Dethioacylation by Sirtuins 1–3: Considerations for Drug Design Using Mechanism-Based Sirtuin Inhibition

Nima Rajabi, Alexander L. Nielsen, and Christian A. Olsen*

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ABSTRACT: The sirtuin enzymes are potential drug targets for intervention in a series of diseases. Efforts to inhibit enzymes of this class with thioamide- and thiourea-containing, substrate-mimicking entities have produced a number of high-affinity binders. However, less attention has been dedicated to the investigation of the stability of these inhibitors under various conditions. Here, we provide evidence of an unprecedented degree of cleavage of short-chain ϵ -N-thioacyllysine modifications meant to target these sirtuins and further provide insights into the serum stability of compounds containing both thioamides and thioureas. Our study questions the utility short-chain thioamide-based inhibitors of sirtuins for drug development and points to monoalkylated thiourea-based chemotypes as being more stable in human serum.

KEYWORDS: Sirtuins, histone deacetylases, enzyme inhibitors, mechanism-based inhibition, posttranslational modifications

The sirtuin (SIRT) enzymes belong to a family of NAD^+ -dependent deacylases, which catalyzes the removal of acyl-based posttranslational modifications (PTMs) on lysine residues in proteins. The human enzyme family comprises seven isoforms (SIRT1–7), which differ in subcellular localization and substrate specificity.¹ Sirtuins 1 and 2 are both cytosolic and nuclear,^{2,3} SIRT3–5 are primarily mitochondrial, and SIRT6–7 are nuclear.⁴ Sirtuins 1–3 and 6 have been shown to primarily target N^ϵ -acetyllysine-containing (Kac) substrates in vivo;⁵ however, these enzymes also catalyze the removal of medium- and long-chain acyl groups in vitro.^{6–8} Sirtuins 4 and 5 have a preference for negatively charged PTMs, arising from acylation with dicarboxylic acids.^{9–11} The activity of SIRT7 is still not as well understood, with reports suggesting various deacylase activities.^{12–15} Numerous studies have suggested that modulating sirtuin activities has therapeutic potential for treating various cancers, metabolic disorders, and neurodegenerative diseases.^{5,11,16,17} Additionally, such compounds can be used as tools to further study the enzymes in the family and their individual roles in the cell. A number of developed inhibitors are designed to exploit the NAD^+ -dependent deacylation mechanism, hence the term “mechanism-based inhibitors”. The most commonly used group of compounds that fall under this category are thioamide- and thiourea-based sirtuin inhibitors. These inhibitors mimic the natural N^ϵ -acetyllysine substrates and are processed by the sirtuins in a similar manner, albeit at a significantly slower rate.^{18,19} Originally, Fatkins et al. showed that a thioacetylated 27-mer peptide

derived from the tumor suppressor p53 was deacylated at a ~ 400 -fold slower rate than its acetylated analog.¹⁸ Similarly, Smith and Denu showed that the yeast sirtuin HST2 could catalyze dethioacylation of a H3-derived 11-mer peptide, at an ~ 80 -fold lower rate compared to its acetylated counterpart.¹⁹ This has been attributed to the formation of so-called stalled intermediates, several of which have been cocrystallized with different sirtuins (Figure 1).^{16,20–22} Similarly, we have previously cocrystallized a thiourea-based SIRT5 inhibitor, which shows the same type of intermediates.²³ In fact, several highly potent and selective mechanism-based inhibitors have been developed over the past decade.^{1,23–25}

Based on these insights, the rate of conversion of thioamide and thiourea-based sirtuin inhibitors has been considered slow enough to be disregarded as a potential issue. Consequently, considerable efforts have been made in the development of mechanism-based sirtuin inhibitors.^{24,26–30} Here, we investigate a series of moderately selective SIRT1 inhibitors, based on a dipeptide scaffold that was previously developed.²³ Combining this scaffold with acyl motifs, which have previously been applied to target SIRT1, furnished potent inhibitors of this isozyme.

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Figure 1. (A) Sirtuin catalyzed deacylation mechanism of thioamide/thiourea based inhibitors. (B) X-ray crystal structures of intermediates I,²¹ II,²² and III²⁰ of different thioamide-based inhibitors bound to sirtuins.

Importantly, we present novel findings suggesting that thioamide-based inhibitors can be cleaved by SIRT1–3 to a higher extent than previously anticipated. These observations highlight the importance of in-depth compound assessment beyond measurement of potency.

In previous efforts to develop inhibitors against SIRT5, inhibitor **1** was synthesized as a control compound, which showed no inhibitory activity against SIRT5.²³ In a subsequent study, we showed that compound **1** was a moderately potent inhibitor of Sir2La, a deacetylase/depropionylase from the Gram positive *L. acidophilus*, while compound **2** was less potent.³¹ Given that thioacetamide- and methylthiocarbonyl-based compounds have also been shown to inhibit SIRT1–3,^{1,25,33} we measured the potencies of **1** and **2** against these enzymes together with control compounds TA,^{24,33} TB,²⁴ and EX-527³² (Figure 2A). The nonmechanism-based SIRT1 inhibitor EX-527 was included because it is one of the most potent and selective SIRT1 inhibitors reported and a commonly used tool compound.³² Furthermore, EX-527 has completed phase I and II clinical trials for the treatment of Huntington's disease.¹⁶ Indeed, both compounds **1** and **2** exhibited inhibitory activity against all three enzymes, with moderate selectivity toward SIRT1 over SIRT2 and higher selectivity for SIRT1 over SIRT3. With compounds **1** and **2** serving as lead chemotypes, a series of thioamide- and thiourea-based compounds was synthesized with varying acyl chain lengths (3–7). Furthermore, α -fluoroacetyl compounds **8** and **9** were included in the series, because these motifs have previously been shown to inhibit sirtuins. Albeit, lower potencies have generally been reported for these compared to their thioamide and thiourea-based counterparts.^{19,23,34} Consistent with previous studies, the thioamide- and thiourea-based inhibitors **1–7** were significantly more potent against SIRT1 than the α -fluoroacetyl inhibitors **8** and **9**. Intriguingly, inhibitors **1–7** were all equipotent to EX-527 against SIRT1 and some of these inhibitors showed similar selectivity profiles compared to EX-527. While compounds **1–7** showed

Figure 2. Structure–activity relationship study, measuring compound potencies against recombinant SIRT1–3. (A) Previously reported compounds.^{23,24,31–33} (B) Inhibitors developed in this study. Potencies are given as IC₅₀ values or %-inhibition at the indicated concentrations (see Supporting Figure S1 for dose–response curves and Figure S2 for bar graphs).

Figure 5. (A) Chemical structures of fluorogenic substrates **10–12** and **oxo-10–oxo-12**. Deacylation of compounds **10–12** and **oxo-10–oxo-12**, by recombinant SIRT1–3 (100 nM or 250 nM) and cell lysate (HEK293, ~65 μ g). Error bars represent the standard deviation based on two independent experiments performed in duplicate.

SIRT2 could not hydrolyze **oxo-1**, **1**, or **3** but efficiently cleaved **oxo-10**, **10**, and **11** at the applied enzyme concentrations. The **TA** compound was not hydrolyzed by SIRT1 or SIRT3 either, which could also be explained by poor affinity. To examine the degree of deacylation in a more complex sample, substrates **10–12** and **oxo-10–oxo-12** were mixed with fresh cell lysates from HEK293 cells.

Notably, the conversion of all substrates was increased significantly (Figure 5B). Presumably the increased deacylation of the substrates is due to the presence of multiple deacylases in the cell lysate, capable of hydrolyzing short chain acyllysines, i.e. the HDAC1–3, HDAC6, HDAC8, SIRT1–3, and SIRT6.^{6,7,35,36} Moreover, it has previously been shown that HDAC8 and HDAC6 can catalyze dethioacetylation, which could explain the substantial increase in conversion of substrate 10 (approximately 70–6-fold compared to SIRT1–3).^{18,37} Although, expression levels of the various deacylases in the cell of course have a major impact on the degree of conversion as well.

To ensure that the observed dethioacylations were not artifacts arising from inhibitor/substrate degradation or thioamide to oxoamide conversion, followed by standard siirtuin deacylation, we performed compound stability tests in assay buffer. These experiments indicated no spontaneous

conversion to the free lysine product nor could thioamide to amide conversion be detected even after 24 h in buffer at 37 °C (Figures S11–S19). Thus, SIRT1–3 can indeed deacylate thioamide-based inhibitors and substrates more efficiently than previously anticipated, indicating that thiourea-based inhibitors should be more promising. To further address the stability of thioamide- and thiourea-based SIRT inhibitors beyond the deacylation, we analyzed the stability of inhibitors **1–6** in human serum, because this is an important factor to consider in drug development. Compound **1** and its thiourea analog **5** exhibited similar stabilities in serum with half-lives above 7 h. On the other hand, the dialkyl thiourea-based compounds **2** and **6** were highly labile, with half-lives of less than 1 h and full degradation observed after 4 and 6 h, respectively. In contrast, their respective thioamide analogs **2** and **3** were significantly more stable with half-lives of 6 and 7 h, respectively. The observation that the dialkyl thiourea compounds **2** and **6** are rapidly degraded in serum, whereas the monosubstituted thiourea **5** is relatively stable, led us to examine the stability of previously reported inhibitors SIRT5 as well (S5–S7).²³ Although more stable than **2** and **6**, the thiourea **S5** was substantially less stable than the corresponding thioamide **S6**, consistent with the above observations. The urea analog **S7** was particularly stable in human serum, showing only a slight degradation after 24 h (Figure S20). Unfortunately, we were unable to determine how the thiourea-based inhibitors were degraded as the chromatograms did not reveal any distinct byproduct peaks. Thus, it remains to be investigated in detail how the substituted thiourea-based inhibitors are degraded.

Finally, inhibitors **1**, **2**, **5**, and **9** (25 μM) were evaluated for their ability to inhibit SIRT1 in HEK293 cells, to test their ability as tool compounds in cellular assays. Thus, acetylation levels of the tumor suppressor p53 (K382) were analyzed, which is a well-documented substrate of SIRT1.^{24,33,38} It has previously been shown that p53 acetylation (K382) is also affected by SIRT2 under certain assay conditions.³⁹ Therefore, a potent and selective SIRT2 inhibitor, **SirReal2**, was included to ensure that the effect on p53 acetylation (K382) was SIRT1 dependent.⁴⁰ Gratifyingly, compounds **1** and **2** exhibited a significant increase in the acetylation of p53 (K382) compared to vehicle-treated control (Figure 6). Interestingly, the increases in p53 acetylation by **1** and **2** were equivalent, and even higher than that of **EX-527**. Neither **SirReal2** nor the *in vitro* inactive compound **9** resulted in significant upregulation of p53 acetylation (K382). However, somewhat surprisingly to us, compound **5** also had a lower effect on p53 acetylation, suggesting that this inhibitor may be less cell permeable than compounds **1** and **2**. Taken together, these control experiments suggest that the effects of compounds **1** and **2** on p53 (K382) acetylation are indeed a result of inhibition of SIRT1.

In summary, we have evaluated a series of mechanism-based inhibitors of SIRT1–3. The thioamide- and thiourea-based inhibitors exhibit potent inhibition of SIRT1, comparable to that of EX-527. While most of these inhibitors are selective toward SIRT1 over SIRT3, more moderate selectivity was generally achieved for SIRT1 over SIRT2.

More notably, we show that short-chain thioamide-based inhibitors **1** and **3** can be deacylated by SIRT1–3 to an unprecedented degree. The extent of dethioacylation appears to decrease upon extension of the acyl chain length, as SIRT1–3 showed higher activity for dethioacetylation than dethiopropionylation and dethiobutyrylation. Furthermore, thioamide-based compounds are rapidly deacylated by whole cell lysate

Figure 6. (A) Chemical stability of compounds 1–6 in human serum (see Figure S20 for full stability curves, including 24 h measurements). (B) Western blot analysis of whole-cell lysates (HEK293) after 6 h treatment with inhibitors EX-527, 1, and 2, 5, and 9, and SirtReal2 (25 μ M), cotreated with TSA (1 μ M). (C) Relative levels of Ac-p53 (K382) to p53, normalized to DMSO control. The error bars represent the standard error of the mean (SEM) based on data from two independent experiments performed in duplicate (see Figure S21 for the full immunoblots and additional image quantification).

(HEK293), suggesting that they are also likely to be short-lived in cells, though inhibitors 1 and 2 both gave rise to similar effects on p53 acetylation in HEK293 cells, presumably by inhibition of SIRT1, indicating a potential utility as tool compounds for in vitro studies.

Nevertheless, the substantial dethioacylation calls into question the utility of short chain thioamide-based sirtuin inhibitors, especially thioacetyl-containing compounds, in medicinal chemistry efforts to target sirtuins.^{1,25} Thiourea-based inhibitors, on the other hand, are not deacylated by SIRT1–3, suggesting that these inhibitors are more suitable for cellular studies. Furthermore, thiourea-based compounds are synthetically more readily accessible and allow for late-stage introduction of the lysine modification, which provides an additional advantage over thioamides. One major drawback is the limited stability in serum when elongating the side chain by *N'*-alkylation, which limits the potential utility of such thiourea-based chemotypes in drug development. Although compound 5 did not exhibit as strong an effect on p53 acetylation as 1 and 2, monoalkylated thiourea-containing compounds may offer an alternative motif for future efforts to inhibit SIRT1–3, due to their extended lifetime in serum.

The collective insight gained in this study is essential for future sirtuin inhibitor design and underscores the crucial need for comprehensive compound assessment when targeting sirtuins with mechanism-based inhibitors. We conclude that short-chain thioamide- and thiourea-based inhibitors may serve as tool compounds for cellular studies, but for drug development efforts alternative chemotypes are needed.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsmchemlett.9b00580>.

Additional figures supporting the manuscript, experimental details, compound characterization data, as well as copies of ¹H, ¹³C, and ¹⁹F NMR spectra (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Christian A. Olsen – Center for Biopharmaceuticals & Department of Drug Design and Pharmacology, Faculty of Health and Medical Sciences, University of Copenhagen DK-2100 Copenhagen, Denmark; orcid.org/0000-0002-2953-8942; Email: cao@sund.ku.dk

Authors

Nima Rajabi – Center for Biopharmaceuticals & Department of Drug Design and Pharmacology, Faculty of Health and Medical Sciences, University of Copenhagen DK-2100 Copenhagen, Denmark; orcid.org/0000-0002-9509-7540

Alexander L. Nielsen – Center for Biopharmaceuticals & Department of Drug Design and Pharmacology, Faculty of Health and Medical Sciences, University of Copenhagen DK-2100 Copenhagen, Denmark; orcid.org/0000-0003-1195-0143

Complete contact information is available at: <https://pubs.acs.org/doi/10.1021/acsmchemlett.9b00580>

Notes

The authors declare no competing financial interest.

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