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Inhibition of the human deacylase Sirtuin 5 by the indole GW5074

Benjamin Suenkel, Frank Fischer, Clemens Steegborn *

Department of Biochemistry, University of Bayreuth, Universitaetsstrasse 30, 95447 Bayreuth, Germany

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ABSTRACT

Sirtuins are NAD^+ consuming protein deacylases involved in many cellular processes from DNA-repair to metabolism. Their contribution to age-related and metabolic diseases makes them attractive pharmaceutical targets. Few pharmacological inhibitors have been reported yet for human Sirt5 since substrates and assays for reliable testing of its activity were unavailable until recently, and most modulators of other Sirtuins were not tested against Sirt5 and therefore have only partially characterized isoform selectivities. We used here improved substrates and assays for testing of known Sirtuin inhibitors for their effects on two activities of human Sirt5, the generic Sirtuin activity deacetylation and the more pronounced Sirt5 activity desuccinylation. Our tests show that most of the compounds have no significant effect on either Sirt5 activity. The indole GW5074, however, was found to be a potent inhibitor for Sirt5's desuccinylation activity, identifying a first pharmacological scaffold for development into Sirt5-specific inhibitors. Interestingly, the compound showed weaker effects in Sirt5 deacetylation assays and also varying potencies against different peptide sequences, indicating a substrate-specific effect of GW5074.

Sirtuins are a unique family of protein deacetylases due to their consumption of NAD^+ as a co-substrate.[1] The seven mammalian Sirtuin isoforms differ in the targets they control: The nuclear isoforms Sirt1, 6, and 7 contribute to functions such as transcription control and DNA-repair,[2,3] and cytosolic Sirt2 deacetylates α-tubulin and signaling proteins such as the acetyltransferase p300.[4] The mitochondrial isoforms Sirt3, 4 and 5 are the only known mitochondrial deacylases[5] and target predominantly metabolic enzymes, such as glutamate dehydrogenase (Sirt3 and Sirt4),[6,7] complex 1 of the respiratory chain (Sirt3),[6] or carbamoylphosphate synthetase 1 (CPS1; Sirt5).[8] Sirt5-dependent deacetylation activates CPS1 and thereby the urea cycle, and these effects are amplified under caloric restriction,[9] indicating that Sirt5 might contribute to the beneficial effects of this treatment.[2,10] More recently, Sirt5 was further found to display a desuccinylase and demalonylase activity that is more pronounced than its weak deacetylase activity, and CPS1 as a first in vivo desuccinylation substrate,[11] indicating that it might function as a more general deacylase and suggesting that these stronger activities will enable to identify additional physiological substrates.

Sirtuins have been implicated in stress responses and several aging-related diseases, such as Parkinson's disease, diabetes, and cancer.[12,13] These findings have stimulated intensive research into compounds modulating their activity,[3,6,10,14] which has resulted in a large number of small-molecule Sirtuin modulators.[15,16] However, no or few modulators have been reported yet for several human isoforms, including Sirt5, also due to a lack of suitable substrates and assays for testing their activity. For the same reason, the isoform specificities of modulators reported for other Sirtuins are only partially described. Thioacylated suicide substrate peptides are the only known Sirt5 inhibitors with a pronounced isoform specificity.[17] During catalysis, which normally proceeds via formation of an alkylimidate intermediate under nicotinamide release followed by hydrolysis of the intermediate,[1,18] the thioacyl-peptides form a stable thioalkyl-imidate that blocks further catalysis. Despite previous first successes in converting, for example, a Sirt1-targeting thioacyl-peptide into a cell-active derivate with mM potency and moderate isoforms selectivity,[19] peptide derivative inhibitors are generally of limited pharmacological relevance due to their size, poor membrane permeability, limited biostability, and the difficulties in converting them into stable non-peptide derivatives.[15,16] Suramin, an anti-parasitic agent, is one of very few known pharmacological Sirt5 inhibitors but inhibits Sirtuins non-specifically in the low μM range by blocking substrate and co-substrate binding.[5,20–23] Additional Sirt5 inhibitors are the non-specific co-product nicotinamide (see below) and thiobabiturate-based compounds that are not yet Sirt5 specific but show some discrimination against Sirt3, another mitochondrial isoform.[24] Of the many other Sirtuin inhibitors described,[15,16] to our knowledge only the weak Sirt1 inhibitor sirtinol and the indole EX-527, a potent Sirt1 inhibitor with weaker effects on Sirt2 and Sirt3,[25] were tested against Sirt5 deacetylation activity and found to be ineffective against this isoform.[26,27] For other potent Sirtuin inhibitors, such as the μM Sirt2 inhibitors GW5074 and AGK2, no information is available for their effects on most mammalian

* Corresponding author. Tel.: +49 921552420; fax: +49 921552432.
E-mail address: clemens.steegborn@uni-bayreuth.de (C. Steegborn).

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isoforms, including Sirt5.[15,16] In general, most published compounds were only tested against Sirt1 and Sirt2, sometimes also Sirt3, for which well-established assays are available. Only recently, improved Sirt5 substrates and assays have been reported based on fluorogenic peptides[23] and on non-modified peptides using a mass spectrometry (MS)-based assay.[28] A further challenge in developing Sirtuin inhibitors stems from the complex enzymatic mechanism of the Sirtuin reaction[1] and a lack of knowledge on the inhibition mechanism for most compounds. While some inhibitors are competitive with one or both substrates, such as suramin, for most compounds competition has not been observed or even been tested. The Sirtuin reaction product nicotinamide, which is assumed to act as general Sirtuin inhibitor by enhancing the reverse reaction of the alkylimidate intermediate, acts non-competitively, as does EX-527 on Sirt1.[25] Furthermore, nicotinamide-dependent inhibition of Sirt5 depends strongly on the acyl group to be removed, and also slightly on the substrate polypeptide sequence.[28] Thus, information on compound effects on the less well studied Sirtuin isoforms, such as Sirt5, and on their inhibition mechanisms are needed to develop a set of potent and isoform-specific compounds.

To improve our knowledge on isoform selection of available Sirtuin inhibitors and as a first step toward the development of pharmacological Sirt5 inhibitors, we tested a series of inhibitors previously reported for other Sirtuins for their effects against Sirt5 activities (Fig. 1): AGK2, a frequently used Sirt2-selective inhibitor; EX-527, a moderately Sirt1-selective indol derivative; GW5074, a Sirt2 inhibiting indole; HR73, a splitomicin derivative yet only tested against Sirt1; salermide, a moderate Sirt2 inhibitor; sirtinol, a weak Sirt1 inhibitor; SRT1720, which activates Sirt1 but inhibits Sirt3; and suramin, which inhibits Sirt1 stronger than Sirt2 and Sirt5, for comparison. To overcome the problems associated with the low Sirt5 deacetylation activity in the established Fluor-de-Lys deacetylation assay[29] and against known substrate peptides, we used recently identified better substrate peptides for Sirt5 (Rauh et. al., submitted)[28] in a continuous coupled assay[30] and in a more sensitive MS-based assay.[28] Since Sirt5 inhibition by nicotinamide was acyl group specific, we tested them against both, Sirt5's deacetylation and desuccinylation activity.

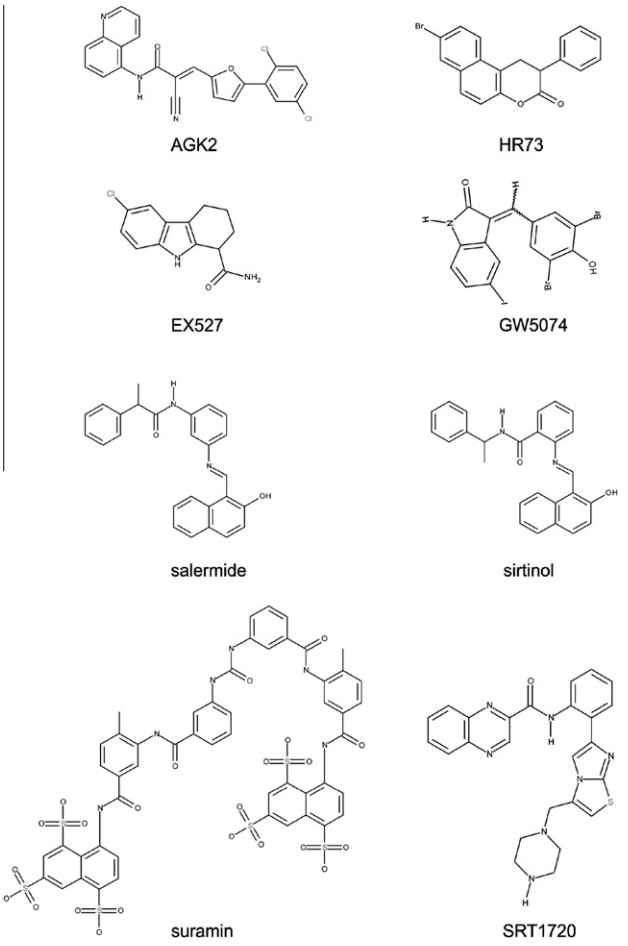

Figure 1. Known Sirtuin modulators tested here for their effects on Sirt5 activity.[38]

We first screened the panel of compounds against the more prominent Sirt5 activity, desuccinylation, in a coupled enzymatic activity assay[30,31] using a succinylated peptide derived from peroxiredoxin 1 (Prx1; succPrx1, SKEYFS-succinylLys-QK)[28] as a substrate. Since GW5074 is incompatible with this assay due to inhibition of the coupled enzyme glutamate dehydrogenase,[32] we tested this compound in a MS-based assay[28,33] against the same substrate peptide. Assays in absence or presence of 10 or 100 μM compound, respectively, revealed no significant effects on Sirt5's desuccinylation activity except for GW5074 (Fig. 2a). While 100 μM compound had no effect for several substances, such as HR73, or reduced Sirt5 activity by ~20–30%, as observed for salermide and suramin, 100 μM GW5074 reduced Sirt5 desuccinylation activity to ~15%. We therefore performed a GW5074 titration experiment which yielded an IC_{50} value of 19.5 ± 7.3 μM for inhibition of the desuccinylation of succPrx1 by Sirt5 (Fig. 2b).

We next tested the same set of compounds, again at 10 and 100 μM, respectively, for their inhibitory effects on the Sirt5 deacetylation activity. Since acetylated Prx1 peptide is a weak Sirt5 substrate, we used an acetylated peptide derived from CPS1 (acCPS1, FKRGVL-acetylLys-EYGVKV) as a substrate in the MS (GW5074) or continuous assay (all other compounds). Interestingly, Sirt5's deacetylation activity was only reduced to ~30% in presence of 100 μM GW5074 (Fig. 2c). Analysis in a dose–response experiment with the GW5074 concentrations used above revealed an IC_{50} of 97.8 ± 18.6 μM for inhibition of acCPS1 deacetylation, a 4-fold higher value compared to inhibition of Sirt5's desuccinylation activity (Fig. 2d). Suramin, on the other hand, showed significant inhibition of the Sirt5 deacetylation activity, in contrast to the result in the desuccinylation assay. All other compounds showed no significant effect on Sirt5 deacetylation activity even at 100 μM concentration, similar to their negligible effects in the desuccinylation assay. The IC_{50} for inhibition of the deacetylation of acCPS1 by suramin is 14.2 ± 5.7 μM (Fig. 2e), in the order of magnitude previously reported for this effect.[22]

The differing suramin effects were surprising considering its assumed competitive mode of action.[22] Likewise, significantly different effects of GW5074, depending on the substrate tested, were unexpected. The compound was assumed to inhibit kinases competitively by blocking their ATP site.[34] It was identified as a μM Sirt2 inhibitor through screening of such adenosine analogs based on the rational that they should also block NAD^+ binding sites through competition with its adenosine moiety.[34] However, we find that the effect of GW5074 on Sirt5 differs depending on the peptide substrate, which is not easily explained through a competitive mechanism (see below). An acyl moiety dependent inhibitory effect on Sirt5 was observed for nicotinamide,[28] which could indicate a similar mechanism for GW5074 based on binding to the nicotinamide-accommodating so-called C-site.[18] However, our deacetylation assays were not only performed with a differing acyl modification, but also with a different peptide sequence. Although differing peptide sequences caused only small differences in the inhibitory effect of nicotinamide,[28] we tested the compounds' effect using an acetylated Prx1 peptide, a poor Sirt5 substrate, to analyze which substrate feature causes the different compound

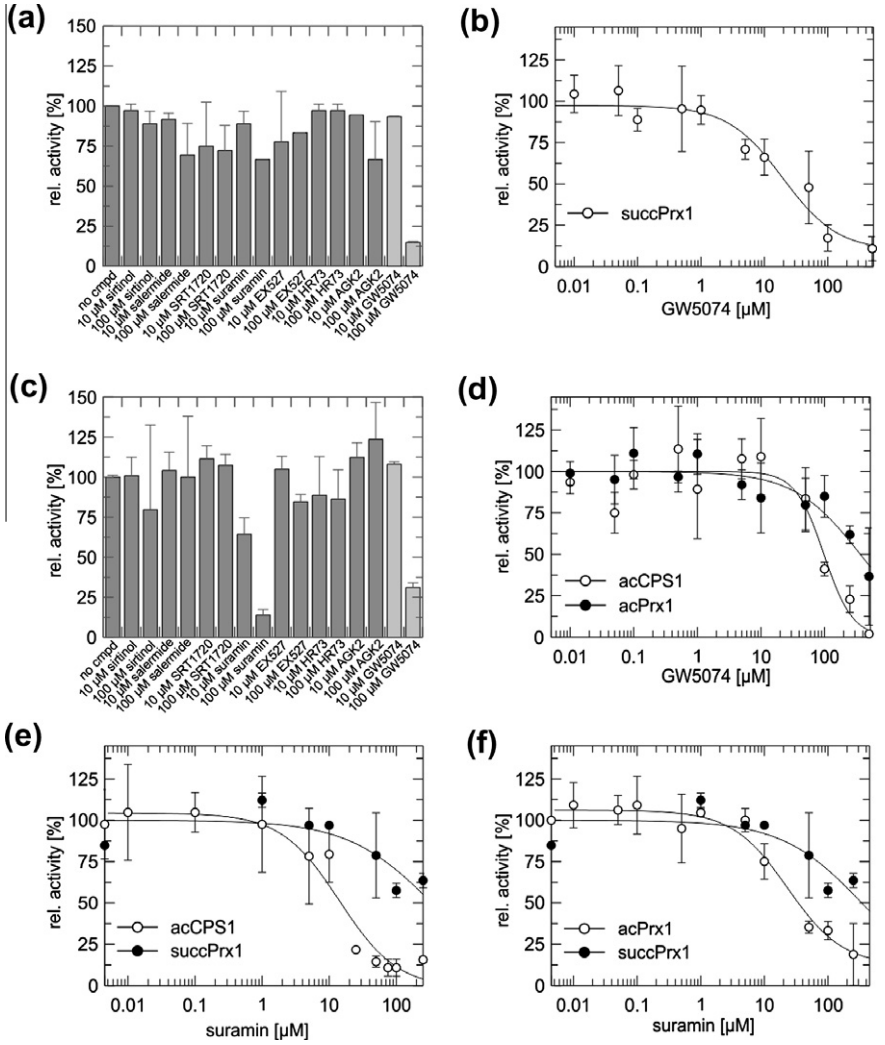

Figure 2. Inhibition of Sirt5 deacylation activities. (a) Relative Sirt5 desuccinylation activity in presence of 10 or 100 µM of the compounds tested. (b) Inhibitor titration experiment confirming potent inhibition of the Sirt5 desuccinylation activity by GW5074. (c) Relative Sirt5 deacetylation activities in presence of 10 or 100 µM compound, respectively. (d) GW5074 titrations show that deacetylation of acCPS1 (○) is only weakly inhibited and deacetylation of acPrx1 (●) is almost insensitive to GW5074 inhibition. (e) Suramin inhibits deacetylation of acCPS1 (○) with an IC_{50} of 14.2 ± 5.7 µM but does not significantly inhibit desuccinylation of succPrx1 (●). (f) Differential suramin inhibition of succPrx1 (●) and acPrx1 (○) deacylation by Sirt5. Data for succPrx1 (●) are those from panel (e) and are included for comparison. All Sirt5 activity tests were done using a coupled enzymatic assay (dark grey bars) except for GW5074, which was tested using a mass spectrometry based assay (light grey bars). For the weak Sirt5 substrate acPrx1, the more sensitive mass spectrometry based assay was used for all measurements.

sensitivity, its sequence or its acyl modification. Dose–response experiments using acPrx1 revealed a higher IC_{50} value for GW5074 (estimated IC_{50} of 200–400 µM) against Sirt5's deacetylation activity (Fig. 2d). Comparing the IC_{50} values for both acetylated substrate peptides thus shows that the substrate sequence influences the compound potency. Consistently, slightly more potent inhibition of Sirt5 desuccinylation activity by suramin and sirtinol (IC_{50} values around 50 µM) than in our assays was reported recently based on a non-physiological, fluorogenic substrate that differs from our substrate in the peptide/peptide mimetic part.[24] However, comparing inhibition of the two deacylation activities against the same peptide sequence (Fig. 2b and d) also shows differences in potency. Thus, peptide sequence as well as its acyl modification influence the sensitivity to inhibition by GW5074.

The substrate-sequence specific potency effects indicate the challenging but also exciting possibility to develop compounds that affect not all substrates of a given Sirtuin equally, but in a substrate (group) specific manner. Our results further provide evidence for activity specific inhibition. Of all tested compounds only suramin was able to inhibit the deacetylation by Sirt5 as already reported,[22] but it did not significantly inhibit Sirt5's desuccinylation activity (Fig. 2e and f). Higher acetyl-peptide than succinyl-peptide concentrations were used for deacylation reactions since it is known that more acetyl-peptide is needed to reach saturation.[11,28] However, K_M values for acetyl-peptides in the range of several hundred µM[11] suggest that Sirt5 is not saturated even at these high concentrations, while it is likely saturated in the desuccinylation assays. Considering the bulky nature and known Sirt5 complex structure of suramin, which shows that the compound acts competitively,[22] differences in acyl-peptide substrate affinities are likely the reason for its stronger effect against Sirt5 deacetylation compared to desuccinylation. GW5074, in contrast, only inhibited desuccinylation by Sirt5 significantly, which thus cannot be caused by weaker competition. Our results therefore indicate that GW5074 does not act as an adenosine analog, blocking competitively the NAD^+ site. Docking a GW5074 related compound into a Sirt2 apo structure indeed suggested its binding to the nicotinamide accommodating C-pocket.[35] Our similar previous observation that nicotinamide inhibits Sirt5-dependent desuccinylation potently but deacetylation weakly appears to support that the

compound binds to the C-pocket. Although such a binding could lead to competition with NAD^+, the large set of data available for nicotinamide inhibition indicate that it only binds significantly after intermediate formation and thus primarily inhibits by preventing hydrolysis of the intermediate (and in fact reverses intermediate formation). It is tempting to speculate that GW5074 binds to the, according to kinetic data long-lived,[36] Sirtuin intermediate complex, and it will be interesting to learn the molecular basis of this substrate-specific mechanism from future structural studies. Structural data should also reveal which GW5074 stereoisomer is responsible for the inhibitory effect, which cannot easily be tested in assays due to the rapid isomerization to a ~1:1 equilibrium in aqueous solutions.[37]

GW5074 is one of the first Sirt5 inhibitors with pharmacologically suitable properties. The compound inhibits Sirt2 deacetylation activity with a potency comparable to Sirt5 (reduction to less than 60% activity with 12.5 μM GW5074)[34] and kinases even more potently,[37] rendering it not suitable for in vivo Sirtuin studies, but its properties make it an interesting starting point for the development of Sirt5 specific inhibitors. Our results also highlight the importance of testing known Sirtuin inhibitors against less characterized Sirtuin isoforms. Our data further show that compounds can act selectively, depending on the activity and substrate tested. Although the Sirt5 desuccinylation activity is more prominent and is likely to be dominant in vivo, a physiological relevance of Sirt5's deacetylation activity cannot be excluded. Our data show that both activities can be inhibited selectively and demonstrate the importance of testing compounds against the Sirt5 activity supposed to be inhibited, and also against different substrate sequences.

In summary, we show here the effects of various known Sirtuin modulators on the two Sirt5 deacylation activities, deacetylation and desuccinylation. Our results identify GW5074 as a lead for further development of pharmacological Sirt5 inhibitors and provide evidence for an activity and substrate sequence specific compound effect.

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