyperinsulinemic−euglycemic clamp study in Zucker fa/fa rats, **49** increased the glucose infusion rate, the glucose disposal rate, and the insulin-stimulated glucose disposal rate after 4 weeks of dosing.

In the study by Feige et al.,[124] **49** was added to the chow at equivalents of 100 and 500 (mg/kg)/day in mice fed a high-fat diet. The compound was found to protect from diet induced obesity by preventing fat accumulation and by increasing energy expenditure. Lipid profiles (triglycerides and cholesterol) were also improved by **49**. In agreement with the results from Milne et al.,[105] **49** reduced glucose and insulin levels and increased metabolic parameters in the hyperinsulinemic−euglycemic clamp paradigm. Compound **49** significantly increased muscle performance as measured by treadmill testing, grip strength, and rotarod performance. There was a switch in the contractile phenotype of gastrocnemius muscle from fast to slow twitch accompanied by an increase in genes controlling fatty acid oxidation (FAO). The livers from **49**-treated animals had reduced fat and increased expression of genes involved in mitochondrial metabolism and FAO. Compound **49** also stimulated energy expenditure in brown fat and again increased genes involved in mitochondrial function and FAO. In this study, **49** was shown to increase deacetylation of known SIRT1 targets, including p53, PGC-1α, and FOXO1.

In addition to the positive metabolic effects of SIRT1 activation produced by compounds like **49**, it is also possible that SIRT1 activators could affect insulin sensitivity by inhibiting inflammation.[27,125] The treatment of Zucker fa/fa rats with the SIRT1 activator SRT2379 (100 mg/kg) (structure not disclosed) improved glucose tolerance, reduced hyperinsulinemia, and enhanced glucose uptake during a hyperinsulinemic−euglycemic clamp study.[27] The metabolic improvement was accompanied by a reduction in inflammatory cytokines (TNFα, IL-6) and chemokines (MCP-1) in adipose tissue and a reduction in the inflammatory state of macrophages in adipose tissue. In this same study, it was shown that knockdown of SIRT1 increased the activation of the JNK and IKK inflammatory pathways and increased LPS-induced TNFα release in primary macrophages and in macrophage cell lines. Activation of SIRT1 by **49** had the opposite effect to SIRT1 knockdown and inhibited LPS-stimulated inflammatory pathways and TNFα release. The SIRT1 dependence of the effect of **49** was demonstrated by the observation that **49** no longer inhibited LPS-induced effects on JNK, IKK, and TNFα release when SIRT1 was knocked down using siRNA.[27]

Further anti-inflammatory effects of SIRT1 activation with **49** have been demonstrated in adipocytes.[125] SIRT1 knockdown in adipocytes inhibited insulin-stimulated glucose uptake and increased activation of inflammatory pathways as evidenced by increased phosphorylation of JNK and insulin receptor substrate 1 (IRS-1). The opposite effect was seen with the SIRT1 activators **49** and SRT2530 (structure not disclosed).[125] In mice fed a high-fat diet, **49** (100 mg/kg) decreased the amount of acetylated NF-κB and reduced the

expression levels of the NF-κB target genes JNK, iNOS, and TNFα receptor-associated factor 2.

Consistent with the effects on liver seen by Feige et al.,[124] **49** has also been shown to ameliorate fatty liver in monosodium glutamate (MSG) mice, a model of nonalcoholic fatty liver disease (NAFLD).[126] Compound **49** (200 mg/kg) inhibited triglyceride accumulation in the livers of MSG mice and reduced the elevated levels of aminotransferase seen in control mice. This positive impact on NAFLD by **49** was accompanied by a decrease in the hepatic expression of lipogenic genes as well as genes involved in inflammation (inflammatory cytokines, macrophage infiltration) and oxidative stress. Serum levels of triglycerides, free fatty acids, and cholesterol were also reduced by **49**.

SIRT1 activation may also produce beneficial metabolic effects through changes in energy balance. Energy balance during fasting is controlled by a fasting-inducible switch consisting of the protein lysine acetyltransferase p300 and the deacetylase SIRT1 which controls energy balance through the regulation of TORC2 and FOXO1. Liver-specific knockout of SIRT1 or a SIRT1 inhibitor increases TORC2 activity and glucose output.[127] Overexpression of SIRT1 or administration of **49** to hepatocytes decreased TORC2 activity. Compound **49** (100 mg/kg) also decreased the amount of acetylated TORC2 in Zucker fa/fa rats.

The positive effect of **49** on mitochondrial biogenesis seen by Milne et al.[105] and Feige et al.[124] has also been seen in renal proximal tubule cells (RPTC).[128] The compound increased mitochondrial biogenesis through deacetylation of the SIRT1 target PGC1α, and this increase in mitochondrial biogenesis was able to prevent oxidant-induced injury of the RPTC. Compound **49** has also been shown to prevent renal fibrosis in the mouse model of unilateral ureteral obstruction (UUO).[129] Compound **49** (100 mg/kg) reduced apoptosis and fibrosis in the UUO model potentially through an increase in COX2 expression. The effect of **49** is in direct contrast to what is observed in mice heterozygous for SIRT1 which show increased apoptosis and fibrosis in the UUO model.[129]

SIRT1-Dependence of SIRT1 Modulators

The SIRT1 dependence of **49** has recently been called into question by Pacholec et al., who were unable to show activation of nonfluorometric SIRT1 substrates by **49** in enzymology studies and demonstrated a lack of selectivity against a panel of nonrelated molecular targets.[116] As discussed earlier, it may not be possible to replicate the cellular milieu of the SIRT1 enzyme in a test tube. Therefore, the SIRT1 dependence can only be studied in cell based systems using appropriate inhibitors and/or siRNA and ultimately in animals in which SIRT1 has been conditionally knocked out. Thus, there have been multiple studies with **49** that have consistently been able to demonstrate the SIRT1 dependence of this compound and other SIRT1 activators in cell-based assays,[124,128,27,125] thereby confirming the primary molecular target as SIRT1.

In the study by Feige et al. the ability of **49** to stimulate oxygen consumption in muscle cells was abolished in cells in which SIRT1 had been knocked down by shRNA.[124] In the study of the anti-inflammatory effects of **49** in macrophages,[27] the ability of **49** to inhibit LPS-induced effects on JNK and IKK phosphorylation and TNFα release was abolished in cells in which SIRT1 had been knocked down using siRNA targeting. In addition CD11c expression, which represents the activated proinflammatory phenotype of RAW cells, was inhibited by **49** in contrast to the effects seen following SIRT1 knockdown which had the opposite effect as well as eliminated the impact of **49**.[27] In the paper by Funk et al., the SIRT1 dependence of **49** was demonstrated using the putative SIRT1 inhibitors sirtinol (**8**) and nicotinamide.[128] Sirtinol (**8**) and nicotinamide are weak, relatively nonspecific SIRT1 inhibitors, so other biological processes may be at play, but the inhibitor data in conjunction with the above-mentioned knockdown data provide strong evidence of SIRT1-dependence for **49**. This SIRT1 dependence shown for **49**[124,27,128,125] suggests that the off-target binding activity reported by Pacholec et al.[116] does not influence the biological effects seen by the different investigators and highlights the importance of checking any pharmacological tool for on-target effects by using appropriate knockdown or knockout techniques.

Pacholec et al. were unable to show efficacy with **49** (30 and 100 mg/kg) in ob/ob mice aside from a reduction in insulin levels.[116] This is in contrast to numerous studies from multiple laboratories that have shown in vivo efficacy with **49** in multiple models of diabetes,[105,124,27] inflammation,[125] and fatty liver disease.[126] The lack of efficacy observed by Pacholec et al. may have been due to the toxicity that was observed in their studies. This toxicity is puzzling, as other laboratories[105,126] have seen no impact on body weights or food intake or other obvious toxicity. Indeed, in a study at the National Institute on Aging, **49** (30 and 100 mg/kg in chow for up to 2 years) has been shown to increase longevity and to improve metabolic parameters in mice fed a high fat diet.[130]

A systems biology approach has also been used to look at the signaling pathways that are activated by calorie restriction, resveratrol, and **49**.[43] Signaling pathways involved in mitochondrial biogenesis, metabolic pathways, and inflammatory pathways were all similarly affected by resveratrol, **49**, or calorie restriction. This provides strong evidence of a common molecular target involved with the three interventions, with SIRT1 being the most likely candidate based on the known biology associated with **49**, resveratrol, and calorie restriction.

Consistent with the systems biology data described above, the SIRT1 dependency of resveratrol has been shown in a number of different contexts. In yeast, resveratrol extends lifespan, but this effect is lost in Sir2 null mutant yeast.[31] Kaeberlein et al.[114] reported that resveratrol was unable to activate Sir2 or increase lifespan in yeast. This inability to see lifespan extension by resveratrol is in contrast to the original paper by Howitz et al.[31] and subsequent work in yeast with resveratrol[131,132] and resveratrol derivatives.[132] In addition, resveratrol has been shown to produce lifespan extension in multiple species including worms,[133,134] flies,[135,134] and mice on a high fat diet.[32] In mouse embryonic fibroblasts (MEFs) isolated from wild type mice, resveratrol increases expression of PGC1α and cytochrome *c*, an effect that is absent in MEFS from SIRT1 knockout mice.[33] In 3T3-L1 adipocytes, resveratrol inhibits adipogenesis, an effect that is lost in SIRT1 RNAi treated cells.[136] Further studies in adipocytes have shown that resveratrol's effects on glucose uptake, de novo lipogenesis, and cytokine expression are also SIRT1-dependent.[137] In an in vivo setting, Boily et al.[138] show that the antitumorigenic effect of resveratrol in a classical two-stage carcinogenesis model is significantly reduced in SIRT1 knockout mice.

SIRT1 and SIRT1 modulation continues to be an extremely vital area of research in both academic and pharmaceutical research settings. This is not surprising given the wealth of

biochemical pathways through which SIRT1 exerts its effects and the numerous disease states that can potentially be positively affected through direct or indirect SIRT1 modulation.[139] At present, a clear and direct causal connection cannot be demonstrated linking biochemical SIRT1 activation by small molecules and the in vivo effects produced by those molecules. This may not be surprising given the complexity of the mechanism of activation, though recent results clearly demonstrate that small molecules can activate SIRT1 to increase deacetylation of a variety of substrates.[140] History shows that the unambiguous assignment of a molecular target and/or mechanism to the direct effects of a small molecule modulator is rare, particulary in cases where multiple and overlapping biochemical pathways are involved. Nevertheless, mounting evidence of SIRT1-dependent modulation by small molecule activators resulting in multiple, distinct pharmacological effects (e.g., metabolic versus anti-inflammatory) suggests that alternative explanations for those effects are increasingly less likely.

Biographies

Charles A. Blum received his Ph.D. in Organic Chemistry in 1990 from Wesleyan University, CT, in the area of natural product total synthesis working with Professor Peter A. Jacobi. This was followed by a postdoctoral fellowship in the laboratory of Professor Harry H. Wassermann at Yale University, CT, working on new synthetic methodologies for the preparation of indoles. Subsequently, he joined Neurogen Corporation and worked on a number of GPCRs and ion channels targeting CNS-related disorders including GABAa modulators (anxiolytics/hypnotics/cognition enhancers), NPY1- and NPY5-receptor antagonists for eating disorders (in collaboration with Pfizer), and TRPV1 antagonists for the treatment of pain (in collaboration with Merck). He is currently Associate Director of Chemistry at Sirtris Pharmaceuticals, a GSK company.

James L. Ellis is Vice President of Preclinical Research at Sirtris Pharmaceuticals, a GSK company. He obtained his B.Sc. in Pharmacology from the University of Aberdeen, Scotland, and his Ph.D. in Neurobiology from the University of London and did postdoctoral work at the Johns Hopkins Asthma and Allergy Center, MD, before moving onto the faculty there. Prior to joining Sirtris, he held a number of positions in biotechnology companies in the Boston area including UCB Pharma, NitroMed, and Surface Logix. In his current role he is responsible for directing and managing the Pharmacology, Target Research, and Preclinical Safety/Toxicology groups as well as overseeing many of the company's collaborations both internal and external to GSK.

Christine Loh earned her Ph.D. in Immunology in Professor Anjana Rao's laboratory at Harvard Medical School, MA. She completed a postdoctoral fellowship at Massachusetts Institute of Technology, MA, under Professor Jun Liu, working on macrophage and T cell signaling pathways, then joined ICOS Corporation in Bothell, WA, investigating small molecule modulation of adaptor protein−enzyme interactions. She subsequently moved to Pfizer and worked on and led several drug discovery programs targeting GPCRs and enzymes, including 11β-HSD and several kinases, for diabetes, oncology, and inflammation. She is now Associate Director of Target Research at Sirtris, a GSK company.

Pui Yee Ng received her A.B. in Chemistry from Harvard University, MA, and a Ph.D. in Organic Chemistry from Columbia University, NY, in 2003. Her graduate work was in the development of asymmetric methodologies, including silane reagents for asymmetric allylation reactions, under the direction of Professor James L. Leighton. Her postdoctoral work at the Broad Institute with Dr. Jared T. Shaw included DOS (diversity-oriented synthesis) library design and natural products synthesis. She joined Sirtris Pharmaceuticals in 2006 and is currently Senior Scientist in the Medicinal Chemistry group.

Robert B. Perni did his graduate studies at Dartmouth College, NH, under Professor Gordon W. Gribble in the area of indole alkaloid synthesis and was awarded a Ph.D. in 1984. Following a postdoctoral stint at the University of Rochester, NY, with Professor Robert K. Boeckman, Jr., working on the total synthesis of macrocyclic natural products, Dr. Perni joined Sterling Winthrop where he worked in the areas of anti-infectives and oncology. He then joined Avid Therapeutics in 1995 working on antihepatitis B agents before joining Vertex Pharmaceuticals in 1998 where he was head of the hepatitis C protease inhibition research program. He joined Sirtris Pharmaceuticals in 2007 where he is currently Vice President, Chemistry.

Ross L. Stein received his Ph.D. in Physical Organic Chemistry from Indiana University, IN, in 1978, after which he conducted postdoctoral research in mechanistic enzymology at the University of Kansas, KS. His career has included positions at Merck, where he headed the Department of Enzymology, and ProScript (acquired by Millennium in 1999) as Vice President of Biochemistry. At ProScript, Dr. Stein was instrumental in the preclinical development and co-inventor of the drug VELCADE, a proteasome inhibitor used to treat multiple myeloma. In 2001, Dr. Stein accepted a position at Harvard Medical School, MA, to establish and head the Laboratory for Drug Discovery in Neurodegeneration. In 2009, Dr. Stein joined Sirtris Pharmaceuticals as Vice President of Discovery Research.

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