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Novel Lysine-Based Thioureas as Mechanism-Based Inhibitors of Sirtuin 2 (SIRT2) with Anticancer Activity in a Colorectal Cancer Murine Model

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Abstract

Sirtuin 2 (SIRT2) is a protein lysine deacetylase that has been indicated as a therapeutic target for cancer. To further establish the role of SIRT2 in cancers, it is necessary to develop selective and potent inhibitors. Here, we report the facile synthesis of novel lysine derived thioureas as mechanism-based SIRT2 inhibitors with anticancer activity. Compounds **AF8**, **AF10**, and **AF12** selectively inhibited SIRT2 with IC₅₀ values of 0.06, 0.15, and 0.08 μ M, respectively. Compounds **AF8** and **AF10** demonstrated broad cytotoxicity amongst cancer cell lines, but minimal toxicity in noncancerous cells. **AF8** and **AF10** inhibited the anchorage-independent growth of human colorectal cancer cell line HCT116 with GI₅₀ values of $\sim$ 7 μ M. Furthermore, **AF8** potently inhibited tumor growth in a HCT116 xenograft murine model, supporting that SIRT2 is a viable therapeutic target for colorectal cancer.

Graphical Abstract

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Author Contributions

A.S.F., J.Y.H., and J.C. contributed equally to this work. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Supporting Information. The following files are available free of charge.

In vitro inhibition assays of SIRT1–3 with compounds **AF8** – **AF12**, cell cytotoxicity assay of various cell lines comparing compounds **AF8**, **AF10**, and **TM**, and ¹H and ¹³C NMR spectra of all synthesized intermediates and final compounds. (PDF)

Molecular Formula Strings (CSV)

qNMR of synthesized compounds (PDF)

INTRODUCTION

Sirtuins are a class of nicotinamide adenine dinucleotide (NAD⁺) dependent enzymes with lysine deacetylase activities.^{1–2} They are also referred to as class III histone deacetylases, but can catalyze the deacylation of various non-histone substrates.³ In mammals, there are seven sirtuins (SIRT1–7) that are involved in the regulation of cellular metabolism, transcription, and differentiation.^{4–5} Specifically, SIRT1, SIRT2, and SIRT5 have been implicated in promoting cancer cell proliferation and tumorigenesis.^{6–8} While SIRT1 and SIRT2 have also been contradictorily shown to be tumor suppressors^{7, 9}, the therapeutic potential of targeting SIRT2 has been demonstrated by isoform specific inhibition in breast cancer cell lines.¹⁰ Recently, SIRT2 was shown to promote tumor growth by regulating oncogenes *c-Myc* and *KRas*.^{6, 11} SIRT2 also promotes basal like breast cancer phenotype and tumor growth through the deacetylation and stabilization of transcription factor Slug.¹² As such, SIRT2 regulates multiple oncogenic pathways and is a promising therapeutic target for cancer. However, there is a need for more studies in animal models of cancer to convincingly demonstrate the therapeutic potential of SIRT2 inhibition.

Several SIRT2-selective inhibitors, such as AGK2, Tenovin-6, **TM**, and SirReal2, have been reported.^{10, 13–15} Among these, the thiomristoyl lysine compound **TM** is a SIRT2 mechanism-based inhibitor that displayed cancer cell-selective toxicity and is active in many human cancer cell lines.^{10, 16} The sirtuin deacylation mechanism involves nucleophilic attack on the NAD⁺ anomeric position by the carbonyl of the acyl group to release nicotinamide (Scheme 1).¹⁷ **TM** contains a thiomristoyl lysine moiety, which can attack the anomeric position of NAD⁺ in the presence of SIRT2, forming a stable covalent intermediate to potentially inhibit SIRT2.¹⁰

While **TM** has promising anticancer activity, it is poorly soluble in water and the synthesis of the thioamide bond is difficult and results in poor yield. We therefore sought to develop similar mechanism-based inhibitors that are less hydrophobic and easier to synthesize. In a previous study by Hirsch, et. al, it was shown that a peptidomimetic thiourea containing compound could mechanistically inhibit SIRT1 and lead to the general inhibition of SIRT1–3.¹⁸ Modifying **TM** with a thiourea moiety could similarly result in improved mechanism based inhibition and facilitate a simplified synthesis route. Here, we present the development of novel lysine-based thioureas as potent and selective SIRT2 inhibitors with anticancer activity in various cancer cell lines and a colorectal cancer xenograft murine model.

RESULTS AND DISCUSSION

Design and synthesis of new SIRT2 inhibitors.

Two different classes of thiourea inhibitors with varying alkyl chain lengths were synthesized through a divergent synthesis route (Scheme 2). The **C** class of inhibitors **C1–12** contained a 3' hydroxyl group on the C-terminal while the **AF** class of inhibitors **AF6–12** did not. Modeling the inhibitors into the known SIRT2 structure suggested the 3' hydroxyl group of the **C** inhibitors could facilitate additional hydrogen bonding interactions with the backbone of residues Val266 or Gln267 (Fig. 1).¹⁹ The **C** inhibitors could therefore have improved potency and solubility as compared to the **AF** inhibitors. SIRT2 contains a

hydrophobic pocket for acyl group binding^{15, 20}, and this pocket accommodates the thiomristoyl group present in **TM**. Towards the goal of reducing the hydrophobicity of the inhibitors, we decided to synthesize thiourea compounds with various alkyl chain lengths.

Commercially available Cbz-L-lysine(Boc)-OH was converted to the corresponding isothiocyanate intermediates **2a** and **2b** in two steps with 54% and 83% yield respectively. The isothiocyanate compounds were then coupled with varying alkyl chain primary amines to form the resultant thiourea compounds, with up to 94% yield. In total, the six **C1-C12** inhibitors varied in chain length from one to twelve carbons and the five **AF6-AF12** inhibitors varied from six to twelve carbons.

AF8, AF10, and AF12 are potent and selective SIRT2 inhibitors *in vitro*.

We then measured the ability of these compounds to inhibit SIRT1, SIRT2, and SIRT3. Because SIRT1, SIRT2, and SIRT3 are similar structurally and comprise the class I sirtuins²¹, it was important to test for selectivity among these three isoforms. A liquid chromatography based assay with acetyl H3K9 peptide² and purified sirtuins was used to test the compounds for SIRT1–3 inhibition. The previously reported **TM** compound was also assayed and used as a reference compound. Nicotinamide, a non-specific sirtuin inhibitor, was used as a positive control for SIRT3 inhibition and the SIRT1 selective inhibitor EX527 used as a positive control for SIRT1 inhibition.^{22–24} Nicotinamide weakly and non-specifically inhibited SIRT3 (IC₅₀ 76 ± 30 µM) and SIRT2 (IC₅₀ 70 ± 5 µM) while EX527 more potently inhibited SIRT1 (IC₅₀ 0.085 ± 0.01 µM) over SIRT2 (IC₅₀ 1.80 ± 0.03 µM), consistent with reported literature values.^{22, 25}

As shown in Table 1, none of the synthesized inhibitors demonstrated significant inhibition of SIRT3 with IC₅₀ values of 50 µM or greater. Except for inhibitors **C1** and **C3**, all inhibitors had micromolar (**C5**) or submicromolar IC₅₀ values for SIRT2. The lack of SIRT2 deacetylase inhibition for compounds **C1** and **C3** is likely due to the shorter side chains of one and three carbons, respectively, which led to decreased interactions in the sirtuin hydrophobic pocket. A trend of increased chain length and increased SIRT2 inhibition was observed, suggesting that a longer alkyl chain length facilitates increased binding in the hydrophobic pocket.

Compounds **C8-C12** were potent for SIRT2 with IC₅₀ values of 0.12 µM or lower, but also inhibited SIRT1 with IC₅₀ values of 1.0, 0.45, and 1.0 µM for **C8**, **C10**, and **C12**, respectively. The decreased selectivity is likely because the additional hydrogen bonding capability of **C8-C12** increases the SIRT1 inhibition more than the SIRT2 inhibition. The resultant 8 to 17-fold selectivity for SIRT2 over SIRT1 was not adequate to ensure SIRT2-specific inhibition for biological testing. As a result, the non-selective inhibitors **C8-C12** were not used in further biological activity assays.

AF6, AF7, AF8, AF10, and AF12 inhibited SIRT2 deacetylase activity with IC₅₀ values of 0.64, 0.16, 0.061, 0.15, and 0.081 µM respectively (Table 1). **AF8** slightly inhibited SIRT1 deacetylase activity (IC₅₀ 11 ± 5 µM) while **AF10, AF12**, and **TM** showed no inhibition. The inhibition of SIRT3 was weakest and only **AF8** showed slight inhibition of SIRT3. The selectivity for SIRT2 over SIRT1 correlated with the alkyl chain length of the inhibitors, as

demonstrated by the 47, 137, 180, >1000, and >1000-fold selectivity of **AF6**, **AF7**, **AF8**, **AF10**, and **AF12** respectively. This trend suggests that longer alkyl chains can exploit isoform specific binding for selective SIRT2 inhibition, despite the fact that SIRT1–3 have similar hydrophobic acyl pockets.²⁶ **AF8** inhibition of SIRT2 (IC_{50} 0.061 ± 0.03 μ M) was the most potent amongst the thiourea inhibitors and comparable to **TM** (IC_{50} 0.034 ± 0.01 μ M).

AF8 was confirmed to work through mechanism-based inhibition of SIRT2. **AF8** was incubated with an excess of SIRT2 and NAD^+ and the reaction mixture analyzed by LC-MS. As shown in Fig. 2, the covalent **AF8**-ADP-ribose intermediate formed by the release of nicotinamide was detected. When **AF8** was removed from the reaction mixture, the intermediate mass was not detected. This result confirmed that **AF8**, and likely the other thiourea **AF** inhibitors, could covalently react with NAD^+ . This is consistent with the mass spectrometry detection of the stalled S-alkylamidate intermediate of a previously reported, non-specific lysine-based thiourea sirtuin inhibitor upon incubation with SIRT1.¹⁸

AF8 and AF10 demonstrated cytotoxicity against human colon, lung, breast, and pancreatic cancer cell lines.

The thiourea modification and shorter alkyl chains of compounds **AF10** and **AF8** were predicted to improve solubility as **TM** had a calculated cLogP value of 8.8 while the thiourea analogues **AF10** and **AF8** had calculated cLogP values of 7.04 and 6.13 respectively (Table 1). Thus, the lower cLogP values suggest that **AF8** and **AF10** may have improved solubility while still maintaining reasonable selectivity and potency for SIRT2. Because of this, **AF8** and **AF10** were tested for possible anticancer activity amongst various cell lines.

As shown in Table. 2, toxicity was observed in all cancer lines upon treatment with **AF8**, **AF10**, and **TM**. None of the inhibitors were toxic to noncancerous HME1 epithelial cells. In a normal colon cell line, CCD 841 CoN, the GI_{50} values of **AF8** and **AF10** were also higher in general than those in the cancer cell lines tested. These observations suggested that, while there is variation, cancer cells could rely more on SIRT2-driven pathways for survival and growth. **AF10** was the most potent inhibitor and was approximately two-fold more potent than **TM** in MCF7, MDA-MB-231, A549, SW948, and HCT116 cells (Table 2). **AF8** was comparable to **TM** in most cell lines except the pancreatic BxPC-3 cell line which showed little inhibition by **AF8**. Both **AF8** and **AF10** were twice as potent in the colorectal HCT116 cell line as compared to **TM** (Table 2). **AF8** demonstrated minimal inhibition of noncancerous colon cell line CCD 841 CoN, suggesting that **AF8** could be used to selectively target cancerous colon cells. Consistent with its increased potency, **AF10** showed higher toxicity in CCD 841 CoN cells, which indicated it may have a lower therapeutic index (Table 2).

AF8, AF10, and TM inhibited the anchorage independent growth of HCT116 cells.

The ability of cancer cells to survive and proliferate without adhesion to an extracellular matrix is one of the hallmarks of oncogenic transformation.^{27–28} To examine anchorage independent growth, HCT116 cells were suspended in a soft agar matrix and incubated until

colony growth was observed.²⁹ Slightly less overall growth was observed in the **TM** treated group, likely due to a smaller number of cells seeded per well. Treatment with **AF8** and **AF10** potently inhibited colony formation with GI₅₀ values of 7.4 and 7.0 μ M respectively while **TM** had a weaker effect with a GI₅₀ value of 16.7 μ M (Fig. 3). The potency of the compounds in the soft agar colony formation assay shows that they can inhibit anchorage independent growth better than normal 2D growth (cytotoxicity assay described in the previous section), suggesting that SIRT2 inhibitors preferentially target the transformed phenotype of cancer cells. This is also consistent with the fact that the inhibitors exhibit less toxicity towards noncancerous cells in the 2D growth assay shown in Table 2.

AF8 and AF10 inhibit SIRT2 but not SIRT1 in cells.

We next sought to confirm that **AF8** and **AF10** could selectively inhibit SIRT2 deacetylase activity in cells. Compounds **C3**, **C5**, and **C10** were also tested to determine if our *in vitro* sirtuin inhibition assay correlated with SIRT2 inhibition in cells. We monitored α -tubulin acetylation as a readout for SIRT2 inhibition as α -tubulin is a well-known SIRT2 deacetylation substrate.³⁰ HCT116 cells were incubated with DMSO alone (control) or varying concentrations of inhibitor for six hours before α -tubulin acetylation was detected by immunofluorescence. As shown in Fig. 4A and Fig. 4C, acetyl α -tubulin levels of HCT116 cells increased upon treatment with **TM** at 25 and 50 μ M, suggesting the inhibition of SIRT2 activity. **AF8** and **AF10** treatment at 25 and 50 μ M also resulted in extensive α -tubulin hyperacetylation as compared to DMSO. Thus, **AF8** and **AF10** can also inhibit SIRT2 activity in cells.

SIRT2 inhibition in cells as measured by α -tubulin acetylation was consistent with calculated *in vitro* SIRT2 inhibition results. As shown in Fig. 4B and Fig. 4D, treatment with 25 or 50 μ M of **C3** did not increase the acetylation of α -tubulin, corroborating the lack of *in vitro* SIRT2 inhibition (IC₅₀ >100 μ M). **C5** slightly increased α -tubulin acetylation at 25 μ M and more robustly increased acetylation at 50 μ M, consistent with moderate *in vitro* SIRT2 inhibition (IC₅₀ 1.0 μ M). **C10** increased α -tubulin acetylation at both 25 and 50 μ M, consistent with more potent *in vitro* SIRT2 inhibition (IC₅₀ 0.036 μ M) (Fig. 4B and Fig. 4D). These observations helped to further validate the results from the *in vitro* sirtuin inhibition assay.

We next tested whether **AF8** and **AF10** inhibit SIRT1 deacetylase activity in HCT116 cells by monitoring the acetylation of p53, a well-known SIRT1 deacetylation substrate.³¹ We also tested compounds **C3**, **C5**, and **C10** to determine if our *in vitro* SIRT1 inhibition results were consistent with SIRT1 inhibition in cells. HCT116 cells were treated with Trichostatin A, an inhibitor of the zinc-dependent HDACs, and with or without 25 μ M of our sirtuin inhibitors. The SIRT1-selective inhibitor EX527 increased p53 acetylation, consistent with previous reports (Fig. 5).^{25, 32} **TM**, **AF8**, and **AF10** did not increase the levels of acetyl p53, indicating that they inhibit SIRT2 but not SIRT1 in cells. Compound **C3** also did not increase the acetylation of p53, consistent with the lack of *in vitro* SIRT1 inhibition (IC₅₀ >100 μ M). Inhibitors **C5** and **C10** moderately increased the acetylation of p53, consistent with the lack of significant SIRT2 selectivity for the compounds (34 and 12-fold respective SIRT2/SIRT1 selectivity). To more closely examine the inhibition of SIRT1 in cells,

HCT116 cells were co-treated with Trichostatin A and 10–50 μ M of **AF8**, **AF10**, and **C10**. Compound **C10** demonstrated a dose dependent increase in acetyl p53 levels, consistent with *in vitro* SIRT1 inhibition (IC_{50} 0.45 μ M) and lack of significant SIRT2/SIRT1 selectivity (Fig. 5). **AF8** did not increase acetyl p53 levels in a dose dependent manner, indicating a lack of SIRT1 inhibition. A very slight increase in acetyl p53 was observed with **AF10**, but there was no correlation between **AF10** concentration and acetyl p53 level, indicating a lack of meaningful SIRT1 inhibition (Fig. 5). This result suggests that the *in vitro* selectivity of **AF8** and **AF10** (203 and >1000-fold selectivity respectively), is sufficient to ensure SIRT2 specific inhibition in cells.

AF8 and AF10 significantly reduced tumor growth in a HCT116 xenograft mice model of colorectal cancer.

Immunodeficient NOD scid gamma mice (NSG mice) were grafted with HCT116 cells to form tumors and then treated with varying doses of **AF8**, **AF10**, or vehicle by intraperitoneal injection over the course of 11 days before the mice were sacrificed and the tumors weighed. As shown in Fig. 6A, **AF8** at 25 mg/kg slightly decreased tumor growth by volume and weight but did not result in a statistically significant difference. **AF8** at 100 mg/kg significantly ($p < 0.0001$) inhibited tumor growth by 80% as measured by the average tumor volume on the last day of treatment. This was consistent with the significant ($p = 0.0001$) difference in average tumor weight that demonstrated an inhibition of 78%. However, **AF10** was less potent in this xenograft model. At 100 mg/kg, **AF10** inhibited tumor growth significantly ($p = 0.0114$), but only by 37% as measured by final tumor volume (Fig. 6B). This was similar to the significant ($p = 0.0152$) tumor inhibition of 49% as measured by final tumor weight. No significant weight loss was observed in either the **AF8** or **AF10** treatment groups.

HCT116 xenograft tumor data suggest that thiourea SIRT2 inhibitors presents a possible new therapeutic approach for treating colorectal cancers. Targeted therapies for colorectal cancer are generally more limited to antibodies targeting angiogenesis through VEGF/VEGFR and growth through EGFR.^{33–34} Such therapies have met moderate success due to the high mutation load found in colorectal cancer, and are frequently less effective against colorectal cancers with constitutively active KRas mutations.^{35–37} The potent inhibition of HCT116 (KRAS^{G13D}) xenograft mice tumor growth by **AF8** suggests that targeting SIRT2 activity can be a viable therapeutic strategy in colorectal cancers containing a KRAS mutation. Given the broad toxicity of **AF8** and **AF10** in other cancer cell lines, it is also possible that SIRT2 inhibitors can be useful for treating other type of cancers.

CONCLUSION

We have developed thiourea mechanism-based inhibitors for SIRT2. Among them, **AF8**-**AF12** are highly selective and potent at inhibiting SIRT2 *in vitro*. Compared with the previous thioamide type inhibitor **TM**, these thiourea compounds are much easier to synthesize, which allowed us to quickly prepare many compounds. In addition, **AF8** is less hydrophobic than **TM** and thus is more desirable as a therapeutic candidate.

AF8 and **AF10** showed cytotoxicity across a variety of breast, lung, pancreatic, and colorectal cancer cell lines, but showed generally less toxicity in normal cells which suggested that cancer cells could have an increased dependence on SIRT2 activity. **AF8** and **AF10** also inhibited SIRT2, but not SIRT1 in cells. **AF8** at 100 mg/kg inhibited tumor growth by 80% in a HCT116 xenograft mouse model. Although in cell culture **AF10** is more potent than **AF8**, in the mouse xenograft model **AF8** is better than **AF10**. This is likely because other properties, such as solubility (**AF8** predicted cLogS is -6.5 , **AF10** predicted cLogS is -7.0), are more important for *in vivo* efficacy, which should be useful for the future development of new SIRT2 inhibitors as anticancer therapeutic candidates. This represents one of the few studies examining the anticancer effect of SIRT2 inhibition in a mouse xenograft model of tumors. The results further support that SIRT2 inhibition is a promising cancer treatment strategy and that developing new and improved SIRT2 inhibitors is warranted, a task that will be facilitated by the facile synthesis procedure of the thiourea type of SIRT2 inhibitors.

EXPERIMENTAL

General.

All reagents were purchased from commercial sources and used without further purification. Reactions were checked for completion by LC-MS and compounds purified through flash chromatography (SiliaFlash Silica Gel, P60). The Cornell University NMR Facility was used to obtain NMR spectra. ^1H spectra (500 MHz, Chloroform-*d*) and ^{13}C NMR spectra (126 MHz, Chloroform-*d*) were obtained on a Bruker 500 MHz with cryoprobe. High Resolution Mass Spectrometry was obtained using either an ESI-Orbitrap mass spectrometer or DART-Orbitrap mass spectrometer. The purity of synthesized compounds was confirmed to be 95% purity by qNMR.

LC-MS System.

Analysis of reaction mixtures and purified compounds was carried out using a Shimadzu HPLC LC20-AD system connected to a Thermo Scientific LCQ Fleet Ion Trap mass spectrometer, Rugged Ion Max Source (electrospray ionization), reverse phase Phenomenex Kinetex EVO C18 column (30×2.1 mm, $5 \mu\text{M}$). Samples were diluted in water/acetonitrile (1:1) and analyzed using water with 0.1% acetic acid (Solvent A) and acetonitrile with 0.1% acetic acid (Solvent B) at a constant flow rate of 0.2 mL/min, recording UV absorbance at 215 and 260 nm and positive mode detection of ions.

General Procedure for the synthesis of **1a-b** as shown through the preparation of Benzyl tert-butyl (6-((3-hydroxyphenyl)amino)-6-oxohexane-1,5-diyl)(S)-dicarbamate (**1a**).

Cbz-L-Lys(Boc)-OH (3 g, 7.89 mmol, 1 eq) was dissolved in dichloromethane (48 mL). To this solution, N-methylmorpholine (1.39 mL, 12.6 mmol, 1.6 eq) and isobutyl chloroformate (1.43 mL, 11.0 mmol, 1.4 eq) were sequentially added and the reaction mixture stirred for 1 hr at RT. Then, 3-aminophenol (1.022 g, 9.45 mmol, 1.2 eq) (or aniline for **1b**) was added and the reaction mixture stirred for 16 hr at RT. The reaction was checked for completion by TLC (hexanes: ethyl acetate 1:1) and LC-MS. The reaction mixture was further diluted in dichloromethane (75 mL) and sequentially washed with water (3×75 mL) and saturated

brine (1× 75 mL). The organic layer was collected, dried over Na₂SO₄, and concentrated by rotary evaporation to yield a viscous, yellow oil. The crude product was purified by silica gel column chromatography (hexanes: ethyl acetate 1:1) to yield **1a** as a pale yellow solid in 80% yield (2.98 g). ¹H NMR (500 MHz, Chloroform-*d*) δ 8.74 (d, *J* = 31.9 Hz, 1H), 7.45 (s, 1H), 7.41 – 7.30 (m, 5H), 7.20 – 7.08 (m, 1H), 6.87 (s, 1H), 6.64 (dd, *J* = 8.1, 2.3 Hz, 1H), 5.93 (d, *J* = 23.4 Hz, 1H), 5.18 – 5.02 (m, 2H), 4.75 (s, 1H), 4.37 (d, *J* = 8.4 Hz, 1H), 3.08 (d, *J* = 7.0 Hz, 2H), 1.90 (d, *J* = 13.6 Hz, 1H), 1.82 – 1.64 (m, 1H), 1.56 – 1.32 (m, 13H). ¹³C NMR (126 MHz, Chloroform-*d*) δ 170.9, 157.0, 156.9, 156.6, 138.5, 135.9, 129.9, 128.6, 128.3, 128.2, 112.0, 111.6, 107.4, 79.7, 67.4, 55.9, 39.7, 31.6, 29.5, 28.4, 22.5. HRMS [DART-Orbitrap] *m/z* calculated for C₂₄H₃₄N₃O₄ ([M-CO₂ + H]⁺) 428.2544, observed 428.2567.

Benzyl tert-butyl (6-oxo-6-(phenylamino)hexane-1,5-diyl)(S)-dicarbamate (1b).

White, powdery solid. 94% yield. ¹H NMR (500 MHz, Chloroform-*d*) δ 8.46 (d, *J* = 47.9 Hz, 1H), 7.53 (d, *J* = 7.9 Hz, 2H), 7.44 – 7.29 (m, 7H), 7.17 – 7.08 (m, 1H), 5.71 (d, *J* = 28.3 Hz, 1H), 5.13 (d, *J* = 3.2 Hz, 2H), 4.69 (s, 1H), 4.33 (s, 1H), 3.11 (s, 2H), 2.07 – 1.88 (m, 1H), 1.75 (dtd, *J* = 14.1, 8.6, 5.4 Hz, 1H), 1.44 (s, 14H). ¹³C NMR (126 MHz, Chloroform-*d*) δ 170.2, 156.7, 156.4, 137.7, 136.1, 128.9 (d, *J* = 1.2 Hz), 128.6, 128.3 (d, *J* = 1.4 Hz), 128.1 (d, *J* = 2.7 Hz), 124.4, 120.0 (d, *J* = 2.4 Hz), 79.4, 67.3, 55.6, 39.5, 31.6, 29.5, 28.4, 22.5, 19.1. HRMS [DART-Orbitrap] *m/z* calculated for C₂₄H₃₄N₃O₃ ([M-CO₂ + H]⁺) 412.2595, observed 412.2618.

General Procedure for 2a-b synthesis as shown through the preparation of benzyl (S)-(1-((3-hydroxyphenyl)amino)-6-isothiocyanato-1-oxohexan-2-yl)carbamate (2a).

Compound **1a** (1g, 2.12 mmol, 1 eq) was dissolved in dichloromethane (14 mL) and trifluoroacetic acid (7 mL). Reaction was stirred for 1 hr at RT before being concentrated by rotary evaporation to remove the trifluoroacetic acid. The crude mixture was dissolved in tetrahydrofuran (21 mL) before 1,1'-thiocarbonyl diimidazole (454 mg, 2.54 mmol, 1.2 eq) and triethylamine (0.73 mL, 5.3 mmol, 2.5 eq) were sequentially added. The reaction was stirred for 16 hr at RT and then further diluted in ethyl acetate (100 mL) and washed with water (3× 60 mL) and saturated brine (1× 60 mL). The organic layer was collected, dried over Na₂SO₄, and concentrated by rotary evaporation. The crude product was purified by flash chromatography (hexanes: ethyl acetate 2:1) to afford **2a** as a white solid in 68% yield (594 mg). ¹H NMR (500 MHz, Chloroform-*d*) δ 8.55 (s, 1H), 7.46 (s, 1H), 7.35 (d, *J* = 3.0 Hz, 5H), 7.14 (t, *J* = 8.1 Hz, 1H), 6.79 (d, *J* = 7.9 Hz, 1H), 6.64 (dd, *J* = 8.2, 2.3 Hz, 1H), 5.65 (d, *J* = 7.2 Hz, 1H), 5.15 (q, *J* = 12.2 Hz, 2H), 4.37 (d, *J* = 8.2 Hz, 1H), 3.56 – 3.35 (m, *J* = 6.9, 6.4 Hz, 2H), 2.01 – 1.84 (m, 1H), 1.70 (tdd, *J* = 22.8, 11.4, 6.1 Hz, 2H), 1.61 – 1.35 (m, 2H). ¹³C NMR (126 MHz, Chloroform-*d*) δ 170.5, 156.8, 138.2, 135.7, 130.2, 130.0, 128.6, 128.4, 128.1, 112.2, 111.6, 107.4, 67.7, 55.7, 44.7, 31.4, 29.4, 22.8. HRMS [DART-Orbitrap] *m/z* calculated for C₂₁H₂₄N₃O₄S ([M + H]⁺) 414.1482, observed 414.1507.

Benzyl (S)-(6-isothiocyanato-1-oxo-1-(phenylamino)hexan-2-yl)carbamate (2b).

White solid, 86% yield. ¹H NMR (500 MHz, Chloroform-*d*) δ 8.37 (s, 1H), 7.49 (d, *J* = 7.9 Hz, 2H), 7.42 – 7.25 (m, 7H), 7.13 (t, *J* = 7.4 Hz, 1H), 5.60 (d, *J* = 8.8 Hz, 1H), 5.14 (q, *J* =

12.2 Hz, 2H), 4.38 (q, $J = 7.9, 7.3$ Hz, 1H), 3.62 – 3.40 (m, $J = 7.0, 6.2$ Hz, 2H), 1.98 (ddt, $J = 13.7, 10.1, 6.1$ Hz, 1H), 1.87 – 1.63 (m, 4H), 1.55 (tdd, $J = 16.2, 11.6, 7.0$ Hz, 2H). ^{13}C NMR (126 MHz, Chloroform- d) δ 169.8, 156.7, 137.4, 135.9, 130.4, 129.0, 128.6, 128.4, 128.1, 124.7, 120.1, 67.5, 55.4, 44.8, 31.5, 29.5, 22.7. HRMS [DART-Orbitrap] m/z calculated for $\text{C}_{21}\text{H}_{24}\text{N}_3\text{O}_3\text{S}$ ($[\text{M} + \text{H}]^+$) 398.1533, observed 398.1556.

Benzyl (S)-(6-(3-hexylthioureido)-1-oxo-1-(phenylamino)hexan-2-yl)carbamate (AF6).

White solid, 80% yield. ^1H NMR (500 MHz, Chloroform- d) δ 8.56 (s, 1H), 7.54 (d, $J = 7.9$ Hz, 2H), 7.41 – 7.23 (m, 7H), 7.15 – 7.08 (m, 1H), 6.09 (s, 2H), 5.76 (d, $J = 7.8$ Hz, 1H), 5.20 – 5.03 (m, 2H), 4.39 (t, $J = 7.5$ Hz, 1H), 3.42 (d, $J = 68.8$ Hz, 4H), 1.94 (dt, $J = 14.5, 7.6$ Hz, 1H), 1.83 – 1.71 (m, 1H), 1.64 (d, $J = 7.6$ Hz, 2H), 1.50 (dq, $J = 38.3, 6.9$ Hz, 4H), 1.37 – 1.14 (m, 7H), 0.98 – 0.72 (m, 3H). ^{13}C NMR (126 MHz, Chloroform- d) δ 181.3, 170.2, 156.7, 137.5, 136.0, 129.0, 128.6, 128.3, 128.0, 124.7, 120.1, 67.3, 55.3, 43.8, 31.8, 31.4, 29.7, 28.9, 28.2, 26.6, 22.5 (d, $J = 3.3$ Hz), 14.0. HRMS [DART-Orbitrap] m/z calculated for $\text{C}_{27}\text{H}_{39}\text{N}_4\text{O}_3\text{S}$ ($[\text{M} + \text{H}]^+$) 499.2737, observed 499.2766.

Benzyl (S)-(6-(3-heptylthioureido)-1-oxo-1-(phenylamino)hexan-2-yl)carbamate (AF7).

White solid, 85% yield. ^1H NMR (500 MHz, Chloroform- d) δ 8.51 (s, 1H), 7.54 (d, $J = 8.0$ Hz, 2H), 7.41 – 7.27 (m, 7H), 7.17 – 7.08 (m, 1H), 6.08 (s, 2H), 5.71 (d, $J = 8.0$ Hz, 1H), 5.13 (d, $J = 5.2$ Hz, 2H), 4.39 (d, $J = 8.0$ Hz, 1H), 3.43 (d, $J = 71.3$ Hz, 3H), 1.96 (dt, $J = 14.1, 7.5$ Hz, 1H), 1.76 (dtd, $J = 13.9, 8.6, 5.8$ Hz, 1H), 1.72 – 1.60 (m, 2H), 1.60 – 1.37 (m, 4H), 1.37 – 1.12 (m, 9H), 0.94 – 0.82 (m, 3H). ^{13}C NMR (126 MHz, Chloroform- d) δ 181.1, 170.2, 156.7, 137.5, 136.0, 129.0, 128.6, 128.3, 128.0, 124.6, 120.1, 67.3, 55.2, 43.8, 31.8, 31.7, 28.9, 28.3 – 28.0 (m), 26.9, 22.6, 22.5, 14.1 (d, $J = 3.0$ Hz). HRMS [DART-Orbitrap] m/z calculated for $\text{C}_{28}\text{H}_{41}\text{N}_4\text{O}_3\text{S}$ ($[\text{M} + \text{H}]^+$) 513.2894, observed 513.2930.

General Procedure for C1-C12 and AF6-AF12 synthesis as shown through the preparation of benzyl (S)-(6-(3-octylthioureido)-1-oxo-1-(phenylamino)hexan-2-yl)carbamate (AF8).

Compound **2b** (250 mg, 0.63 mmol, 1 eq) (or **2a** for **C1-C12**) was dissolved in dimethylformamide (4.2 mL). To this mixture, octylamine (0.26 mL, 1.57 mmol, 2.5 eq) and triethylamine (0.17 mL, 1.25 mmol, 2 eq) were sequentially added and the reaction stirred for 16 hr at RT. The reaction mixture was diluted in dichloromethane (50 mL) and washed with water (3 $\times$ 25 mL) and saturated brine (1 $\times$ 25 mL) before being dried over Na_2SO_4 and concentrated by rotary evaporation. The crude mixture was purified by flash chromatography (hexanes: ethyl acetate 2:1) to afford **AF8** as a white, crystalline solid in 74% yield (247 mg). ^1H NMR (500 MHz, Chloroform- d) δ 8.51 (s, 1H), 7.54 (d, $J = 8.0$ Hz, 2H), 7.43 – 7.24 (m, 7H), 7.17 – 7.08 (m, 1H), 6.08 (s, 2H), 5.72 (d, $J = 8.1$ Hz, 1H), 5.22 – 5.02 (m, 2H), 4.39 (d, $J = 8.5$ Hz, 1H), 3.35 (s, 2H), 2.03 – 1.85 (m, $J = 6.8$ Hz, 1H), 1.76 (dtd, $J = 13.9, 8.6, 5.7$ Hz, 1H), 1.66 (h, $J = 6.5$ Hz, 2H), 1.51 (dq, $J = 36.6, 7.0$ Hz, 4H), 1.37 – 1.12 (m, 11H), 0.90 (t, $J = 7.0$ Hz, 3H). ^{13}C NMR (126 MHz, Chloroform- d) δ 181.0, 170.2, 156.7, 137.5, 136.0, 129.0, 128.6, 128.3, 128.0, 124.6, 120.1, 67.3, 55.2, 43.8, 31.8, 29.24, 29.17, 29.0, 28.2, 26.9, 22.6, 22.5, 14.1. HRMS [DART-Orbitrap] m/z calculated for $\text{C}_{29}\text{H}_{43}\text{N}_4\text{O}_3\text{S}$ ($[\text{M} + \text{H}]^+$) 527.3050, observed 527.3078.

Benzyl (S)-(6-(3-decylthioureido)-1-oxo-1-(phenylamino)hexan-2-yl)carbamate (AF10).

White solid, 70% yield. ^1H NMR (500 MHz, Chloroform-*d*) δ 8.51 (s, 1H), 7.54 (d, J = 7.9 Hz, 2H), 7.42 – 7.24 (m, 7H), 7.18 – 7.08 (m, 1H), 6.08 (s, 2H), 5.72 (d, J = 8.1 Hz, 1H), 5.22 – 5.04 (m, 2H), 4.39 (d, J = 7.9 Hz, 1H), 3.42 (d, J = 76.0 Hz, 4H), 1.95 (dt, J = 14.2, 7.5 Hz, 1H), 1.84 – 1.71 (m, 1H), 1.55 (dddq, J = 44.3, 37.4, 14.7, 7.4, 6.9 Hz, 5H), 1.36 – 1.14 (m, 16H), 0.90 (t, J = 6.9 Hz, 3H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 181.0, 170.2, 156.7, 137.5, 136.0, 129.0, 128.6, 128.3, 128.0, 124.6, 120.1, 67.3, 55.2, 44.3, 43.8, 31.9, 31.8, 29.5 (d, J = 2.4 Hz), 29.3 (d, J = 1.9 Hz), 29.0, 28.2, 26.9, 22.7, 22.5, 14.1. HRMS [DART-Orbitrap] m/z calculated for $\text{C}_{31}\text{H}_{47}\text{N}_4\text{O}_3\text{S}$ ($[\text{M} + \text{H}]^+$) 555.3363, observed 555.3394.

Benzyl (S)-(6-(3-dodecylthioureido)-1-oxo-1-(phenylamino)hexan-2-yl)carbamate (AF12).

White solid, 57% yield. ^1H NMR (500 MHz, Chloroform-*d*) δ 8.48 (s, 1H), 7.54 (d, J = 8.0 Hz, 2H), 7.43 – 7.24 (m, 7H), 7.17 – 7.08 (m, 1H), 6.07 (s, 2H), 5.69 (d, J = 8.1 Hz, 1H), 5.14 (d, J = 4.5 Hz, 2H), 4.38 (d, J = 7.9 Hz, 1H), 3.72 – 3.21 (m, 2H), 2.04 – 1.88 (m, 1H), 1.85 – 1.72 (m, 1H), 1.67 (h, J = 6.7 Hz, 1H), 1.60 – 1.39 (m, 4H), 1.37 – 1.13 (m, 20H), 0.94 – 0.86 (m, 3H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 181.1, 170.1, 156.7, 137.5, 136.0, 129.0, 128.6, 128.3, 128.0, 124.6, 120.1, 67.4, 55.2, 43.8, 31.9, 31.7, 29.7, 29.64, 29.59, 29.53, 29.4, 29.3, 29.0, 28.2, 28.0, 26.9, 22.7, 22.4, 19.1, 14.1. HRMS [DART-Orbitrap] m/z calculated for $\text{C}_{33}\text{H}_{51}\text{N}_4\text{O}_3\text{S}$ ($[\text{M} + \text{H}]^+$) 583.3676, observed 583.3704.

Benzyl (S)-(1-((3-hydroxyphenyl)amino)-6-(3-methylthioureido)-1-oxohexan-2-yl)carbamate (C1).

White solid, 48% yield. ^1H NMR (500 MHz, DMSO-*d*6) δ 9.87 (s, 1H), 9.37 (s, 1H), 7.53 (d, J = 7.9 Hz, 1H), 7.47 – 7.28 (m, 5H), 7.23 – 7.14 (m, 1H), 7.07 (t, J = 8.0 Hz, 1H), 6.99 – 6.93 (m, 1H), 6.45 (dd, J = 8.0, 2.3 Hz, 1H), 5.04 (s, 2H), 4.11 (td, J = 8.5, 5.2 Hz, 1H), 3.33 (s, 7H), 2.79 (s, 3H), 1.73 – 1.55 (m, 2H), 1.55 – 1.21 (m, 3H). ^{13}C NMR (126 MHz, DMSO-*d*6) δ 171.4, 158.0, 156.5, 140.4, 137.5, 129.8, 129.4, 128.8, 128.7, 128.3, 128.2, 110.8, 110.4, 106.8, 65.9, 55.9, 32.1, 29.0, 23.6. HRMS [ESI-Orbitrap] m/z calculated for $\text{C}_{22}\text{H}_{29}\text{N}_4\text{O}_4\text{S}$ ($[\text{M} + \text{H}]^+$) 445.1904, observed 445.1908.

Benzyl (S)-(1-((3-hydroxyphenyl)amino)-1-oxo-6-(3-propylthioureido)hexan-2-yl)carbamate (C3).

White solid, 41% yield. ^1H NMR (500 MHz, Chloroform-*d*) δ 8.82 (s, 1H), 7.47 (s, 2H), 7.40 (t, J = 2.2 Hz, 1H), 7.33 (s, 4H), 7.13 (t, J = 8.1 Hz, 1H), 6.93 (d, J = 8.0 Hz, 1H), 6.65 (dd, J = 8.1, 2.3 Hz, 1H), 6.12 (d, J = 50.5 Hz, 1H), 5.89 (d, J = 8.0 Hz, 1H), 5.22 – 5.00 (m, 2H), 4.42 (d, J = 7.7 Hz, 1H), 3.36 (d, J = 19.1 Hz, 3H), 1.86 (s, 1H), 1.70 (s, 5H), 1.56 (h, J = 7.3 Hz, 4H), 1.47 – 1.22 (m, 1H), 0.92 (t, J = 7.4 Hz, 3H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 181.2, 171.0, 156.9, 138.3, 135.8, 130.0, 128.6, 128.4, 128.1, 112.3, 112.0, 107.6, 67.5, 55.6, 43.6, 31.9, 29.7, 28.3, 22.6, 22.3, 11.4. HRMS [ESI-Orbitrap] m/z calculated for $\text{C}_{24}\text{H}_{33}\text{N}_4\text{O}_4\text{S}$ ($[\text{M} + \text{H}]^+$) 473.2217, observed 473.2222.

Benzyl (S)-1-((3-hydroxyphenyl)amino)-1-oxo-6-(3-pentylthioureido)hexan-2-yl)carbamate (C5).

White solid, 94% yield. ^1H NMR (500 MHz, DMSO-*d*6) δ 9.87 (s, 1H), 9.37 (s, 1H), 7.53 (d, J = 7.9 Hz, 1H), 7.37 (d, J = 5.4 Hz, 4H), 7.35 – 7.25 (m, 2H), 7.19 (q, J = 2.8, 2.2 Hz, 1H), 7.07 (t, J = 8.1 Hz, 1H), 6.99 – 6.93 (m, 1H), 6.45 (dd, J = 8.1, 2.3 Hz, 1H), 5.04 (s, 2H), 4.11 (td, J = 8.6, 5.3 Hz, 1H), 3.33 (s, 10H), 1.63 (ddd, J = 18.2, 9.4, 4.4 Hz, 1H), 1.45 (p, J = 7.2 Hz, 3H), 1.41 – 1.16 (m, 5H), 0.87 (t, J = 7.1 Hz, 3H). ^{13}C NMR (126 MHz, DMSO-*d*6) δ 171.4, 158.0, 156.5, 140.4, 137.5, 129.8, 128.8, 128.3, 128.2, 110.8, 110.4, 106.8, 65.9, 55.9, 32.1, 29.1, 28.9, 23.6, 22.4, 14.4. HRMS [ESI-Orbitrap] m/z calculated for $\text{C}_{26}\text{H}_{37}\text{N}_4\text{O}_4\text{S}$ ($[\text{M} + \text{H}]^+$) 501.2530, observed 501.2532.

Benzyl (S)-1-((3-hydroxyphenyl)amino)-6-(3-octylthioureido)-1-oxohexan-2-yl)carbamate (C8).

White solid, 79% yield. ^1H NMR (500 MHz, Chloroform-*d*) δ 9.07 (s, 1H), 8.18 (s, 1H), 7.39 (s, 1H), 7.29 (p, J = 6.8, 5.7 Hz, 5H), 7.05 (t, J = 8.0 Hz, 1H), 6.89 (d, J = 8.0 Hz, 1H), 6.71 – 6.55 (m, 1H), 6.55 – 6.19 (m, 2H), 6.14 (d, J = 7.9 Hz, 1H), 5.21 – 4.87 (m, 2H), 4.39 (q, J = 7.3 Hz, 1H), 3.71 – 2.98 (m, 4H), 2.58 (s, 1H), 1.78 (d, J = 12.0 Hz, 1H), 1.71 – 1.57 (m, 1H), 1.48 (dt, J = 19.7, 8.5 Hz, 3H), 1.39 – 1.04 (m, 14H), 0.88 (t, J = 7.0 Hz, 3H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 180.9, 171.4, 156.9, 138.3, 135.8, 129.9, 128.6, 128.3, 128.0, 112.3, 112.0, 107.8, 67.4, 55.7, 44.2 (d, J = 89.0 Hz), 32.0, 31.8, 29.7, 29.3, 29.2, 29.1, 28.3, 26.9, 22.7, 22.6, 14.1. HRMS [ESI-Orbitrap] m/z calculated for $\text{C}_{29}\text{H}_{43}\text{N}_4\text{O}_4\text{S}$ ($[\text{M} + \text{H}]^+$) 543.3000, observed 543.3004.

Benzyl (S)-6-(3-decylthioureido)-1-((3-hydroxyphenyl)amino)-1-oxohexan-2-yl)carbamate (C10).

White solid, 80% yield. ^1H NMR (500 MHz, Chloroform-*d*) δ 9.04 (s, 1H), 8.09 (s, 1H), 7.39 (s, 1H), 7.29 (s, 5H), 7.06 (t, J = 8.1 Hz, 1H), 6.90 (d, J = 8.0 Hz, 1H), 6.68 – 6.54 (m, 1H), 6.29 (d, J = 69.3 Hz, 2H), 6.10 (d, J = 7.9 Hz, 1H), 5.21 – 4.88 (m, 2H), 4.40 (q, J = 7.4 Hz, 1H), 3.60 – 3.01 (m, 4H), 2.42 (s, 1H), 1.78 (s, 1H), 1.71 – 1.57 (m, 1H), 1.49 (dt, J = 21.7, 7.2 Hz, 4H), 1.26 (d, J = 5.6 Hz, 15H), 0.89 (t, J = 7.0 Hz, 3H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 180.9, 171.4, 156.9, 138.3, 135.8, 130.0, 128.6, 128.3, 128.0, 112.3, 112.0, 107.8, 67.4, 55.7, 44.2 (d, J = 82.2 Hz), 32.0, 31.9, 29.6, 29.4, 29.3, 29.1, 28.4, 27.0, 22.8, 22.7, 14.1. HRMS [ESI-Orbitrap] m/z calculated for $\text{C}_{31}\text{H}_{47}\text{N}_4\text{O}_4\text{S}$ ($[\text{M} + \text{H}]^+$) 571.3313, observed 571.3311.

Benzyl (S)-6-(3-dodecylthioureido)-1-((3-hydroxyphenyl)amino)-1-oxohexan-2-yl)carbamate (C12).

White solid, 82% yield. ^1H NMR (500 MHz, Chloroform-*d*) δ 9.01 (s, 1H), 8.00 (d, J = 8.0 Hz, 1H), 7.39 (s, 1H), 7.30 (s, 5H), 7.08 (t, J = 8.1 Hz, 1H), 6.91 (d, J = 8.0 Hz, 1H), 6.62 (dd, J = 8.0, 2.2 Hz, 1H), 6.54 – 6.10 (m, 2H), 6.06 (d, J = 7.9 Hz, 1H), 5.20 – 4.92 (m, 2H), 4.40 (q, J = 7.4 Hz, 1H), 3.66 – 3.01 (m, 4H), 2.20 (s, 1H), 1.98 – 1.72 (m, 1H), 1.66 (d, J = 11.1 Hz, 1H), 1.50 (dt, J = 16.3, 7.7 Hz, 4H), 1.42 – 1.06 (m, 20H), 0.90 (t, J = 6.9 Hz, 3H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 181.0, 171.3, 156.9, 138.3, 135.8, 130.0, 128.6, 128.3, 128.0, 112.3, 112.0, 107.7, 67.5, 55.7, 44.2 (d, J = 103.5 Hz), 32.0, 31.9, 29.69,

29.67, 29.64, 29.59, 29.4 (d, $J = 1.4$ Hz), 29.1, 28.3, 27.0, 22.7, 14.2. HRMS [ESI-Orbitrap] m/z calculated for $C_{33}H_{51}N_4O_4S$ ($[M + H]^+$) 599.3626, observed 599.3625.

Sirtuin Purification.

Human SIRT1–3 were all expressed and purified as previously reported.¹⁰

Enzyme IC₅₀ Assay.

Inhibitors were stored at -20 °C as stock solutions in DMSO (20 mM – 50 mM) and further serial dilutions were prepared on the day of the assay. Inhibitors were tested in duplicate at final concentrations ranging from 0.002 – 417 μ M with DMSO as control. At 0 °C, sirtuins were diluted in the reaction buffer (20 mM Tris, 1 mM NAD⁺, 1 mM DTT, pH 8.0) to the final concentration (0.05 μ M SIRT1, 0.2 μ M SIRT2, 0.05 μ M SIRT3). Inhibitors were added to the appropriate SIRT1–3 reaction mixture and preincubated at 37 °C for 15 min. Acetyl H3K9 peptide (10 μ M final concentration) was added to start the reaction, and the reaction mixture further incubated at 37 °C until 10–18% of the peptide was deacetylated (3 min for SIRT1, 6 min for SIRT2, and 15 min for SIRT3). The reaction was quenched by the addition of aqueous acid (0.2 M HCl, 6 M acetic acid) at an equal volume, followed by vigorous vortexing. Samples were then centrifuged at 17,000 g x 2 min at RT to pellet the enzyme. The supernatant was then analyzed by analytical HPLC on a Shimadzu LC, Phenomenex Kinetex EVO C18 column (100 $\times$ 4.60 mm, 5 μ M, 100 Å), UV absorbance measurement at 215 nm and 326 nm, 0.1% trifluoroacetic acid in water (Solvent A), 0.1% trifluoroacetic acid in acetonitrile (Solvent B), and 0.5 mL/min flow rate. The peak areas of deacetylated to acetylated H3K9 peptide were used to quantify deacetylase activity. Deacetylase activity was then normalized to the DMSO control, and Log(inhibitor concentration) vs normalized deacetylase activity fitted by nonlinear regression to $Y=100/(1+10^{((X-\text{LogIC}_{50}))})$ on GraphPad Prism version 6.01.

Cell Cytotoxicity Assay.

To a flat bottom 96-well plate, 1000 – 3000 cells were seeded into each well and incubated for 20 hr at 37 °C (1000 cells for HCT-116, 2000 cells for MDA-MB-231, MDA-MB-468, NCI-H23, A549, SW948 and 3000 cells for MCF7, BxPC-3, CCD 841 CoN, and HME1). Inhibitors in varying final concentrations (1 – 100 μ M) were added to each well and the cells incubated for 72 hours at 37 °C. Then, 100 μ L of media was removed from each well and 20 μ L of CellTiter-Blue (Promega) was added to each well. The 96-well plate was then incubated for 4 hours at 37 °C to measure cell viability. The fluorescence of each well (560 nm excitation/590 nm emission) was measured using a Fluoroskan Ascent FL microplate fluorometer. The fluorescence was normalized to DMSO treated cells, and GraphPad Prism software used to plot the cell viability curves and calculate the GI₅₀ value for each cell line.

Soft Agar Assay.

HCT116 cells in a 10 cm dish (70–80% confluent) were rinsed with phosphate buffered saline (PBS), trypsinized for 1 min, resuspended in media (McCoy's Medium + 10% Calf Serum), and counted by hemocytometry. To make the solid base layer, a stock 3% agar solution in water was heated to 42 °C, diluted to 0.6% in media, and mixed with inhibitors to

final concentrations of 2.5 – 50 μ M. To each well in a six well plate, 2 mL of the base layer was added and allowed to solidify for 1 hour at room temperature. To make the growth layer, the stock 3% agar solution was diluted to 0.3% in media and inhibitors were added to final concentrations of 2.5 – 50 μ M. Then, HCT116 cells were added to the growth layer to a concentration of 500 cells/mL. In triplicate, 2 mL of the growth layer containing inhibitor and HCT116 cells was added to each well and incubated at 37 °C. After five days, another 2 mL of growth layer containing inhibitor was added to each well. After 9 to 11 days, 200 μ L of nitro blue tetrazolium chloride in water (2 mg/mL) was added to each well and incubated overnight at 37 °C. The plates were imaged using a ChemiDoc MP Imaging System and colonies were counted with ImageJ software.

Xenograft Mice Study.

All animals used in this study were handled in accordance with federal and institutional guidelines, under a protocol approved by the Cornell University Institutional Animal Care and Use Committee. Immunodeficient female and male NSG mice were purchased from The Jackson Laboratory. When mice were approximately four to eight weeks old, 1×10^6 HCT116 cells were injected on both sides of the abdomen. The cells were allowed to grow for 3 – 5 days until the tumors were just large enough to be visible and measured. Inhibitors were dissolved in solution (80% PBS, 10% DMSO, 10% Kolliphor EL) to a concentration of 10 mg/mL before injection into mice. **AF8** at 25 mg/kg (n=3) and 100 mg/kg (n=4), or vehicle (n=4), was intraperitoneally injected every day over the course of twelve days. **AF10** at 100 mg/kg (n=8) or vehicle (n=6) was also intraperitoneally injected every day over the course of twelve days. Tumor volume was measured daily and mice were sacrificed on the last day and the tumors extracted and weighed.

Immunofluorescence Microscopy.

400,000 HCT116 cells were seeded per dish (MatTek 35 mm, No. 1.5 coverslip, 14 mm glass diameter, poly-D-lysine coated) and incubated for 24 hr at 37 °C. Each well was then treated with DMSO or inhibitor at a final concentration of 50 μ M and incubated for 6 hr at 37 °C. Cells were fixed in ice cold methanol for 10 min. and rinsed 3x in detergent containing buffer (Tris-buffered saline or TBS, 0.1% Tx-100). Cells were blocked in blocking buffer (TBS, 0.1% Tx-100, 5% BSA) for 1 hr at RT and then incubated in primary acetyl α -tubulin antibody (Millipore MABT868 1:100 dilution) overnight at 4 °C. Cells were then washed 3x in detergent containing buffer and incubated with secondary antibody (Cy3-conjugated goat α -mouse, Thermo Fisher A10521, 1:500 dilution) for 1 hr at RT. Cells were rinsed 5x in detergent containing buffer and mounted overnight at RT (DAPI Fluoromount-G, SouthernBiotech). Cells were imaged using a Zeiss LSM880 inverse laser scanning confocal microscope.

P53 Acetylation Assay and Western Blotting.

400,000 HCT116 cells were seeded per well in a six well plate and incubated for 24 hr at 37 °C. Subsequently, each well was co-treated with 400 nM TSA and respective concentration of **AF8**, **AF10**, **TM**, **EX527**, **C3**, **C5**, or **C10** for six hours at 37 °C before cells were collected and lysed in buffer (4% SDS, 150 mM NaCl, 50 mM triethanolamine,

Universal Nuclease, pH 7.4). The protein concentrations of cell lysates were calculated using the BCA assay. To evaluate AcP53 (K382) levels, 50 µg of lysate was ran on a 12% SDS-PAGE gel. To check protein loading, 8 µg of lysate was also loaded on the same gel. The protein gel was transferred to a PVDF membrane, blocked in buffer (TBS, 5% BSA, 0.1% Tween-20) for 1 hr at RT, and incubated overnight at 4°C with AcP53 (K382) antibody (CST 2525S), or with β-actin antibody (SCBT sc-47778) for 1 hr at RT. Membranes were rinsed with TBST (5× 7 min) and incubated with anti-rabbit or anti-mouse secondary antibody (CST 7074S and CST 7076S) for 1 hr at RT. Membranes were rinsed again with TBST (5x, 7 min each) before being incubated with ECL substrate and imaged on a Typhoon FLA 7000.

Cell Culture.

Cell lines were cultured in DMEM with 10% FBS (MCF-7, 293T, MDA-MB-231, MDA-MB-468, HME1, CCD 841 CoN), RPMI with 10% FBS (A549, SW948, NCIH23, BxPC-3), McCoy's Media with 10% Calf Serum (HCT116). Cells were incubated at 37 °C with 5% CO₂.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

BSA	Bovine Serum Albumin
FBS	Fetal Bovine Serum
LC-MS	Liquid Chromatography-Mass Spectrometry
HPLC	High Performance Liquid Chromatography
PBS	Phosphate Buffered Saline
DMSO	Dimethyl sulfoxide
NAD⁺	nicotinamide adenine dinucleotide
TEA	triethylamine
TSA	Trichostatin A

TBS	Tris-Buffered Saline
NEAA	Non-Essential Amino Acids.

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Scheme 1.

Inhibition of sirtuin lysine deacylation by thiourea compounds

Scheme 2. Synthesis of thiourea compounds with varying chain length^a

^a Reactions and conditions: (a) (i) Dichloromethane (DCM), N-methylmorpholine, isobutyl chloroformate, rt, 1 h; (ii) addition of 3-aminophenol, 16 h. (b) (i) DCM, N-methylmorpholine, isobutyl chloroformate, rt, 1 h; (ii) addition of aniline, 16 h. (c) (i) DCM/Trifluoroacetic acid, rt, 1 h; (ii) Tetrahydrofuran, triethylamine (TEA), 1,1'-thiocarbonyldiimidazole, rt, 16 h. (d) Dimethylformamide, TEA, addition of corresponding primary alkylamine, rt, 16 h.

“AF” Inhibitor

“C” Inhibitor

Figure 1.

The **C** class inhibitors modeled into the peptide binding site of SIRT2 (PDB code 4X3O) show additional possible hydrogen bonds as compared to the **AF** inhibitors. For both classes of inhibitors, hydrogen bonds are predicted to form between the lysine carbonyl and the Gly236 backbone nitrogen, between the N-terminus lysine backbone nitrogen and the Glu237 backbone carbonyl, and between the C-terminus backbone nitrogen and the Gln267 backbone carbonyl. The 3' hydroxyl of the **C** class inhibitors (circled in green) could make

additional hydrogen bonds with the Gln267 backbone nitrogen or the Val266 backbone carbonyl or nitrogen (circled in pink).

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Figure 2. Detecting the covalent intermediate formed between **AF8** and NAD^+ in the presence of SIRT2 by liquid chromatography-mass spectrometry (LC-MS). When **AF8** was removed from the reaction mixture, the intermediate mass was not detected as shown through the 1067.5 – 1068.5 m/z ion trace.

Figure 3.

AF8, AF10, and TM inhibited the anchorage-independent growth of HCT116 cells in a soft agar colony formation assay. At a concentration of 10 μM and greater, **AF8** and **AF10** completely inhibited the growth of HCT116 cells. The GI₅₀ values for **AF8** and **AF10** were 7.4 and 7.0 μM respectively whereas the GI₅₀ for **TM** was 16.7 μM.

Figure 4.

AF8, AF10, TM, C5, and C10 inhibit SIRT2 activity in HCT116 cells as shown through an increase in α -tubulin acetylation. HCT116 cells were incubated with 25 or 50 μ M of inhibitor for six hours at 37 $^{\circ}$ C and α -tubulin acetylation detected by immunofluorescence. (A) At 25 μ M, **TM, AF8, and AF10** increased α -tubulin acetylation in HCT116 cells. (B) At 25 μ M, **C3** did not increase α -tubulin acetylation, **C5** slightly increased α -tubulin acetylation, and **C10** more strongly increased α -tubulin acetylation. (C) At 50 μ M, **TM**,

AF8, and **AF10** also increased α -tubulin acetylation. (D) At 50 μ M, **C3** did not increase α -tubulin acetylation, but **C5** and **C10** increased α -tubulin acetylation.

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Figure 5.

AF8 and **AF10** did not noticeably increase p53 acetylation. HCT116 cells were co-incubated with 400 nM TSA and 25 μM of **TM**, **AF8**, **AF10**, **C3**, **C5**, **C10**, and EX527 for 6 hours and p53 K382 acetylation levels analyzed by western blot. Known SIRT1 inhibitor EX527 dramatically increased acetyl p53 levels, while compounds **C5** and **C10** moderately increased acetyl p53 levels. A dose dependent increase of acetyl p53 was observed upon treatment with **C10**, but not **AF8** and **AF10**.

Figure 6.

AF8 and **AF10** significantly inhibit tumor growth by volume and weight in a HCT116 xenograft murine model of colorectal cancer. (A) Tumor volume, tumor weight, and mouse body weight for mice treated with vehicle (n = 4), **AF8** 25 mg/kg (n = 3), **AF8** 100 mg/kg (n = 4). (B) Tumor volume, tumor weight, and mouse body weight for mice treated with vehicle (n = 6) or **AF10** 100 mg/kg (n = 8). Statistical results were analyzed with an unpaired, two tailed t-test.

In vitro IC₅₀ values of inhibitors for SIRT1-3 and relative selectivity for SIRT2 over SIRT1.

^bClogP values computed using DataWarriors software.

Table 2.GI₅₀ values of SIRT2 inhibitors across a variety of cell lines.

Cell Line	Type	TM GI ₅₀ (μM)	AF8 GI ₅₀ (μM)	AF10 GI ₅₀ (μM)
MCF7	Breast Cancer	37.0 ± 4.5	42.6 ± 18.5	18.4 ± 6.0
MDA-MB-468	Breast Cancer	15.7 ± 2.5	26.1 ± 6.4	13.4 ± 1.7
MDA-MB-231	Breast Cancer	42.8 ± 6.5	27.2 ± 8.1	14.8 ± 4.1
BxPC-3	Pancreatic Cancer	13.3 ± 3.5	72.4 ± 25.5	24.3 ± 5.0
NCI-H23	Lung Cancer	16.4 ± 1.8	35.8 ± 14.2	15.6 ± 3.5
A549	Lung Cancer	17.3 ± 6.0	20.8 ± 6.9	9.0 ± 3.9
SW948	Colorectal Cancer	19.2 ± 3.5	30.0 ± 8.9	12.8 ± 3.5
HCT116	Colorectal Cancer	131.6 ± 54.0	58.9 ± 26.5	54.4 ± 12.6
CCD 841 CoN	Colon Cells	n.d. ^a	136.8 ± 53.5	47.4 ± 7.0
HME1	Epithelial Cells	n.i. ^b	n.i.	n.i.

^a nd: not determined.^b ni: no inhibition.